University of Algarve
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Master's thesis in Marine Biology

Phylogeography and phylogeny of European gorgoniids (Gorgoniidae): genera *Eunicella* and *Leptogorgia*

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As gorgónias, antozoários marinhos (Sub-classe Octocorallia) ubiquamente distribuídos por todo o mundo, são ecologicamente importantes e amplamente conhecidas por acolherem vastas comunidades de organismos associados. Tipificadas pela sua arquitectura arborescente, as gorgónias possuem um esqueleto axial de origem proteica (gorgonina) que confere suporte às colónias e pode ser de diferentes naturezas. Apesar da sua incontestável importância ecológica, a taxonomia e relações filogenéticas das gorgónias, e octocorais na generalidade, são alvo de aceso debate e pouco consenso entre taxonomistas. Na verdade, até o reconhecimento de limites taxonómicos ao nível das espécies é complicado e muitas espécies são subdivididas em sub-espécies ou variantes locais ao longo de amplas escalas geográficas. Na base desta controvérsia taxonómica estão diversos factores entre os quais: a variabilidade inter e intra-específica dos caracteres morfológicos usados na identidficação das espécies; homoplasia e falta de caracteres morfológicos diagnosticantes adequados; ausência de registos fósseis; resolução limitada dos marcadores moleculares usados até à data ao nível das espécies; e baixos níveis de variabilidade dos genes mitocondriais nos octocorais.

De acordo com o presente sistema taxonómico a subclasse Octocorallia está subdividida em três ordens: Helioporacea, Pennatulacea e Alcyonacea. A ordem Alcyonacea engloba todas as gorgónias com esqueleto axial, assim como todos os corais moles que não possuem um esqueleto de suporte. Entre os diversas espécies que compõem a ordem Alcyonacea, apenas dois grupos representam entidades morfológicas discretas (sub-ordens Holaxonia e Calcaxonia) pelo que os restantes, interligados por formas intermediárias, são classificados como grupos sub-ordinais ao invés de sub-ordens (Protoalcyonaria, Stolonifera, Alcyoniina e Scleraxonia). Apesar da monofilia da sub-classe Octocorallia ser suportada por análises moleculares e pelo menos uma sinapomorfia (pólipos com oito mesenterias), nenhum dos grupos taxonómicos enumerados acima traduz as relações filogenéticas reveladas por marcadores moleculares e muitas das famílias actualmente reconhecidas são parafiléticas.

A família Gorgoniidae (Holaxonia) contém diversos géneros de octocorais de baixa profundidade entre os quais *Eunicella* e *Leptogorgia* claramente dominam a fauna de octocorais na margem Este do Oceano Atlântico. A taxonomia de ambos os géneros baseia-se na combinação do modo de crescimento das colónias e forma dos escléritos (espículas de origem calcária), mas os padrões de cor das colónias também podem ser usados no género *Leptogorgia*. Nas costas europeias o género *Eunicella* encontra-se representado por cinco espécies para o qual poucos caracteres diagnosticantes existem, enquanto que para o género *Leptogorgia*, a espécie *Leptogorgia sarmentosa* é a única actualmente reconhecida como

válida. Apesar de durante muitos anos se ter considerado *L. sarmentosa* e *Leptogorgia lusitanica* (espécie característica da costa Ibérica) espécies distintas, actualmente estão englobadas numa única espécie com variantes/sub-espécies geográficos. Para as costas europeias são reconhecidos o variante *lusitanica*, que ocorre tipicamente na costa portuguesa e apresenta diversos padrões de cor em púrpura e amarelo, e o *sarmentosa* característico do Mediterrâneo e que ocorre em tons terracota. Não obstante, a taxonomia de *L. sarmentosa* ainda hoje continua a invocar controvérsia.

Perante a actual condição taxonómica das espécies de *Eunicella* e *Leptogorgia* que habitam as margens costeiras europeias, os objectivos do presente estudo incidiram no 1) uso de sequêncicas nucleotídicas de genes mitocondriais para determinar os limites ao nível das espécies para os gorgonídeos (Gorgoniidae) europeus; 2) clarificar o *status* taxonómico das sub-espécies/variantes de *L. sarmentosa* ao longo da transição Portugal-Mediterrâneo através de marcadores nucleares; e 3) analizar o nível de conectividade entre as populações Atlânticas e Meiterrânicas de *L. sarmentosa* e *Eunicella verrucosa* (as duas únicas espécies de gorgonídeos a co-ocorrerem em ambos os locais) também com recurso a marcadores nucleares.

Os dados genéticos de quatro marcadores mitocondriais (COI, ND2, ND6 e *msh1*, um homólogo do gene *mutS* encontrado na "MSLH mismatch repair pathway" das bactérias, e exclusivo ao genoma mitochondrial dos octocorais) não revelaram variabilidade suficiente para diferenciar as diferentes espécies de *Eunicella* sequenciadas (*E. verrucosa*, *Eunicella gazella* e *Eunicella labiata*), asim como qualquer dos variantes geográficos de *L. sarmentosa*, i.e., *lusitanica* ou *sarmentosa*. A falta de variabilidade dos genes mitocondrias para análises intra-específicas é amplamente reconhecida e suportada por diversos estudos moleculares. No caso do género *Eunicella*, por exemplo, o gene COI não revelou diferenciação intra ou inter-específica para as espécies Mediterrânicas *Eunicella cavolini* e *Eunicella singularis*.

A posição filogenética de ambos os géneros (*Eunicella* e *Leptogorgia*) na sub-classe Octocorallia foi inferida através uma análise *maximum-likelihood* (ML; gene *msh1*) em que se usou espécies representativas da grande maioria das ordens e sub-ordens presentemente reconhecidas pela taxonomia tradicional, e as sequências de octocorais mais similares às de *E. verrucosa*/*L. sarmentosa* obtidas através da ferramenta BLAST do GenBank. No total, incluiram-se espécies de 48 géneros diferentes assim como 17 espécies de *Leptogorgia* e um único representativo de *Eunicella* (*E. verrucosa*). A árvore filogenética obtida através da análise ML revelou uma divergência transatlântica significativa entre as diferentes espécies de *Leptogorgia* que ocorrem no lado Este do Oceano Atlântico e as que ocorrem no lado

Oeste do Atântico em conjunto com as do Pacífico Este. Conversamente a um evento de especiação transatlântica (vicariância), os dois grupos de *Leptogorgia* podem corresponder a diferentes linhagens com morfologias convergentes ou ancestrais (retidas ao longo da sua evolução). No entanto, análises mais detalhadas são um passo essencial na corroboração destes dados e na inferência de conjecturas definitivas. Tal como em estudos anteriores, a monofilia da família Gorgoniidae e do género *Leptogorgia* foram rejeitadas (teste AU, $p < 0.001$). No que diz respeito às análises filogeográficas, as redes de haplótipos compostos, construidas com o software TCS e com base em dois intrões de genes nucleares (EF1EC1 e i56), não revelaram qualquer nível de estruturação aparente para as populacões de *E. verrucosa* e *L. sarmentosa* ao longo da transição Atlântico-Mediterrâneo. No entanto, o número de indivíduos utilizados foi muito limitado, e apesar de pelo menos um dos intrões (i56) ter mostrado um sinal de diferenciação genética bastante interessante, são necessárias análises mais detalhadas para ter um panorama global dos níveis de diferenciação entre ambas as populacões.

No futuro, os dois marcadores nucleres reportados neste estudo assumem-se como candidatos adequados para melhorar a compreensão da diferenciação e padrões de conectividade das populações de *E. verrucosa* e *L. sarmentosa* ao longo da transição Atlântico-Mediterrâneo, assim como das relações filogenéticas entre as diferentes espécies de *Eunicella* e variantes geográficos de *L. sarmentosa*. Tanto o delineamento de limites ao nível da espécie como a estimação do fluxo de genes entre populações são passos essenciais para a gestão e conservação da biodiversidade.

Palavras-chave: Gorgónia, *Leptogorgia sarmentosa*, *Eunicella verrucosa*, marcadores nucleares, *msh1*, Sistemática molecular, Transição Atlântico-Mediterrâneo, Estrutura populacional

Keywords: Gorgonian, *Leptogorgia sarmentosa*, *Eunicella verrucosa*, nuclear markers, *msh1*, Molecular systematics, Atlantic-Mediterranean transition, Population structure

Octocorals are a diverse group of marine anthozoans (phylum Cnidaria) ubiquitously distributed throughout the world. Their geographic distribution ranges from polar zones to tropics, while bathymetrically they inhabit almost all kinds of reef and hard-bottom substrates across a wide extent of depths (Bayer 1961, 1973; Fabricius and Alderslade 2001). Represented by soft corals, blue corals, sea fans and sea pens (the only group colonizing soft sediments; Bayer 1973), octocorals reach their maximum biodiversity in the shallow waters of the Indonesian-Philippine-New Guinea archipelagos (Fabricius and Alderslade 2001). Whereas soft corals dominate the Octocorallia fauna of the Indo-West Pacific, often occupying from 20 to 50 % of the available coral reefs substrate (e.g., Tursch and Tursch 1982; Dinesen 1983; Fabricius 1997), the gorgonian (sea fans) families Plexauridae and Gorgoniidae represent the great majority of shallow water species from subtropical and tropical regions of both sides of the Atlantic Ocean, and West Americas (Bayer 1953, 1973; Grasshoff 1988). Both soft corals and gorgonians are ecologically important, contributing considerably to the living biomass of coral reefs (Tursch and Tursch 1982) and providing three-dimensional structures that function as microhabitats for several associated organisms (Bayer 1961; Krieger and Wing 2002; Buhl-Mortensen and Mortensen 2005), respectively. Despite their importance, octocorals taxonomy and phylogenetic relationships are still controversial (see further section). Most of the current organization into higher taxonomic levels (Fig.1) is based upon somehow inappropriate morphological characters, which do not reflect their phylogenetic relationships (Bayer 1961, 1981b; France et al. 1996; Bernston et al. 2001; Sánchez et al. 2003a; McFadden et al. 2006a). In fact, some subdivisions may not be valid at all (e.g. Scleraxonia) and the taxonomic organization may be artificial (McFadden et al. 2006a). To date there are over 3200 species of octocorals described worldwide (Williams and Cairns 2009, updated).

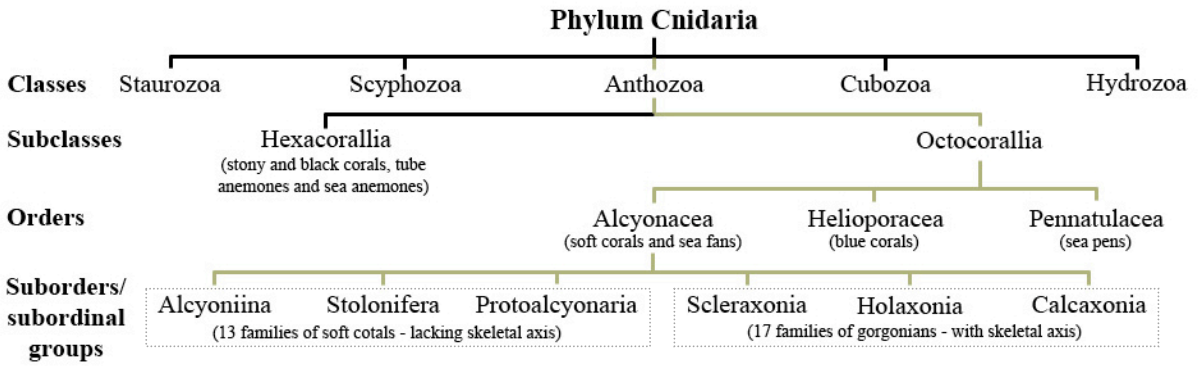

Fig. 1 Taxonomic organization of subclass Octocorallia within the Phylum Cnidaria (mapped by olive lines). Calcaxonia and Holaxonia are classified as suborders while Alcyoniina, Stolonifera, Protoalcyonaria and Scleraxonia represent subordinal groups. Both taxonomic affiliation and number of families of the subclass Octocorallia follows Daly et al. (2007).

2.1 *Biology*

2.1.1 *General anatomy*

The subclass Octocorallia is distinguished from other anthozoans by the presence of polyps bearing eight mesenteries (non-calcareous partitions dividing the gastrovascular cavity of the polyp and joining the pharynx to the body wall, see Bayer et al. 1983) and eight tentacles, one for each mesenterial chamber (Bayer 1961, 1973; Daly et al. 2007). Although several other features such as sedentariness, wide range of colonial organization and integration, pinnated monomorphic (autozooids) or dimorphic (autozooids and siphonozooids) polyps and a skeleton consisting of calcareous sclerites are used to diagnose/identify the subclass (Bayer 1961, 1973), none of them are diagnostic apomorphies†, since they are not exclusive to all octocorals groups (Daly et al. 2007). For example, Alderslade and McFadden (2007) have recently reported the existence of pinnule-less polyps in some octocorals taxa; Bayer and Muzik (1976) described and classified an unusual (rare among octocorals) solitary octocoral species, *Taiaroa tauhou*; and the blue corals (Order Helioporacea) do not produce compacted sclerites, but rather a skeleton of crystalline aragonite (Bayer 1973; Bayer and Muzik 1977). In addition, several molecular studies based either on nuclear (28S rDNA: Chen et al. 1995; 18S rDNA: Bernston et al. 1999; Won et al. 2001) or mitochondrial (16S rDNA: France et al. 1996) markers support the long-standing (e.g. Haeckel 1866 in Daly et al. 2007) monophyly of Octocorallia, corroborating therefore the octamerous polyp structure as a diagnostic apomorphy of the subclass (Daly et al. 2007).

The obsolete Order Gorgonacea (gorgonians), nowadays superseded by the suborders Holaxonia and Calcaxonia, and the subordinal group Scleraxonia (Bayer 1981a; Grasshoff 1999), is typified by the arborescent architecture of its colonies (Bayer 1973). This tree-like structure arises from the presence of a supportive proteinaceous (gorgonin) axial skeleton that can be of different natures. In the Scleraxonian species the different axial structures are composed of free-sclerites deposits and the axis may or not be consolidated. The Holaxonian and Calcaxonian species, on the other hand, are characterized by a solid axial structure, with a soft cross-chambered central core in the former, and solid and calcified in the latter, surrounded by concentric layers of gorgonin with variable degrees of calcification. Both suborders have no free axial sclerites (Bayer 1961, 1973; Williams and Cairns 2009, updated).

The morphology of gorgonian polyps is very consistent among families and the

† Apomorphy – a novel evolutionary trait (derived), which originated as result of mutations or gene transfer in a historical stem lineage or stem population of a monophylum (Wägele 2005).

gastric cavities are typically short. The polyps are united basally by the coenenchyme tissue, a mixture of colonial mesoglea and calcareous sclerites, and connected to each other by a network of stem canals (Bayer 1973). The distribution of polyps is quite variable, ranging from two longitudinal bands arrangement to a scattered disposition along stems and branches. Sclerites form and color vary a lot too, both among species (Bayer, 1961) and among the different layers of coenenchymal cortex (Bayer 1973; Thibaudeau 1983 in Lewis and von Wallis 1991). With the exception for some species within Corallidae and Paragorgiidae families, all other gorgonians have monomorphic polyps, i.e., responsible for all physiological functions (Bayer 1973). A general illustration of gorgonian anatomy is presented in Figure 2.

2.1.2 *Life cycle and reproductive biology*

The life cycle of octocorals is quite simple not implying therefore any complex, although present, larval stage or alternation of generations (Bayer 1973). As anthozoans, octocorals lack the pelagic medusa stage, typical of most of the remaining cnidarians, and adults are found in the sessile polyp form (Collins et al. 2006). Even though there is some dispute on the origin of the medusa stage within Cnidaria, the consistent placement of anthozoans basal to the other cnidarian classes in most phylogenetic analyses (e.g. Bridge et al. 1995; Medina et al. 2001; Collins 2002), and the recent genetic evidence for Stauromedusae (which contains sessile jellyfishes) as the earliest lineage diverging within Medusozoa, suggests that medusa as adult stage is a derived rather than ancestral character

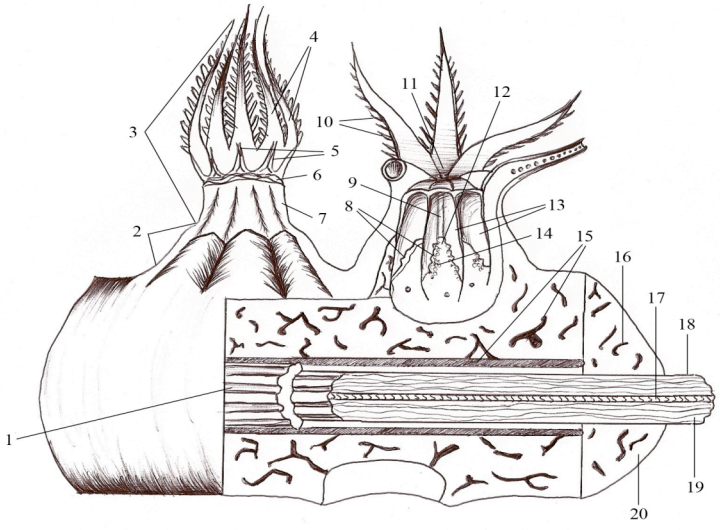

Fig. 2 General anatomy of gorgonians. 1- axial sheath; 2 - anthostele; 3 - anthocodia; 4 - tentacles; 5 - points; 6 - crown; 7 - neck zone; 8 - mesenterial filaments; 9 - pharynx; 10 - pinnules; 11 - peristome; 12 - gastric cavity; 13 - mesenteries; 14 - gonads; 15 - gastrodermal canals; 16 - solenia; 17 - central chord; 18 - axis; 19 - axis cortex; 20 - coenenchyme. Adapted from Bayer et al. (1983).

among cnidarians (Collins et al. 2006). Regarding gorgonians, Weinberg and Weinberg (1979) studied the life cycle of the temperate species *Eunicella singularis* and reported a 4-5 days period since the release of the free larval stage (planula) until its settlement and complete metamorphosis to polyp (see the cited authors for a description). A similar period was also described for the gorgonian *Acabaria biserialis* (Ben-Yosef and Benayahu 1999). As recruits/planulae and juvenile colonies, gorgonians are subject to high mortality rates (Weinberg and Weinberg 1979; Lasker et al. 1998; Coma et al. 2001 in Linares et al. 2007; Bramanti et al. 2005). Possible causes may include: predation on planktonic planulae; settlement of larvae on unfavorable substrata (e.g. soft sediments); and damages due to sediment/water currents abrasion or algae smothering (Weinberg and Weinberg 1979; Coma et al. 2001 in Linares et al. 2007). Adults, on the other hand, are typically long-lived and have low rates of natural mortality (Weinberg and Weinberg 1979; Coma et al. 2004 in Linares et al. 2007; Santangelo et al. 1993 in Tsounis et al. 2006).

The reproductive biology of sessile marine invertebrates plays a crucial role in our understanding of many aspects of their population dynamics and life history (Hughes 1992 in Gutiérrez-Rodríguez and Lasker 2004a). Octocorals can reproduce both sexually and asexually (Coma et al. 1995; Bastidas et al. 2002). Unlike scleractinian corals that have a wide variety of sexual reproductive strategies (for review see Fadlallah 1983), the great majority of octocorals, in particular gorgonians, have been reported as either internal (e.g. Weinberg and Weinberg 1979; Benayahu 1991; Vighi 1972 in Santangelo et al. 2003) or external/surface gonochoric brooders (e.g. Brazeau and Lasker 1990; Coma et al. 1995; Bastidas et al. 2002; Gutiérrez-Rodríguez and Lasker 2004a). Nevertheless, other reproduction strategies such as gonochoric broadcasters (Benayahu and Loya 1986; Brazaeau and Lasker 1990; Lasker et al. 1996; Munro 2004) and the formerly uncommon hermaphroditism have been described (Benayahu 1991; Benayahu 1997 in Ben-Yosef and Benayahu 1999; Schleyer et al. 2004).

The gametogenic cycles of octocorals, i.e., oogenesis and spermatogenesis vary in timing and frequency. For instance, in the temperate internal brooders *Corallium rubrum* and *Eunicella singularis*, while oocytes have a multi-year development, always with two cohorts of immature and mature oocytes, spermaries develop annually. Both maturation cycles end with a short spawning event from late May to July (Tsounis et al. 2006; Ribes et al. 2007). This kind of gametogenic cycles is also present among broadcaster spawners from the family Alcyoniidae (Benayahu et al. 1990 in Dahan and Benayahu 1997). Other species such as the soft corals *Dendronephtya hemiprichi* (Dahan and Benayahu 1997) and *Heteroxenia*

fuscescens (Benayahu 1991) show year-round (i.e. occurring throughout the year) cycles of maturation and spawning. As a general pattern, spermatogenesis is shorter than oogenesis (Coma et al. 1995; Ben-Yosef and Benayahu 1999; Ribes et al. 2007).

Besides gametes development, octocorals can vary in colony size at first reproduction (Benayahu and Loya 1986; Santangelo et al. 2003), size of mature oocytes, number of produced eggs per polyp (Brazeau and Lasker 1989; Coma et al. 1995; Beiring and Lasker 2000) and fertilization success (Lasker et al. 1996; Lasker 2006). Sex ratios of 1:1 are quite frequent (e.g. Benayahu and Loya 1986; Coma et al. 1995; Orejas 2002; Ribes et al. 2007), although male and female skewed ratios have been reported (Brazeau and Lasker 1989, 1990; Ben-Yosef and Benayahu 1999).

Octocorals can reproduce asexually by several different mechanisms, which include fragmentation (Walker and Bull 1983; Lasker 1984; Coffroth and Lasker 1998), colony fission (Farrant 1987), stolonization (Karlson et al. 1996), budding (Dahan and Benayahu 1997) and parthenogenesis mediated planulae (Walker and Bull 1983). Among gorgonians fragmentation and stolonization are the most common (Coma et al. 1995). Clonal propagation through asexual reproduction can enhance the reproductive output of a genetic individual, allowing therefore the establishment of successful genotypes. In fact, for some species asexual propagation has profound effects on populations' structure, dynamics and maintenance (Hughes et al. 1992 in Coffroth and Lasker1998). Nevertheless, it may be quite rare or quantitatively insignificant among temperate gorgonians (e.g. *Paramuricea clavata*, Coma et al. 1995; *Eunicella cavolini*, Weinbauer and Velimirov 1996; *Corallium rubrum*, Russo et al. 1999 in Garrabou et al. 2001; and *Eunicella verrucosa*, Munro 2004).

2.2 *Ecology*

2.2.1 *Nutrition*

Like many other cnidarians, octocorals are benthic suspension feeders obtaining nutrition from suspended particulate matter (Jørgensen 1955 in Sponaugle and LaBarbera 1991). Many of them, in particular gorgonians, act as passive feeders, thereby depending on water current regimes to capture their food (Lasker 1981; Sponaugle and LaBarbera 1991). Although some octocorals, especially the tropical shallow-water species, possess symbiotic zooxanthellae (Ribes et al. 1998), the energetic budget arising from this metabolic pathway may not be enough, and hence heterotrophic supply of nutrients like nitrogen and phosphorous is required (Muscatine & Porter 1977 in Coma et al. 1994; Muscantine 1973 in

Ribes et al. 1998). The balance between these two nutrition pathways is however unclear (Muscantine 1973 in Mitchell et al. 1992).

Cnidarians have been traditionally characterized as carnivorous (Lasker 1981; Brusca and Brusca 1990 in Fabricius et al. 1995b; Hyman 1940 in Ribes et al. 1998). Several studies on octocorals (e.g. Fabricius et al. 1995ab; Ribes et al. 1998, 1999; Migné and Davoult 2000) proved however that such a generalization is incorrect, since phytoplankton is often part of their natural diet. Laboratory manipulations successfully fed octocorals with zooplankton like *Artemia* nauplii, diatoms and brine shrimp cysts (Leversse 1976; Patterson 1984; Migné and Davoult 2000). Nevertheless, *in situ* studies on natural feeding indicate that capture of zooplankton may be restricted to small-size and low-motility preys (e.g. invertebrate eggs), since unlike scleractinians, octocorals have low nematocyst densities and varieties (Mariscal and Bigger 1977 in Fabricius et al. 1995b). Moreover, octocorals can also utilize coral mucus aggregates and detrital particulate organic carbon as nutrition source (Coffroth 1984; Fabricius and Dommisse 2000; Ribes et al. 2003). Feeding rates and efficiencies are quite variable depending upon factors such as water flow velocities, colony orientation to prevailing currents, colony size, polyps surface area and prey densities (Leversee 1976; Patterson 1984, 1991; Sponaugle and LaBarbera 1991; Coma et al. 1994; Fabricius et al. 1995).

2.2.2 *Secondary production and growth rates*

Suspension feeder's communities are extremely active and dynamic boundary systems in terms of energy transfer and succession (Gili and Coma 1998). Among them, gorgonians can play an important role in the energetic exchanges between planktonic and benthic communities (Coma et al. 1994; Mistri and Ceccherelli 1994). Reported estimates of secondary production, though scarce, indicate values ranging from 0.26 to 10.5 g ash-free dry mass (AFDM) m^{-2} yr^{-1} for several species of gorgonians (Mitchell et al. 1992; Mistri and Ceccherelli 1994; Weinbauer and Velimirov 1995).

Intraspecific growth rates have long been assumed as extremely variable due to intrinsic and external factors (Weinberg and Weinberg 1979; Yoshioka and Yoshioka 1991; Marschal et al. 2004). In fact, even long-term observations (hence statistically significant) showed large variations in individual growth rates (e.g. Yoshioka and Yoshioka 1991). In the Mediterranean species *Corallium rubrum* for instance, Marschal et al. (2004) found a 6-fold range, i.e., between 0.1 and 0.6 mm yr^{-1}, of the intraspecific growth rates (estimated through basal diameter measurements). Interspecific growth rates, on the other hand, can be similar

for the same regions. For example, Weinberg and Weinberg (1979) obtained average growth rates (length increment from the foothold to the most distant branch tip) of 22.4 mm yr^{-1} for *Eunicella singularis*, 28.5 mm yr^{-1} for *Lophogorgia ceratophyta* (now *Leptogorgia sarmetosa*, Grasshoff 1992) and 12.5 mm yr^{-1} for *Paramuricea clavata* in the Mediterranean Sea; for the same region Mistri and Ceccherelli (1994) reported rates of 27-30 mm yr^{-1} for *P. clavata*; in the shallow-waters of southwest Puerto Rico (Caribbean) Yoshioka and Yoshioka (1991) found mean growth rates varying between 14 to 26 mm yr^{-1} for the majority of the 13 gorgonian species monitored. All these studies registered the occurrence of negative growth, probably due to external factors such as abrasion and or predation.

2.2.3 *Ecological interactions*

Octocorals are well known for housing vast assemblages of associated organisms with variable degrees of intimacy (Bayer 1961; Wendt et al. 1985). Regardless of the habitat, e.g. tropical shallow-waters or temperate deep-waters, understanding the dependency of such associations is of great value in order to access their ecological importance and develop sustainable management strategies (Buhl-Mortensen and Mortensen 2004). Commensalism, perhaps the commonest kind of interaction among octocorals, may encompass epizoites such as asterozoans echinoderms (brittle stars), galatheid decapods, amphipods, isopods, anomuran crustaceans, sessile ctenophores and cirripedes (Bayer 1961; Wendt et al. 1985; Tahera 2001; Buhl-Mortensen and Mortensen 2004; Myers and Hall-Spencer 2004; Lin et al. 2007; Kumagai 2008); mesobionts like hydroids and syllid polychaetes (Fig.3c, d) partially or completely embedded in the octocoral tissue (Bayer 1961; San Martín and Nishi 2003; Gili et al. 2006); or other associates like fishes, shrimps and crabs taking shelter among the branches (Bayer 1961; Wendt et al. 1985; Buhl-Mortensen and Mortensen 2004).

Poecilostomatoid copepods (Bayer 1961; Buhl-Mortensen and Mortensen 2004; Conradi et al. 2004), along with snails of the genus *Rapa* and barnacles of the order Ascothoracica (Bayer 1961), are notorious parasites inhabiting octocorals. With respect to pathogens affecting marine taxa and thus octocorals, even information on basic properties such as host specifies and transmission mode is lacking (Kim and Harvell 2004). Still, Geiser et al. (1998) and Kim and Harvell (2004) reported a fungal epizootic (aspergillosis) affecting gorgonians of the genus *Gorgonia*; Harvell et al. (2001) isolated a cyanobacterium (*Scytonema* sp.) as the probable causative agent of necrosis in the Caribbean gorgonian *Briareum asbestinum*; Martin et al. (2002) analyzed the ability of 11 *Vibrio* strains to induce tissue necrosis in *Paramuricea clavata* and *Eunicella cavolini*, and highlighted its potential to

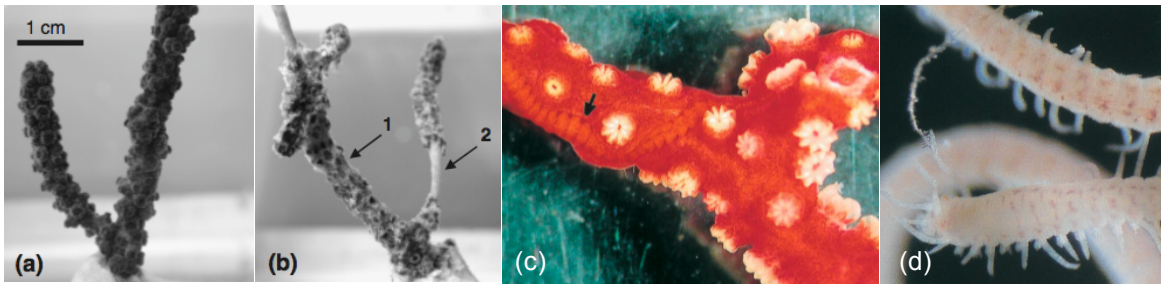

Fig. 3 Healthy (a) and diseased (b) colonies of *Paramuricea clavata* from aquarium infection experiments; (b1) damaged coenenchyme and (b2) denuded skeletal axis (Bally and Garrabou 2007). Commensalist polychaete *Alcyonosyllis glasbyi* embedded in the gorgonian *Melithaea flabellifera* indicated by the arrow (c) and a non-type specimen of *A. glasbyi* (d) (Martín and Nishi 2003).

enhance necrosis crisis at increased seawater temperatures like the ones noted during the 1999 mass mortality event in the French Mediterranean coast; Hall-Spencer et al. (2007) obtained similar results with *Vibrio* bacteria isolated from diseased *Eunicella verrucosa* at the cold waters of SW England; and the Indo-Pacific scleractinian coral pathogen *Vibrio coralliilyticus*, was shown for the first time to be involved in disease outbreaks of a temperate octocoral, the gorgonian *P. clavata* (Fig.3a, b; Bally and Garrabou 2007).

2.2.4 *Predation*

Predation on octocorals is limited to a few groups of specialized predators. Generalist predators, particularly fishes, are incapable of consuming octocorals because of their production of secondary metabolites that function as feeding deterrents (e.g. van Alstyne and Paul 1992; Gerhart and Coll 1993; Epifanio et al. 2007; for review see Coll 1992 or Sammarco and Coll 1992). Sclerites have also been suggested to have deterrence potential (Harvell et al. 1988), and along with chemical metabolites may form the baseline of octocoral evolutionary success in areas of high predation such as coral reefs (Tursch et al. 1978 in Kelman et al. 1999; Sammarko and Coll 1992). Moreover, it is also noteworthy that these chemicals can effectively inhibit the settlement and development of fouling algae and sessile epifauna on gorgonians (Targett et al. 1983; Gerhart et al. 1988).

Although few, there are some organisms that can feed on octocorals. Among them, the ovulid gastropods *Cyphoma gibbosum* (Gerhart 1986, 1990; Lasker et al. 1988; Ruesink and Harvell 1990) and *Cyphoma signatum* are respectively generalist and specialist (*Plexaura* spp.) predators feeding on gorgonians in the Caribbean (Ruesink and Harvell 1990). Other known predators include nudibranchs such as *Tritoniopsis elegans*, *Phyllodesmium poindimiei* (Wagner et al. 2007) and *Tritonia bollandi* (Smith and Gosliner 2003), and the bristleworm *Hermodice carunculata* (Souza et al. 2007).

2.3 *State of the art*

2.3.1 *Taxonomy and phylogeny*

Octocorals taxonomy and phylogenetic relationships are poorly understood, and subject to little consensus among taxonomists (Bayer 1961, 1981b; France et al. 1996; Bernston et al. 2001; Sánchez et al. 2003a; McFadden et al. 2006a). Several factors account for this condition, particularly the intra and interspecific variability of morphological characters (e.g. sclerites) used to diagnose species (Bayer 1961); scarcity of useful morphological features and widespread homoplasy (Williams 1997 in McFadden et al. 2006a); lack of paleontological evidence (Bayer 1973); limited resolution of molecular markers used to date at the species level (France et al. 1996; Bernston et al. 2001; Sánchez et al. 2003a; McFadden et al. 2006a); and low levels of mitochondrial genes variability in octocorals (France et al. 1996; France and Hoover 2001, 2002; McFadden et al. 2004).

During most of the last century, the subclass Octocorallia was subdivided into seven orders: Helioporacea (blue corals), Pennatulacea (sea pens), Protoalcyonaria, Stolonifera, Telestacea, Alcyonacea and Gorgonacea (soft corals and sea fans) (e.g. Hyman in Bernston et al. 2001). However, only two of these groups are clearly distinct in terms of morphology. The order Helioporacea is unique in producing a massive and rigid skeleton composed of aragonite crystals completely lacking sclerites (Bayer and Muzik 1977); and the order Pennatulacea, the most complex group with respect to functional specialization of polyps, is characterized by the differentiation of a primary polyp (oozooid) into a barren stalk (peduncle) that anchors the colony to soft sediments and a distal rachis from which secondary polyps arise (Bayer 1961, 1973). All the remaining orders fall into intergrading forms for which a concise ordinal boundary is very difficult to define solely on the basis of morphological structures (Bayer 1973, 1981a). As a consequence, Bayer (1981a) assigned these five orders to the new Protoalcyonaria, Stolonifera, Alcyoniina, Scleraxonia and Holaxonia suborders, combining all of them into a single order, Alcyonacea. Some families of the suborder Holaxonia (gorgonians) that do not form a chambered central core were later reassigned to the suborder Calcaxonia (Grasshoff 1999). The present taxonomic subdivision of order Alcyonacea is still lacking a clear morphological boundary between the six suborders. As a matter of fact, the suborders Protoalcyonaria, Stolonifera, Alcyoniina and Scleraxonia are rather classified as subordinal groups since they are linked by intermediate forms (Fabricius and Alderslade 2001). Moreover, unless the monophyly of these groups can be proved (through molecular tools for instance), the present taxonomic subdivision is very unlikely to persist.

Although the monophyly of the subclass Octocorallia is well supported by molecular data (Chen et al. 1995; Bernston et al. 1999; Won et al. 2001; France et al. 1996), few phylogenetic analyses found molecular support for the monophyly and taxonomic grouping of the previously described orders or suborders. Instead, most of the molecular analyses conducted to date recovered three clades, for which the taxa clustering varies according to the genetic markers utilized, and for which no morphological synapomorphy† have been identified (Sánchez et al. 2003a; Bernston et al. 2001; McFadden et al. 2006a; Daly et al. 2007). Bernston et al. (2001) and more recently McFadden et al. (2006a) presented the most complete data sets. The former authors used nuclear 18S rDNA sequences and did not recover the monophyly of any of the three proposed octocorals suborders (Helioporacea, Pennatulacea and Alcyonacea [Fig.4 left]). They did however recover monophyly for Pennatulacea, and separately for some species of families Alcyoniidae (Alcyoniina), and Coralliidae and Paragorgiidae (Scleraxonia), which all present dimorphism of polyps morphology. Under these results, the authors suggested that the most logical evolutionary explanation would be that dimorphism arose multiple times within Octocorallia, since these dimorphic species share few other morphological traits and are also separated with genetic data.

McFadden et al. (2006a), on the other hand, with the combination of two mitochondrial protein-coding markers (ND2 and the *mutS* homolog *msh1*), obtained the order Pennatulacea to be monophyletic but nested within Calcaxonia. The remaining suborders/subordinal groups were distributed throughout the two other clades (Fig.4 right). Both Calcaxonia and Holaxonia were well supported as discrete entities, and the phylogeny consistent with a single evolutionary origin of their skeletal axis. This was not however the case of the subordinal groups Scleraxonia, Alcyoniina and Stolonifera. The failure of both mitochondrial and nuclear markers used until now to resolve some phylogenetic relationships within Octocorallia, particularly the deeper nodes and its root, led McFadden et al. (2006a) to suggest that octocorals experienced a rapid radiation, and that the morphological characters used to define Scleraxonia, Alcyoniina and Stolonifera are not appropriate since they appear to be prone to homoplasic events.

The mitochondrial DNA (mtDNA) of octocorals show low rates of nucleotide substitution when compared to other animals (e.g. France et al. 1996; France and Hover 2002; McFadden et al. 2004). Actually, this condition is typical of anthozoans species, even tough octocorals show some of the lowest values (for review see Shearer et al. 2002). The

† Synapomorphy – homologous evolutionary novelty which can be used as evidence for a sister-group relationship and that evolved in the last common stem species of these sister taxa (Wägele 2005)

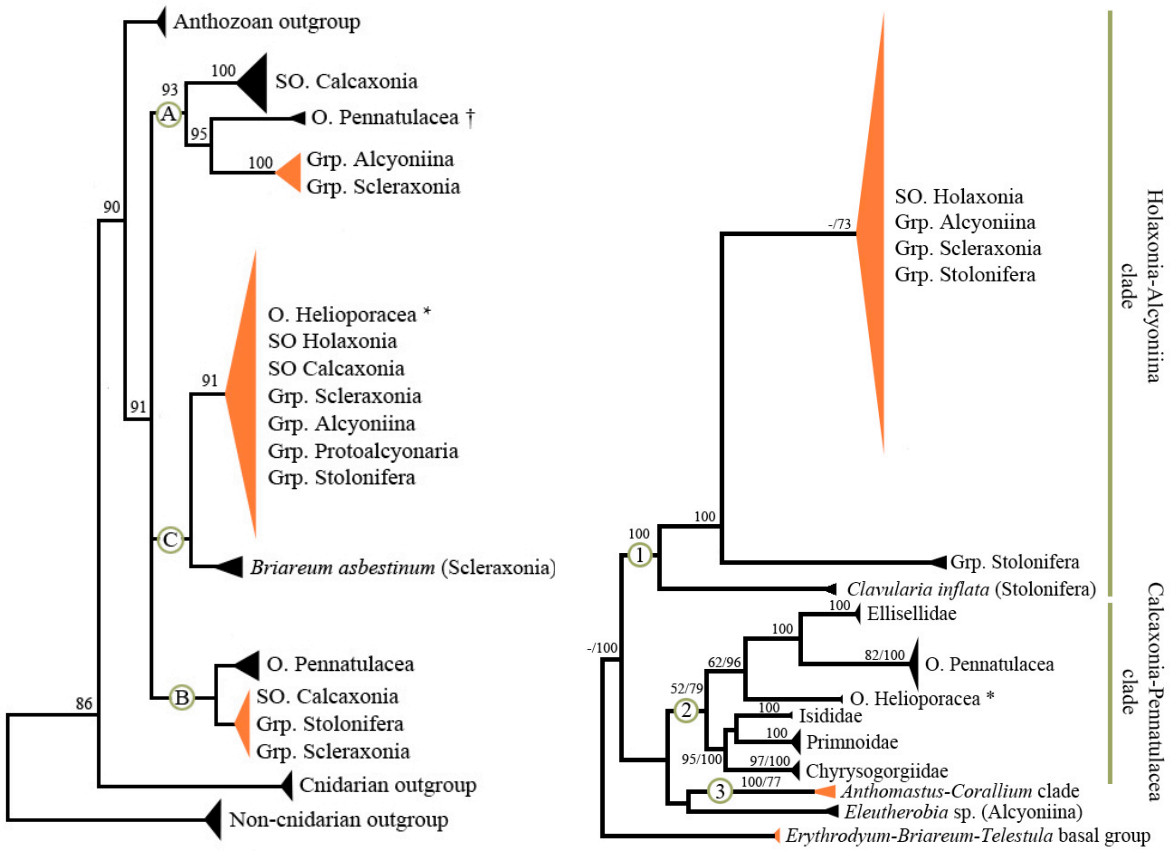

Fig. 4 Phylogenetic relationships among the anthozoan subclass Octocorallia. Left: from Bernston et al. (2001) based on nuclear 18 rRNA sequences; Right: from McFadden et al. (2006) based on mitochondrial protein-coding sequences (ND2 and *msh1*). Numbers at nodes represent percentage bootstrap support (maximum likelihood) and posterior probabilities (Bayesian likelihood: right tree), respectively. Circled letters and numbers indicate the major groups discussed by the authors; orange clades represent groups of mixed taxa; * Order represented by a single species; † single pennatulacean species (*Umbellula* sp.) grouping outside clade B. Branches with large number of species were pruned to facilitate presentation but retain their principal elements.

slow rate of mitochondrial genes evolution found in octocorals may be related to the presence in their mtDNA of a mismatch repair gene (*MSH1*), homolog to the *mutS* gene found in the bacterial *MSLH* mismatch repair pathway (Pont-Kingdon et al. 1995, 1998). This gene was first identified by Pont-Kingdon et al. (1995) in the mitochondrial genome of the soft coral *Sarcophyton glaucum*, but later confirmed to be present in all octocorals tested and in no other metazoans mtDNA (Culligon et al. 2000; France and Hoover 2001; McFadden et al. 2006a; Ledoux et al. unpublished; this study).

Eukaryotes may have at least six different *MSH* families, which apart from octocorals *msh1* are encoded by the nuclear genome (Culligan et al. 2000). In the light of such a fact, the simplest explanation would be that the gene was originally present in the nucleus and later transferred to the mitochondrial genome of octocorals after their divergence from Hexacorallia (Pont-Kingdon et al. 1998). However, the authors did not ruled out the other way around or the gene loss in all eukaryotes. In fact, the monophyly of *MSH* gene families

(both nuclear and mt*msh1*), and the close branching of eukaryotic *MSH1* and eubacteria *Rickettsia prowasekii mutS* sequences, suggests that all nuclear *MSH* families derive from the mt*MSH1*, itself the descent of eubacterial *mutS* genes acquired during endosymbiotic events (Culligan et al. 2000). More recently, the discovery of the first virally encoded MutS homolog, found in the 1.2 Mb genome of the giant Mimivirus, clustering at an intermediate position between a bacterial group of the epsilon division (Nitratiruptor/Sulfurimonas/ Arcobacter) and octocorals, suggests the common origin of this MutS homologues and the possible central role of Mimivirus as the vector of bacterial MutS gene into octocorals mitochondrial genome after their divergence from hexacorals (Claverie et al. 2009).

2.3.2 *Population genetics and phylogeography*

In octocorals, population genetics and phylogeography are among the less studied fields to date. Both research areas rely on the use of molecular markers with polymorphism levels high enough to differentiate populations, which in many organisms have been achieved through mitochondrial markers. Because mtDNA usually tends to have higher rates of mutation than nuclear DNA (nDNA) and is maternally inherited (non-recombinant), it is frequently the most suitable choice for population genetics and phylogeography (Avise 1994; Avise 2000; Beebee and Rowe 2008). Nevertheless, these characteristics and the use of mtDNA as marker of molecular diversity may be discussed (Galtier et al. 2009). For example, octocorals and anthozoans in general, exhibit low mtDNA sequence variability both within species and among species of the same genus (e.g. France et al. 1996; France and Hoover 2002; McFadden et al. 2006b) making it therefore an inappropriate choice for this kind of studies. For instance, Calderón et al. (2006) found no polymorphism for populations of four Mediterranean gorgonian species with the mitochondrial COI gene. Another potential marker, the nuclear ITS2, revealed too much intra-individual variability for the single species analyzed with this marker (*Eunicella cavolini*). Constantini et al. (2007a) used ITS1 for the phylogeography of red coral (*Corallium rubrum*) but they didn't analyze the potentially misleading consequences of intra-individual variability.

Among gorgonians, though few, there are some studies that used molecular markers with higher polymorphism. In one such example, Gutiérrez-Rodríguez and Lasker (2005 *corrigendum*, after 2004b) found high levels of genetic structure and differentiation among Bahamian populations of the gorgoniid (Gorgoniidae) *Pseudopterogorgia elisabethae* (concordant with its life-history traits, i.e., limited dispersal of reproductive propagules, and in part with the geography of Bahamas) by using three microsatellite loci. Generalized

genetic structuring patterns were also found with microsatellite loci for the temperate, and also brooder, *Corallium rubrum* across several different spatial scales in the Mediterranean Sea (Constantini et al. 2007ab; Mokhtar-Jamaï et al. 2009; Ledoux et al. in press). Mokhtar-Jamaï et al. (2009) preliminary results on sequences of a nuclear marker (intron of the Elongation Factor 1 gene), indicated however an absence of deep phylogeographic structure, indicative of a lack of long-term isolation between the most distant populations. Conception et al. (2008) used an intron of a nuclear gene as well, which enabled them to identify a potential cryptic species previously assigned to *Carijoa riisei*, and to explain much of the variation (35% compared to <10 % of mtDNA) between Pacific and Atlantic samples of this species. Finally, Gutiérrez-Rodríguez et al. (2009) analysed the variability in ITS1 and ITS2, and found significant genetic structure and differentiation in populations of *P. elisabethae* across Caribbean (concordant with previous microsatellite data), but no clear correspondence between genetic and morphological variation. The absence of significant genetic differentiation between morphological variants has been reported in hexacorals as well (e.g. intron of nDNA: Mackenzie et al. 2004). Unlike Constantini et al. (2007) ITS2 analysis, Gutiérrez-Rodríguez et al. (2009) analyzed the intra-individual variability of the multi-copy ITS, although the question of intra-individual variability for *P. elisabethae* didn't seem so problematic as for *C. rubrum*.

2.3.3 *The Atlantic-Mediterranean transition: a phylogeographic perspective*

From the standpoint of phylogeography, the transition between Atlantic Ocean and Mediterranean Sea has been widely analyzed in several marine taxa: fishes (Naciri et al. 1999; Daeman et al. 2001; Gysels et al. 2004; Bargelloni et al. 2005; Charier et al. 2006); mammals (Bérubé et al. 1998; Natoli et al. 2005); turtles (Encalada et al. 1996, 1998); sponges (Duran et al. 2004ab); crustaceans (Zane et al. 2000; Stamatis et al. 2004; Remerie et al. 2006); mollusks (Pérez-Losada et al. 2002; Wilke and Pfenninger 2002; Nikula and Väinölä 2003); chaetognath (Peijnenburg et al. 2004); seagrasses (Coyer et al. 2004; Alberto et al. 2008); and echinoderms (Baus et al. 2004; Caldéron et al. 2008). However there is no clear pattern of biological characteristics which might explain the Atlantic-Mediterranean transition, as there are contradicting results. Whereas for some taxa there are a significant genetic differentiation between Mediterranean and Atlantic populations, suggesting the presence of phylogeographic breaks (e.g. Strait of Gibraltar and Almeria-Oran Front), for others there are few or no signs of population structure at all. In fact, even closely related species display this discordant differentiation pattern (Borsa et al. 1997; Patarnello et al.

2007). Regarding corals, particularly gorgonians, to my knowledge there is no single study analyzing the phylogeographical patterns across East Atlantic and Mediterranean Sea.

2.3.4 *Case study: genera* Eunicella *and* Leptogorgia *(Gorgoniidae)*

The holaxonian family Gorgoniidae contains several important genera of shallow-water octocorals (Bayer 1961; Grasshoff 1988). The family is characterized by the small dimension of it sclerites (< 0.3 mm long) that can be regularly sculpted or ornate, and by the thin coenenchyme (Bayer 1961; Grasshoff and Alderslade 1997 in Sánchez 2007). Several authors have already recognized the ambiguity surrounding the family description, which include diagnostic traits that are not well defined at the generic level and may therefore lead to the classification of some species in either Gorgoniidae or Plexauridae (Sánchez et al. 2003b; Aguilar and Sánchez 2007; Sánchez 2007). Additionally, molecular analyses have strongly supported the paraphyly of Gorgoniidae and the basal placement of the genus *Plexaurella* (Plexauridae) relative to gorgoniids (Sánchez et al. 2003b; Wirshing et al. 2005; McFadden et al. 2006a). Based on ITS2 sequences and their predicted RNA secondary structure, Aguilar and Sánchez (2007) presented new phylogenetic hypotheses for most gorgoniids, identifiyng four distinct clades that even displaying competing conjectures for the relationships of basal nodes, conflicted with current taxonomy. Although these results were later corroborated with morphological synapomorphies from surface layer sclerites (Fig. 5; Sánchez 2007), the authors analyzed relatively few species and didn't analyze the intra-individual variability of the marker (ITS2).

Within the family Gorgoniidae, the genera *Eunicella* and *Leptogorgia* dominate the East Atlantic octocorals species (Grasshoff 1992). The taxonomy of both genera is based mainly on the combination of colonies growth form and sclerites shape, but the coloration patterns of colonies and sclerites can also be used in *Leptogorgia*. *Eunicella* species are distinguished by their club-shaped sclerites, which form dense pavements at the surface of

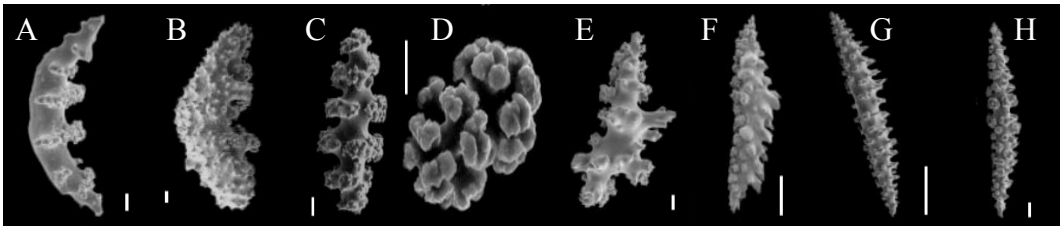

Fig. 5 Scanning electron microscopy images of different types of sclerites from the surface coenenchyme layer of gorgoniid species. (A, B) scaphoid sclerites of *Pseudopterogorgia acerosa* and *Pterogorgia citrina*; (C, D) capstan sclerites of *Leptogorgia virgulata* and *Pacifigorgia stenobrochis*; (E, F) asymmetrical spiny sclerites of *Muriceopsis sulphurea*; (G, H) long and spiny sclerites of *Filigorgia angolana* and *Filigorgia ridouri*. Scale bars: (A-D) 0.01 mm; (E, H) 0.02 mm; (F, G) 0.1 mm (Sánchez 2007).

coenenchyme, and by the straight and warty spindles found at interior layers. Although most species are ramified and attached to hard substrates some are free living and unbranched (Grasshoff 1988, 1992). The genus *Leptogorgia* is characterized by having a coenenchyme with spindles symmetrically ornamented by whorls of tubercles (some species have asymmetrical spindles) and by the diversity of growth forms that its colonies display (e.g. branching in one plan and dichotomous branching). Colony branches do not anastomose and like *Eunicella* most species grow attached to hard bottoms by a holdfast. Unlike other goorgoniids, in *Leptogorgia* spindle types differ slightly across different layers of tissue (Grasshoff 1988).

In his revision of the gorgonian fauna of Europe and West Africa, Grasshoff (1992) described 19 species of *Eunicella* and 29 of *Leptogorgia* in this area. Five of the 29 *Leptogorgia* species have recently been assigned to the genus *Filigorgia* Stiasny, 1937 (Sánchez 2007). Within the genus *Eunicella*, *Eunicella verrucosa* (Fig.6A) is one of the most common shallow-water species of European ecosystems, which may include other species

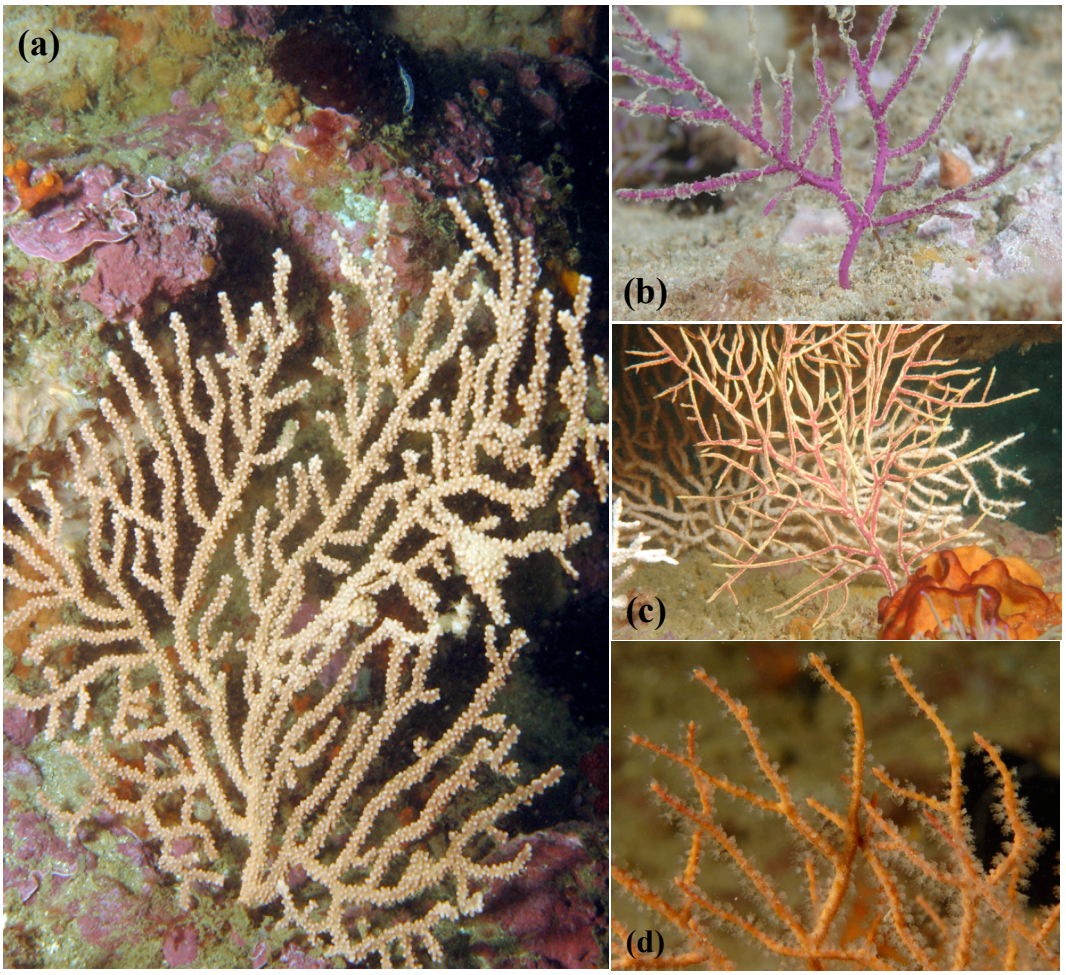

Fig.6 European gorgoniids in their natural habitat: *Eunicella verrucosa* (a), and Portuguese (b, c) and Mediterranean (d) variants of *Leptogorgia sarmetosa*. Photos: courtesy of Pedro A. Neves (a, b, and c) and F. Zuberer (d).

such as *Eunicella gazella*, *Eunicella labiata*, *Eunicella singularis* and *Eunicella cavolini*. Although the last two species have been reported to be restricted to the Mediterranean Sea (Grasshoff, 1992), *E. singularis* has been sampled in the South coast of Portugal (Vieira, 2008). Contrarily to other *Eunicella* species, *E. singularis* presents some photosynthetic symbionts (*Symbiodinium sp.*). Inside *Leptogorgia sarmentosa* there are several potential different forms flourishing across the European coasts, such as the *sarmentosa* and *lusitanica* forms (Carpine and Grasshoff 1975; Grasshoff 1988 [Fig.6B, C, D]). Regarded as different species for many years (e.g. Carpine and Grasshoff 1975) the nomenclature status of these two entities is controversial and insecure because of their resemblance (Grasshoff 1992). Despite the fact of being much alike *lusitanica* differs from *sarmentosa* by the single-plane branching pattern of its colonies and by the higher number of color variants. Differences between sclerites are poorly marked but in average *lusitanica* sclerites are not so slender and small. Whereas *sarmentosa* is commonly found in the Mediterrean Sea, *lusitanica* is found in the Ibero-Morroco region, Portugal and Bay of Biscay (Carpine and Grasshoff 1975; Grasshoff 1988). In 1992 Grasshoff concluded that according to his taxonomic analyses both forms should be apprehended as regional subspecies and assigned to a single species, *Leptogorgia sarmentosa*.

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Phylogeography and phylogeny of European gorgoniids (Gorgoniidae): genera *Eunicella* and *Leptogorgia*

December 2009

Abstract Gorgonians are marine anthozoans (Octocorallia) ubiquitously distributed worldwide with important ecological functions. Despite their recognizable relevance, the phylogenetic relationships between species are subject to intense debate and even the recognition of species boundaries is often difficult to assess. Here, we analyze the genetic differentiation among European gorgoniids of genera *Eunicella* and *Leptogorgia*, and present some preliminary results on the phylogeography of Atlantic and Mediterranean populations of *Eunicella verrucosa* and *Leptogorgia sarmentosa*, as well as on the phylogenetic position of these species. Genetic data from four mitochondrial loci lacked variability to differentiate species within *Eunicella*, as well as to differentiate the Mediterranean and Atlantic variants of *L. sarmentosa*. A maximum-likelihood analysis (mt *msh1*) focusing on higher taxonomic levels and using more species, including *Leptogorgia* spp., revealed a significant transatlantic divergence between *Leptogorgia* species from East Atlantic, and West Atlantic plus East Pacific. Conversely to a transatlantic speciation event (vicariance) the two *Leptogorgia* groups can correspond to different lineages with convergent or ancestral retained morphologies, though further detailed analyses should be done. Similarly to previous analyses the monophyly of Gorgoniidae and *Leptogorgia* was rejected (AU test, $p < 0.001$). Composite haplotype networks constructed with TCS and based on two nuclear markers did not show any apparent population structuring across the Atlantic-Mediterranean transition for *E. verrucosa* and *L. sarmentosa*, but further analysis are required since the number of individuals used was limited and one of the loci (i56) displayed an interesting signal. In the future, the two nuclear markers reported here are likely to improve our understanding of the differentiation and connectivity patterns of *E. verrucosa* and *L. sarmentosa* populations across the Atlantic-Mediterranean transition, as well as on the phylogenetic relationships between *Eunicella* species, poorly defined at the morphological level. Both species delimitation, and estimation of population's gene flow are essential steps for biodiversity management and conservation.

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Introduction

In the past few decades, the widespread decline of marine biodiversity has reached alarming levels. Broadly acknowledged to result from anthropic pressures, this global trend is best-exemplified by the regression of coral reefs, which rapidly became a top priority for conservation agendas. Gorgonians, marine anthozoans (subclass Octocorallia) ubiquitously distributed worldwide (Bayer 1961), are ecologically important and well known for housing vast assemblages of associated organisms with variable degrees of intimacy (e.g. Wendt et al. 1985; Buhl-Mortensen and Mortensen 2004, 2005). Moreover, gorgonian communities are of great scenic value for underwater activities such as scuba diving (Sánchez et al. 2003b) and some species are important sources of pharmacological products (Mayer et al. 1998; Bruckner 2002). As a whole, gorgonians and octocorals in general represent a considerable value of environmental services.

Despite their recognizable relevance, octocorals taxonomy and phylogenetic relationships are far from being well understood and subject to little consensus among taxonomists (Bayer 1961; France et al. 1996; Bernston et al. 2001; Sánchez et al. 2003a; McFadden et al. 2006a; Aguilar and Sánchez 2007; Sánchez 2007). In fact, even the recognition of taxonomic boundaries at

species level is difficult and many species are split into local variants over wide geographical ranges (Grasshoff 1992, 2001). Several factors account for both these conditions, particularly the intra and interspecific variability of morphological characters (e.g. sclerites) used to diagnose species (Bayer 1961); scarcity of useful morphological features and widespread homoplasy (Williams 1997 in McFadden et al. 2006a); lack of paleontological evidence (Bayer 1973); limited resolution of molecular markers used to date at the species level (France et al. 1996; Bernston et al. 2001; Sánchez et al. 2003a; McFadden et al. 2006a); and low levels of mitochondrial genes variability in octocorals (France et al. 1996; France and Hoover 2001, 2002; McFadden et al. 2004).

The family Gorgoniidae contains several important genera of shallow-water octocorals (Bayer 1961, Grasshoff 1988), among which *Eunicella* and *Leptogorgia* contain most species of East Atlantic biotopes. The taxonomy of both genera is based mainly on the combination of colonies growth form and sclerites shape, but the coloration patterns of colonies and sclerites can also be used in *Leptogorgia* (Grasshoff 1988, 1992). Within the genus *Eunicella* there are five species inhabiting the European coasts for which few morphological diagnostic characters exist (Table 1). *Eunicella verrucosa* is the most common species co-occurring throughout the Mediterranean Sea and Atlantic Ocean. Among the genus *Leptogorgia*, on the other hand, *Leptogorgia sarmentosa* is the only species present across the European borders of both Mediterranean and Atlantic (Grasshoff 1992). According to the last comprehensive revision of European gorgoniids (Grasshoff 1992), *L. sarmentosa* is split into four geographical nominate subspecies/ variants differing mostly on the coloration patterns: *L. sarmentosa* for the terra-cotta brown and yellow variants of Mediterranean; *L. lusitanica* for the yellow and purple variants of Portugal; *L. rhizomorpha* for the brown varieties of Biscay; and *L. alba* for the white varieties of the Atlantic coast of Morocco. Although the author found no morphological support for the assignment of these variants to the species level, *L. lusitanica* and *L. sarmentosa* have been considered different species for many years. Despite the fact of being much alike the former differs from the latter by the single-plane branching pattern of its colonies and by the higher number of color variants. Differences between sclerites are poorly marked but in average *L. lusitanica* sclerites are not so slender and small (Carpine and Grasshoff 1975; Grasshoff 1988).

Table 1 Diagnosing characters of European gorgoniids of the genus *Eunicella*.

Species	Diagnostic characteristics [a]
Eunicella verrucosa Pallas, 1766	White or pinkish, densely ramified w/ short branches; protuberant polyps; sclerites: balloon clubs w/ round spiny edges (lateral view)
Eunicella gazella Studer, 1878	Polyps not or little protuberant; sclerites: clubs broadly circular (lateral view) w/ flat or having small humps in the apical edge
Eunicella labiata Thomson, 1927	Brownish violet/black, protuberant polyps w/ bright edges; sclerites: balloon clubs w/ nearly parallel edges (lateral view)
Eunicella singularis Esper, 1791	Long straight branches; polyps a little protuberant; sclerites: clubs narrowly circular (lateral view) w/ exterior face completely flat; photosynthetic symbionts
Eunicella cavolini (Koch, 1887)	Yellow to light-red, densely branched, polyps little or few protuberant; sclerites: clubs widely circular w/ fine humps in exterior surface

[a] Following Grasshoff (1992)

The correct assessment of species boundaries is crucial not only for understanding the biogeographical patterns of organisms and associated speciation processes, but also for conservation practices (Knowlton 1993; Palumbi 1996; Knowlton and Weigt 1997; Eytan et al. 2009). Gorgonians, and anthozoans in general, are a clear example of organisms in which the use of alternative characters other than morphological is needed. In this sense, genetic analyses can provide some tools for species delimitation (Knowlton 2000; Eytan et al. 2009). However, unlike most animal groups where mitochondrial DNA (mtDNA) generally displays an appropriate resolution and suitable properties for species boundaries inference, population genetics and phylogeography (Avise 1994; Avise 2000; Eytan et al. 2009; but see Galtier et al. 2009 for a critical review on mtDNA prperties), octocorals mtDNA lack polymorphism to reveal population or species differentiation in many genera (France and Hoover 2001, 2002; McFadden 2004; but see Sánchez et al. 2003b, McFadden et al. 2006b and Prada et al. 2008 for exceptions). For instance, among temperate gorgonians of Mediterranean, the mitochondrial COI gene, designated for use as universal DNA barcode in animals (Hebert et al. 2003ab), failed to identify both intra and interspecic (for congeners species) variation at distant regions (Calderón et al. 2006; Mokhtar-Jamaï et al. 2009). Low levels of intraspecific polymorphism and slow evolution of the mitochondrial genome are not however exclusive to octocorals, and have been reported in other anthozoans (see Shearer et al. 2002 for an overview), plants (Wolfe et al. 1987) and sponges (Duran et al. 2004; Wörheide 2006). Among anthozoans, this situation is clearly stronger in octocorals, a fact that could be related to the presence of a mismatch repair gene (*MSH1*), homolog to the *mutS* gene found in the bacterial MSHL mismatch pathway, and exclusive to octocorals mtDNA (Pont-Kingdon et al. 1995, 1998; Culligon et al. 2000; France and Hoover 2001; McFadden et al. 2006a; Ledoux et al. unpublished). However this hypothesis has not been tested.

Nuclear markers are then necessary for assessing both species and population boundaries. Few studies have adopted this alternative approach on gorgonians, but their results so far appear promising. For example, microsatellite loci revealed strong genetic structuring among populations of the Bahamian *Pseudopterogorgia elisabethae* (Gutiérrez-Rodríguez and Lasker 2004b)

and the Mediterranean *Corallium rubrum* (Constantini et al. 2007ab; Mokhtar-Jamaï et al. 2009; Ledoux et al. in press) at different spatial scales; ITS's proved to be useful for phylogeography analysis and to assess the congruence between genetic and morphological variation at species level (*P. elisabethae*: Gutiérrez-Rodríguez et al. 2009), but the question of concerted evolution of ITS is sometimes problematic (Calderón et al. 2006); and two introns of nuclear genes suggested respectively, lack of long term isolation for populations of *C. rubrum* across its Mediterranean distribution (Mokhtar-Jamaï et al. 2009), and a potential cryptic species previously assigned to *Carijoa riisei* (Conception et al. 2008).

Here, we analyze the intra and interspecific polymorphism of four mitochondrial markers in European gorgoniids of genera *Eunicella* and *Leptogorgia*, as well as the sequence variability of three nuclear gene introns for Mediterranean and Atlantic populations of *E. verrucosa* and *L. sarmentosa*. Even though the geological history and current hydrographic patterns of the Atlantic-Mediterranean transition are relatively well known, this transition does not correspond to a clear phylogeographic break for a number of organisms (see Borsa et al. 1997, Patarnello et al. 2007). In fact, even closely related species display this discordant differentiation pattern (e.g. Sparids: Bargelloni et al. 2003, 2005). The particular goals of the present study were: (1) to use mtDNA sequences of European gorgoniids in order to precise species limits, (2) clarify through the usage of nuclear markers the taxonomic status of *L. sarmentosa* subspecies/variants across the Portugal-Mediterranean transition, and (3) analyze with nuclear markers the degree of connectivity between Atlantic and Mediterranean populations for *E. verrucosa* and *L. sarmentosa*.

Materials and Methods

Sampling

Specimens used in this study were collected by scuba diving in three different regions, Arrábida Marine Park and Armação de Pêra, Portugal (NE Atlantic), and Marseilles, France (NW Mediterranean). Harvested specimens were stored in 95 % ethanol after collection and identification, and kept at 4 or -20 ºC. The gorgonian species sampled included *Eunicella verrucosa* (Pallas, 1766), *Eunicella gazella* Studer, 1878, *Eunicella labiata* Thomson, 1927 and *Leptogorgia sarmentosa* (Esper, 1791). *Leptogorgia sarmentosa* specimens from Portugal and Mediterranean were treated as the subspecies/variants *lusitanica* and *sarmentosa*, respectively, following Grasshoff (1992). GPS coordinates of sampling sites and number of samples obtained is presented in Table 2.

DNA extraction

Genomic DNA was extracted from specimens either with a QIAmp® DNA Mini Kit (Qiagen) according to manufacturer's protocols, or with a standard 5M NaCl procedure. Briefly, on the latter case DNA was extracted by grinding the tissue in 600 µl of extraction buffer [0.05 M Tris-HCl (pH 8), 0.1 M EDTA (ethylenediaminetetraacetic acid)], 70 µl of 10% SDS (sodium dodecyl sulfate) and 10 µl of 10 mg ml^{-1} proteinase K. After a 3h and 30min incubation period at 55 ºC with constant mixing and occasional vortex of the samples, 200 µl of 5M NaCl was added, followed by a brief (20 min) incubation at 37 ºC and centrifugation at 13000 rpm (20 min). The resulting supernatant (~ 700 µl) was then transferred to a clean tube, gently mixed with an equal volume of cold isopropanol and centrifuged for 15 min at the same conditions. Supernatant was discarded and washed with cold 70% ethanol, followed by a 15 min centrifugation; the supernatant was again discarded and the pellet air-dried overnight. Finally, the pellet was resuspended in 100 µl of ddH_2O. On both extraction methods the DNA was derived from small pieces of polyp, or in some cases (e.g. some *Leptogorgia*) from branch tissues, since the isolation of individual polyps was not always easy to accomplish. Extracted DNA was kept at -26 ºC until amplification.

DNA amplification

DNA amplifications focused on four mitochondrial markers, cytochrome *c* oxidase 1 (COI), NADH-dehydrogenase subunits 2 and 6 (ND2 and ND6), and the *mutS* homolog *msh1*; and three nuclear markers: two introns (EF1EC1 and EF1EC4) of the Elongation Factor 1 gene (EF1) and one intron (i56) of the Glutamyl-prolyl-tRNA-synthetase gene. Part of the COI gene was amplified with the primers COI Cni F and R developed by Calderón et al. (2006), while the fragment of the *msh1* gene was obtained with the primers ND42599F (5'-GCCATTATGGTTAACTATTAC-3') (France and Hoover 2002) and Mut-3458R (5'-TSGAGCAAAAGCCAYTCC-3') (adapted from Sánchez et al. 2003). Parts of the ND2 and ND6 genes were amplified with the primers developed by McFadden et al. (2004) (ND6-1487F: 5'-TTTGGTTAGTTATTGCCTTT-3'; ND3-2126R: 5'-CACATTCATAGACCGACACTT-3'; 16S-647F: 5'-ACACAGCTCGGTTTCTATCTACAA-3'; ND2-1418R: 5'-ACATCGGGAGCCCACATA-3'). The primers used for introns EF1EC1 and EF1EC4 were initially developed by Aurelle et al. (unpublished data) for *Eunicella cavolini* (Koch, 1887). The intron i56 was amplified with the primers developed by Chenuil et al. (in prep.) based on sequences from several metazoan phyla.

With exception for the cloning reactions, all polymerase chain reactions (PCRs) had a final volume of 25 µl which either followed the concentrations reported by Cunha et al. (2008) or included: 9.9 µl of H_2O, 5 µl of 5x Go*Taq*® Flexi buffer (Promega), 2.5 µl of 25 mM $MgCl_2$ (Promega), 2.5 µl of 1.25 mM dNTPs mix, 1.25 µl of both forward and reverse primers at 10 µM, 0.1 µl of 5 $U.\mu l^{-1}$ Go*Taq*® DNA Polymerase (Promega) and 2.5 µl of template DNA. The thermocycler profile of ND2 and ND6 genes followed Sánchez et al. (2003b), whereas all other molecular markers had similar profiles differing only in the annealing temperatures and number of cycles: initial denaturation of 94 ºC for 3 min, followed by 35 cycles (30 cycles in some EF1EC1 samples) of 1 min at 94 ºC (denaturation), 1 min at annealing temperature and 1 min at 72 ºC (extension); and a final extension of 10 min at 72 ºC. Annealing temperatures were 50 ºC for COI, 53 ºC for i56, 60 ºC for EF1EC1 and *msh1*, and 64 ºC for EF1EC4. PCRs were performed either in a Mastercycler ep*gradient S* (Eppendorf) or in a TPersonal Thermocycler (Biometra). PCR products were visualized on ethidium bromide (EtBr)-stained 1.5 % agarose gel and sequenced at the Sequencing Core Facility of Roscoff Marine

Station (France) or at the Molecular Biology Services of the Centre of Marine Sciences (University of Algarve – Portugal). For EF1EC4 sequencing was carried out in both forward and reverse directions due to the large size of PCR products.

Cloning of nuclear markers

All the nuclear markers for which direct sequencing was undertaken contained multiple peaks, evidencing the presence of heterozygous individuals. In order to solve this problem, the final PCR product of some individuals was cloned with pGEM®-T Easy cloning kit (Promega) using DH5α *Escherichia coli* chemically competent cells (Invitrogen). Cloning procedure was performed in a class II microbiological safety cabinet (Thermo Scientific). The PCR product precipitation and cloning protocols are described in Appendix 1. Pre-cloning amplifications were conducted under the same conditions as described above (later reactions) but for a final volume of 50 µl. For post-cloning reactions, the bacterial colonies containing the insert were directly amplified with T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-TATTTAGGTGACACTATAG-3') promoter primers according to the following cycling parameters: 94 ºC for 3 min; 30 cycles at 94 ºC (1 min), 50 ºC (1 min) and 72 ºC (2 min); and a final step at 20 ºC for 15 min. Reactions were carried out in a 25 µl volume containing 11.9 µl H_2O, 5 µl of 5x Go*Taq*® Flexi buffer, 1.5 µl of $MgCl_2$ 25 mM, 4 µl of dNTPs mix 1.25 mM, 1.25 µl of each primer 10 µM, and 0.1 µl of 5 $U.\mu l^{-1}$ Go*Taq*® DNA Polymerase. PCR products visualization and sequencing were performed as specified previously.

Data analysis

In order to assess the taxonomic position of *Eunicella* and *Leptogorgia* species within Octocorallia we used a maximum-likelihood (ML) approach to construct a phylogenetic tree. Basically, starting from McFadden's et al. (2006a) *msh1* phylogeny, we selected representatives of most of the major subdivisions and added the most similar octocoral sequences relative to *E. verrucosa*/*L. sarmentosa* obtained through a nucleotide BLAST search of GenBank. Following these authors, we used *Erythropodium caribaeorum* and *Briareum polyanthes* to root the tree as well. In addition, we performed a maximum parsimony (MP) analysis. A detailed list of specimens acquired, with information on sampling sites and accession numbers is shown in Appendix 2.

Nucleotide sequences of all molecular markers were aligned with Bioedit (Hall 1999) or Geneious (Drummond et al. 2009) software and adjusted by eye. Insertion/deletion mutations (indels) were processed/treated in two different modes, one for the *msh1* phylogeny (ML and MP) and another for the phylogeographic analysis (EF1EC1 and i56). In the former case, indels with different start and/or end positions were recoded to the nucleotide matrix and considered a single mutation event, i.e., for an indel starting at the same but ending at a different position in two sequences, the first gap would be recoded as a nucleotide other than the correspondent for the same position of the inserted motif (e.g. A and T, respectively for an insertion starting with a G) (but see Simmons and Ochoterena 2000). In the subsequent analysis (i.e. ML and MP) gaps were treated as missing characters. For the phylogeographical analysis, all indels were treated as missing because indels patterns were too complex. The best-fit model of nucleotide substitution for the *msh1* phylogeny was selected based upon a ModelTest search (Posada and Crandall 1998). ML phylogenetic analysis was performed using PHYML v2.4.4 (Guindon and Gascuel 2003) with a GTR + Γ model (1000 bootstrap) set to fixed proportion of invariant sites and empirical estimation of base frequencies. Likewise, the gamma distribution parameter was set to as estimated. An additional ML analysis was run without gaps recoding. Maximum-parsimony heuristic searches were conducted with 10 rounds of random sequence addition and tree bisection-reconnection branch swapping. Nodal support was estimated using the bootstrap approach (Felsenstein 1985) with 1000 replicates. The MP analyses were performed with PAUP* v4b10 (Swofford 2002). Statistical support for our best-fit ML tree and alternative topologies where specific groups (e.g. Gorgoniidae) were constrained to be monophyletic, were tested using the approximately unbiased test (AU) (Shimodaira 2002). The AU test adjusts tree selection bias ignored by other methods (e.g. KH; Kishino and Hasegawa 1989) and is less conservative than the Shimodaira-Hasegawa test (SH) (Shimodaira and Hasegawa 1999). Confidence on tree selection and/or proposed hypotheses (e.g.

Table 2 Summary of samples collection information.

Species	Collection locality	GPS coordinates	Samplers	Date	#	General description
Eunicella verrucosa	Maire island, Marseilles	43°12'28" N & 5°20'19" E	F. Zuberer & S. Sartoretto	Feb, 2009	17	Bright pink color, sometimes white; white in ethanol; polyps in general, densely crowded in ticker branches; protuberant polyps
	Três Marias, Arrábida	38°27'26" N & 09°00'11" W	Author, A. Engelen & O. Diekmann	Nov, 2008	10	
Eunicella gazella	Três Marias, Arrábida	38°27'26" N & 09°00'11" W	Author, A. Engelen & O. Diekmann	Nov, 2008	2	White color; branches generally tick and cylindrical; polyps little or not protuberant
	Jardim do Vieira, Armação de Pera	37°05'422" N & 8°20'751" W	Author	Jul, 2009	31	
Eunicella labiata	Poço, Armação de Pera	37°03'108" N & 8°21'170" W	Author	Jul, 2009	6	Brown to black brownish color w/ white/bright polyps; remaining traits are similar to *E. verrucosa*
Leptogorgia sarmentosa: variant *lusitanica*	Três Marias, Arrábida	38°27'26" N & 09°00'11" W	Author, A. Engelen & O. Diekmann	Nov, 2008	17	Color: yellow, bright purple, purple w/ yellow polyps; purple w/ white polyps; sometimes a yellowish carmine; slim "filiform" branches
	Jardim do Vieira, Armação de Pera	37°05'422" N & 8°20'751" W	Author	Jul, 2009	33	
Leptogorgia sarmentosa: variant *sarmentosa*	Maire island, Marseilles	43°12'28" N & 5°20'19" E	F. Zuberer	Apr, 2009	9	Terra-cotta color; sometimes yellowish terra-cotta; slim "filiform" branches

monophyly of a specific group) is assessed through calculation of an approximately unbiased *P*-value. The AU test was executed using the software CONSEL (10 sets of 10000 bootstrap replicates; Shimodaira and Hasegawa 2001) in conjunction with the phylogenetic programs MacClade v4.08 (Maddison and Maddison 2001), PAML (Yang 1997) and PAUP* v4b10 (Swofford 2002).

Because of sample size limitations, the phylogeographical analysis was restricted to the reconstruction of haplotype networks. For each intron and species, parsimony networks were estimated with TCS v1.21 (Clement et al. 2000) using a fixed connection limit of 20 steps and as previously referred, gaps as missing. Whenever possible, polymerase errors derived from the cloning procedure were checked and corrected by comparison of the cloned sequences with the original obtained by direct sequencing. Among introns of nuclear genes it is quite frequent to obtain unphased chromatograms for diploid organisms because of length variant heterozygosity (LVH; e.g. Creer et al. 2005; Foltz 2007). Although there are several methods for phase and haplotype determination in nuclear DNA analyses (see Zhang and Hewitt 2003 for review), some of them, particularly the statistical approaches based upon algorithms (e.g. "PHASE method" of Stephens et al. 2001), require large amounts of individual sequence data (genotypes) in order to perform efficiently (Fallin and Schork 2000; Zhang and Hewitt 2003; Flot et al. 2006). Since the number of individual sequences/haplotypes obtained per intron in this study was too small, we opted not to use such approaches for haplotype determination. In addition, the term "composite haplotype" will be used instead of haplotype because the network reconstruction was conducted using sequences obtained both by direct sequencing and post-cloning sequencing, that may not reflect the true and/or all alleles. Network ambiguities (loops) were resolved following the criteria proposed by Crandall and Templeton (1993) based on predictions from the coalescent theory.

Results

DNA sequences

With exception for the 16S-rRNA fragment and the ND6-ND3 non-coding intergenic spacer (IGS), all mitochondrial sequences had the same length across the different species sequenced. We obtained sequences for a 527 bp long fragment from the coding region of COI; 25 bp from the 3' end of ND4L, 13 bp from the ND4L-*msh1* IGS, and 772 bp from the 5' end of *msh1*; 384 bp from the 3' end of ND6, 43-55 bp of the ND6-ND3 IGS, and 99 bp from the 5' end of ND3; and the last 150-151 bp from the 3' end of 16S, and 583 bp from the 5' end of ND2. The positions of our sequences in the mitochondrial genome were inferred from the combined fragments obtained by Pont-Kingdon et al. (1998) and Beaton et al. (1998) for *Sarcophytum glaucum* as follows: COI (positions 15,165-15691); ND4L-*msh1* (positions 2,654-3,426); ND6-ND3 (positions 1,601-2,138); and 16S-ND2 (positions 7,425-8,159) (Fig. 7). Because the final part from the 3' end of 16S from *Eunicella* spp. and *Leptogorgia* spp. contained a 28 bp insertion relative to *S. glaucum*, all reference positions upstream the 3' end of this gene incorporated an indel mutation. The number of used individuals varied from 1 (*sarmentosa* variant) to 9 (*lusitanica* variant) according to quality of returned sequences and intraspecific variability.

Among the nuclear markers, we were unable to amplify the EF1EC1 fragment for any of the *Leptogorgia* variants, despite the consistent amplification in *Eunicella*. *Leptogorgia* samples also failed to amplify for EF1EC4, another intron of EF1 for which we obtained low levels of intraspecific polymorphism in *E. verrucosa* (data not shown) and no PCR product for *E. gazella* (the other *Eunicella* species tested) as well. The EF1EC1 fragments of *E. verrucosa* and *E. gazella* varied in length from ~ 541 bp to ~ 800 bp corresponding respectively to two sets of sequences differing in an ~ 260 bp indel. In the longest sequences, after the first ~ 345 bp, the chromatograms loose signal and become unreadable, even for individuals not displaying any apparent heterozygous PCR product respective to the ~ 260 bp indel. Cloning of most of these individuals confirmed that the unreadable part corresponded to the heterozygous position of the indels. Nevertheless, we did not recover the full sequences and the analysis of EF1EC1 sequences was therefore limited to the first ~ 160 bp plus the last ~ 380 bp. Regarding i56 nuclear marker, all species were successfully amplified and sequences were of high quality. The i56 fragments differed considerably in length between *E. verrucosa* individuals (787-532 bp), but not much between *Leptogorgia* spp. specimens (482-457 bp). In the former case most of the length variation was attributable to the variable size of the tandem repeats present across the sequences. In the later case, on the other hand, the length variability corresponded to a 28 bp insertion present in two individuals but no other, and the reverse situation (i.e., present in all the other individuals but not in those two) for a 3 bp insertion.

ML phylogeny

The final alignment of *msh1* comprised a total of 78 specimens from 48 different Octocorallia genera. We

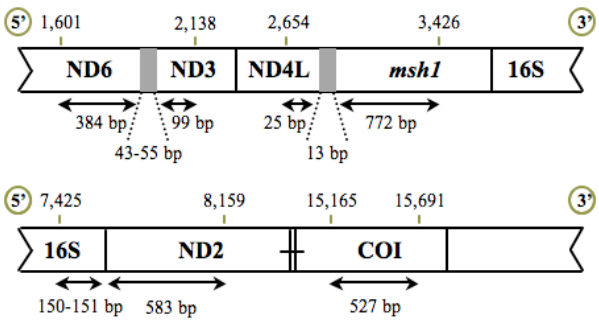

Fig. 7 Schematic showing the size of the mtDNA fragments obtained by sequencing and respective positions in the mitochondrial genome (concatenated fragments of Pont-Kingdon et al. 1998 and Beaton et al. 1998, respectively). IGS's indicated by grey boxes; schematic not to scale Modified from Beaton et al. 1998.

included sequences of 17 species of *Leptogorgia* and a single representative of genus *Eunicella* (*E. verrucosa*). Although the nucleotide sequences of *Leptogorgia* congeners differed considerably, the variability between *Eunicella* species analyzed was inexistent so we used only one representative sequence for the phylogenetic analysis. Additionally, we compared our *Eunicella* spp. sequences with *E. cavolini* and *E. singularis* (data not shown) and found the same sequence. Several indels were present in the alignment, but those did not change the correct reading frame. Among the 857 bp of the alignment (including 20 added positions resulting from gap recode) 222 bp were identical and 374 were parsimony-informative.

Maximum-likelihood and maximum-parsimony (tree not shown) methods recovered very similar topologies with major differences limited to the placement of less supported groups. All the well-supported clades (> 70%) consisted of the same species/specimens in both analyses. The phylogenetic tree obtained with the maximum-likelihood analysis (-logLk = 9370; gamma shape parameter = 0.786) recovered the same three major clades (Fig. 8) obtained by McFadden et al. (2006), but with an overall lower bootstrap support. Base frequencies and relative substitution rates, respectively, were as follows: A = 0.2964, C = 0.1760, G = 0.1988 and T = 0.3287; and (A-C) = 1.8080, (A-G) =8.1242, (A-T) = 0.9536, (C-G) = 0.8474, (C-T) 7.6739, and (G-T) = 1.0 (fixed). Gap recoding had little effect on both tree topology and bootstrap values. Concerning tree topology, the difference consisted in the positioning of *Telestula* sp., which after gap recoding grouped with McFadden's et al (2006a) clade 3 (Fig. 8), instead of grouping with the basal *Erythropodium-Briareum* group. Since the primary goal of this analysis was to focus on *Eunicella* spp. and *Leptogorgia* spp. we kept the gap-recoded tree.

In the ML tree neither family Gorgoniidae nor genus *Leptogorgia* were found to be monophyletic, not conforming therefore to the present taxonomy. Alternative trees in which the monophyly of both Gorgoniidae and *Leptogorgia* were sequentially forced were significantly less likely (AU topology test, $p < 0.001$) (Table 3). Family Gorgoniidae was polyphyletic with *Plexaurella nutans* in a basal position, and *Swiftia* sp. as the closest more internal species (both of family Plexauridae). *E. verrucosa*, *Guaiagorgia* sp. and *Pterogorgia* spp. (Gorgoniidae) were grouped outside the clade containing all other gorgoniids and the two plexaurids, and together with species from the holaxonian families Plexauridae and Acanthogorgiidae for the two former species. This grouping was not, however, well supported by bootstrap values. Similarly to family Gorgoniidae, the genus *Leptogorgia* was also found to be polyphyletic and monophyly was rejected by the AU topological test (Table 3). However, two groups of *Leptogorgia* were clearly distinct and well supported by bootstrap values, one containing all species occurring in the East Atlantic Ocean/ Mediterranean Sea (100%), and another including a mixture of species from East Pacific and West Atlantic Oceans (99%). These two groups, respectively, were placed together with two species of *Pseudopterogorgia* and two of *Pacificogorgia* from different geographical origins (Fig. 8). In the former case, the placement of *Pseudopterogorgia* spp. was not well defined (bootstrap percentage < 50%). The separation between East Pacific and West Atlantic Oceans, on the other hand, was not clearly evident, and consisted of three well-supported clades occurring in the West Atlantic (three species; 89%), East Pacific (five species; 91%) and West Atlantic plus an unknown occurrence region (two species; 100%). *Leptogorgia labiata*, which occurs in the East Pacific, was placed basal to this Pacific/Atlantic group. A tree in which *Leptogorgia* species from the biogeographical regions East Pacific and West Atlantic were constrained to be monophyletic had a significantly lower likelihood value ($p < 0.001$; table 3). Within the polyphyletic Gorgoniidae group, it is interesting to note the intra-generic variability in the substitutions per site (Fig. 9). For instance, *L. chilensis*, *L. alba*, *L. styx*, *L. ramulus* and *L. cuspidata* (all from the East Pacific) have relatively few substitutions per site when compared to *L. labiata* (also from East Pacific) or to *L. violacea* (West Atlantic). Similarly, the species *Pseudopterogorgia bipinnata* has considerably higher number of substitutions per site relative to its congeners species.

The *L. sarmentosa/lusitanica* sequences that we included in this analysis, clustered with the remaining *Leptogorgia* species from East Atlantic. Four distinct haplotypes were found (Figs. 8, 9): haplotype 1, which included five individuals of the purple and yellow variants of Portugal (*lusitanica*) and four of the terra-cotta variants from the Mediterranen Sea (*sarmentosa*); haplotype 2 that included two *lusitanica* specimens; and haplotypes 3 and 4, which included a single individual of *lusitanica* each.

Intra and peri-specific variation in mitochondrial genes

All mitochondrial markers tested in this study failed to reveal any clear differentiation pattern at the species level. However, several genus distinctive mutations were present across the nucleotide sequences (Fig. 10). Among the coding regions of genes partially sequenced, the *msh1* fragment was the most variable with 93.3% identical sites and 49 variable positions (out of 734 bp). ND6, ND2 and COI had 96.6% (371 bp out of 384), 96.7% (564 bp out of 583) and 97% (511 bp out of 527) identical positions, respectively. With exception for 16S (96% identical sites: 145 bp out of 151), the fragments of the adjacent genes, partially amplified and sequenced as a result of primer anchoring (ND3, ND4L), were the most invariant: 99% (98 bp out of 99) and 100% (24 bp) identical sites for ND3 and ND4L, respectively. Compared to *Leptogorgia* species, the 16S fragment of *Eunicella* was one bp shorter. Concerning the non-coding intergenic spacer's, whereas the ND6-ND3 IGS had 80% of identical positions (44 bp out of 55),

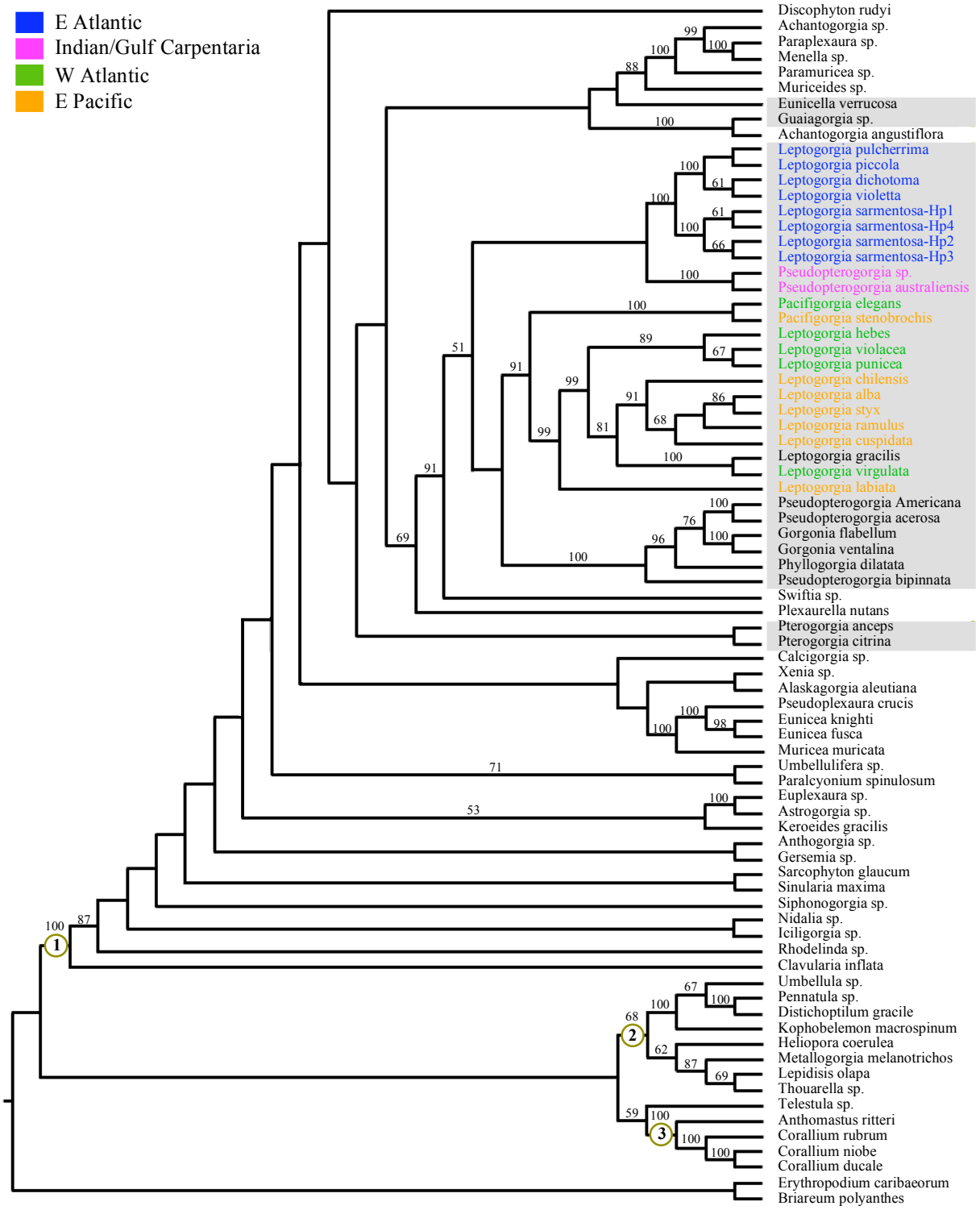

Fig. 8 Maximum-likelihood tree (*msh1*) depicting the phylogenetic relationships among 48 genera of the subclass Octocorallia. Values at nodes are percentages from 1000 bootstrap replicates higher than 50%. Circled numbers represent the three major clades discussed by McFadden et al. 2006a: 1) Holaxonia-Alcyoniina; 2) Calcaxonia-Pennatulacea; and 3) *Anthomastus-Corallium*. Members from family Gorgoniidae are enclosed in grey boxes. Specimen names displayed in colors according to their sampling site/geographical occurrence: blue – East Atlantic Ocean; rose – Indian Ocean/Gulf of Carpentaria; green – West Atlantic Ocean; and orange – East Pacific Ocean.

corresponding to an 11 bp insertion in the 3' end of *Leptogorgia* species IGS, the ND4L-*msh1* IGS was invariant across its entire length (14 bp). It is notable in the striped nucleotide alignment that among COI and ND6 genes there are one and two mutations, respectively, shared by all *E. gazella* individuals and one specimen of *E. verrucosa* (Fig. 10: positions 82, 98 and 103). All the remaining *Eunicella* individuals (both

Table 3 Log-likelihood scores and statistical confidence (AU) of trees with a specific group of gorgoniids constrained to be monophyletic.

Constrained group	-ln*L*	AU
None (best ML tree)	7545.39	1
Gorgoniidae	7654.27	R
Leptogorgia	7664.20	R
Leptgorgia (Biogeographical regions)	7692.22	R

AU (approximately unbiased test; Shimodaira and Hasegawa 2001). R of rejected for $p < 0.001$.

E. verrucosa and *E. labiata*) are invariant at these positions.

Nuclear markers

Considering both nuclear markers, a total of 24 individuals (15 *E. verrucosa*, 1 *E. gazella*, 4 *L. sarmentosa* variants of each sampled area) were sequenced and 43 sequences obtained (Table 4). For intron EF1EC1, whereas the 5' end fragments analyzed consisted of PCR products directly sequenced for which ambiguous positions (possibly corresponding to allelic distinctive positions) were treated as missing, the 3' end fragments comprised sequences obtained by both post-cloning and direct sequencing. Among the acquired sequences, 7 corresponded to individuals with the ~ 541 bp fragment (~ 260 bp deletion relative to the other individuals) obtained by direct sequencing and where it was possible to match the 5' and 3' ends. However, for the remaining individuals (8 *E. verrucosa* + 1 *E. gazella*), the 3' end fragments were obtained by cloning (sometimes two distinct clones for the same individual) and therefore impossible to match with the 5' end fragment obtained by direct sequencing, without knowing the phases for this part. Moreover, for some individuals (5 *E. verrucosa*), the 5' end fragment was the only one sequenced. For these reasons, the 5' and 3'end fragments of EF1EC1 were analyzed separately. Concerning i56 all sequences were cloning-derived.

The first statistical parsimony analysis (TCS) obtained in this study were constructed by using the default 0.95 probability connection limit. However, some sequences were quite divergent resulting in separate networks. Subsequent analyses were set with a connection limit of 20 steps in order to connect all sequences. The final analysis (Fig. 11) revealed the presence of a single network for each nuclear fragment tested (Fig. 11A), and one loop (4 mutations in length) for the 5' end fragment of EF1EC1 network (Fig. 11A). This loop connected composite haplotype E3 to the first notch after E5-E4 composite haplotypess bifurcating (Fig. 11A, orange arrow). Since according to the topological criterion (Crandall and Templeton 1993), haplotypes are more likely to be connected to interior than to tip haplotypes, the loop was omitted. Over the two populations sampled (Arrábida, Atlantic Ocean; and Maire Island, Mediterranean Sea), 10 (EF1EC1) and 7 (i56) composite haplotypes were observed for *Eunicella* spp., and 11 (i56) for *Leptogorgia sarmentosa*.

For EF1EC1 networks 4 composite haplotypes (E1 and E5: 5' end fragment; and E6: 3' end fragment; Fig, 11A, B) were shared between Atlantic and Mediterranean populations of *E. verrucosa*. The other composite haplotype (E5.4) found in the network of the 3' end fragment that was shared between the two populations, included a sequence of *E. gazella*, in contrast to the 5' end fragment network, where *E. gazella* had a distinct composite haplotype (E4). Shared haplotypes were the most frequent. In terms of geographical occurrence, and based on the limited number of sequences included (e.g. only one for *E. gazella*), there is no apparent diagnosing alleles for Mediterranean and Atlantic populations evidenced by this marker, as well as no obvious distinction between *E. gazella* and *E. verrucosa*. For instance, whereas for the 5' end fragment of EF1EC1, *E. gazella* haplotype (E4) were separated from the composite haplotype E1 by as many mutations (7) as the *E. verrucosa* E5 (9), for the 3' end fragment, they merged to a single composite haplotype (E5.4). Composite haplotypes E3, E4, E5 and E5.4 corresponded to the individuals with the ~ 260 bp deletion.

Among the i56 networks (Fig. 11C, D) none of the two species (*E. verrucosa* and *L. sarmentosa*) tested shared composite haplotypes between populations of the Atlantic Ocean and Mediterranean Sea. Excluding composite haplotypes L1 and L5 (*Leptogorgia* spp.) all sequences obtained corresponded to a distinct sequence. Like EF1EC1, the composite haplotype networks were not separated into any apparently evident geographical/sequence genetic grouping (*sarmentosa* variant, Mediterranean Sea; or *lusitanica* variant, Atlantic Ocean). Both *E. verrucosa* and *Leptogorgia sarmentosa* i56 networks included two distant composite haplotypes, V6 plus V7 and L10 plus L11, respectively.

Discussion

Polyphyly of family Gorgoniidae

The ML phylogenetic analysis of nucleotide sequences from the mitochondrial *msh1* gene supported the inclusion of family Gorgoniidae in the heterogeneous, but well supported clade 1 (100%; Fig. 8), gentically identified as distinct in subclass Octocorallia by most molecular analyses. This group includes all members of the sub-order Holaxonia (sea fans) and a majority of taxa from the sub-ordinal groups Alcyoniina, Scleraxonia and Stolonifera. (18S rDNA: Bernston et al. 2001; 16S + 18S: Sánchez et al. 2003a; and ND2 + *msh1*: McFadden et al. 2006a).

Both MP (tree not shown) and ML methods recovered tree topologies where family Gorgoniidae was polyphyletic. The species *Plexaurella nutans* and

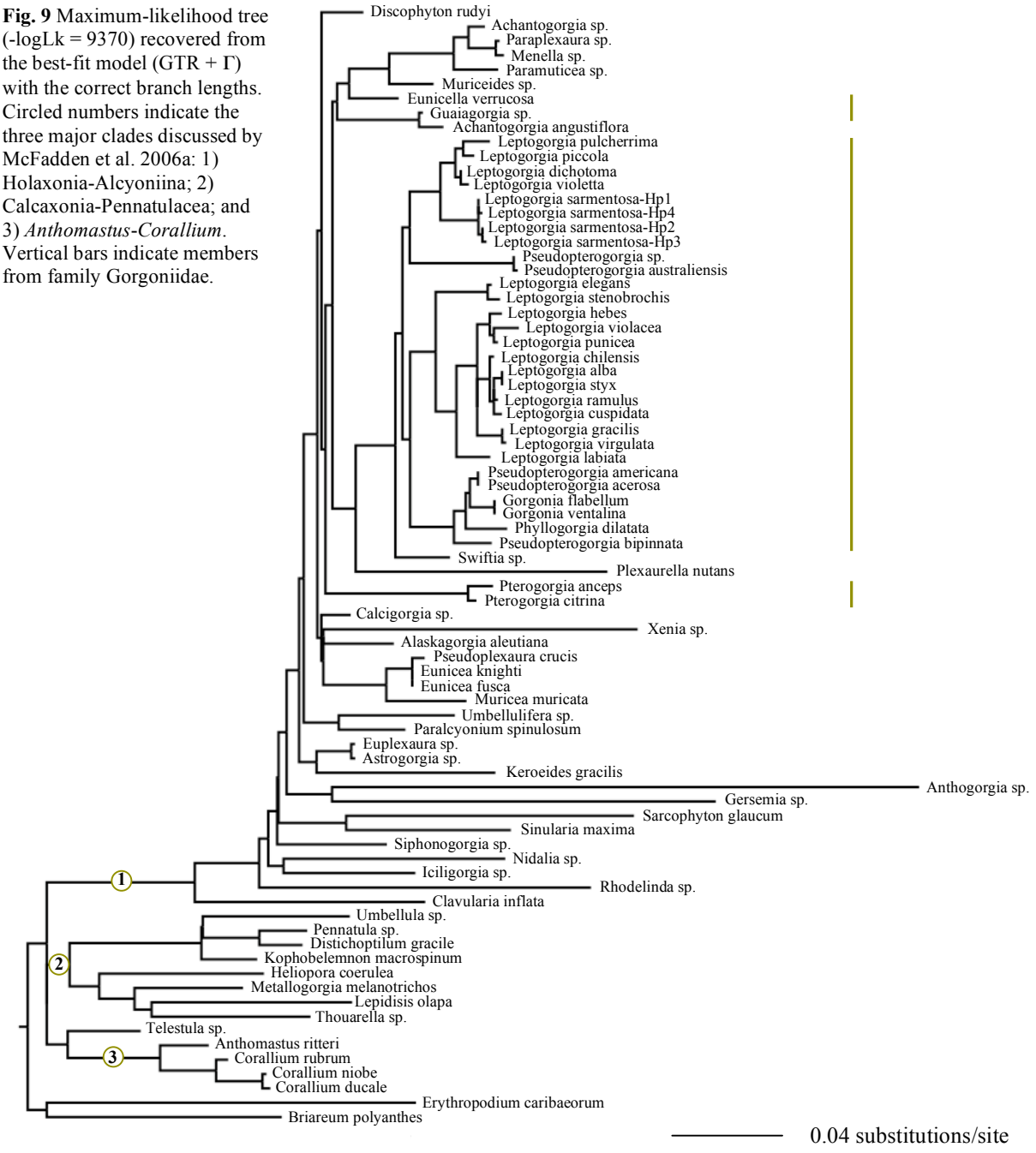

Fig. 9 Maximum-likelihood tree (-logLk = 9370) recovered from the best-fit model (GTR + Γ) with the correct branch lengths. Circled numbers indicate the three major clades discussed by McFadden et al. 2006a: 1) Holaxonia-Alcyoniina; 2) Calcaxonia-Pennatulacea; and 3) *Anthomastus-Corallium*. Vertical bars indicate members from family Gorgoniidae.

Swiftia sp. (traditionally assigned to Plexauridae) were basal to a group comprising most gorgoniids, but that did not include all of them (e.g. *Eunicella verrucosa*). In fact, these two plexaurid species were more closely related to most gorgoniids than were *E. verrucosa* and *Guaiagorgia* sp. (Gorgoniidae as well). Furthermore, the monophyly of Gorgoniidae was rejected by the AU topological test ($p < 0.001$; Table 3). Although these results rely on basal branchings that are not well resolved, they clearly conflict with the present morphology based taxonomy (Bayer 1981a) and corroborate the widely acknowledged ambiguity that surround the description of these two families. The description of families Gorgoniidae and Plexauridae include characters not well defined at the generic level that may led to the classification of some species in either of the two families (Sánchez et al. 2003b; Aguilar and Sánchez 2007; Sánchez 2007). In addition, our results agree with several previous molecular analyses based on both mitochondrial (Sánchez et al. 2003b; Wirshing et al. 2005; McFadden et al. 2006a) and nuclear (Aguilar and Sánchez 2007) markers.

Among the molecular analyses mentioned above, Sánchez et al. (2003b) and Wirshing et al. (2005) presented the most robust analyses focusing on Plexauridae and Gorgoniidae. Both these authors strongly argued the need of revising these families because of their non-monophyly, particularly because

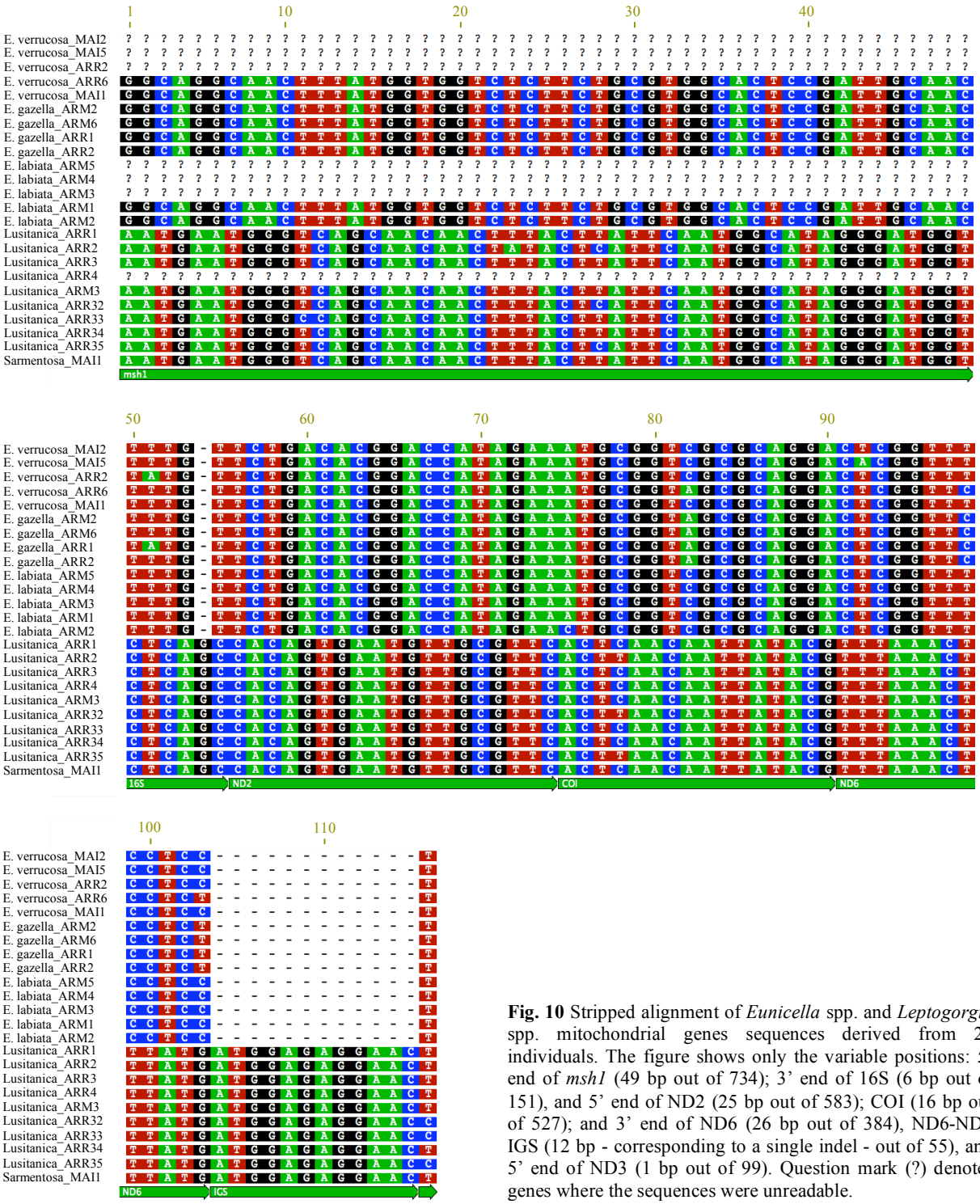

Fig. 10 Stripped alignment of *Eunicella* spp. and *Leptogorgia* spp. mitochondrial genes sequences derived from 25 individuals. The figure shows only the variable positions: 5' end of *msh1* (49 bp out of 734); 3' end of 16S (6 bp out of 151), and 5' end of ND2 (25 bp out of 583); COI (16 bp out of 527); and 3' end of ND6 (26 bp out of 384), ND6-ND3 IGS (12 bp - corresponding to a single indel - out of 55), and 5' end of ND3 (1 bp out of 99). Question mark (?) denotes genes where the sequences were unreadable.

of the basal grouping of some plexaurids (e.g. *Plexaurella* and *Swiftia*) relative to Gorgoniidae. Wirshing et al. (2005) proposed some alternative classification schemes like the inclusion of genera *Plexaurella* (also proposed by Sánchez et al. 2003b) and *Swiftia* in family Gorgoniidae, and the reconsideration of former family designations such as Paramuriceidae for most species of the subfamily stenogorgiinae (Plexauridae; e.g. *Paramuricea* spp.). More recently, new hypotheses on the relationships between Gorgoniidae species have been proposed based on the nuclear ITS2 and its predicted secondary structure (Aguilar and Sánchez 2007). Although a subsequent study provided morphological synapomorphies supporting the four distinct clades genetically identified by Aguilar and Sánchez (2007), several important taxa were not considered in both these analyses as noted by Sánchez (2007) (e.g. *Eunicella*). Moreover, the ribosomal ITS is a multi-copy gene for which intra-individual sequence variability should be

Table 4 Total number of sequences obtained per species for each nuclear marker (EF1EC1 and i56) and location/population sampled.

			Arrábida, Portugal (Atlantic Ocean)			Maire Island, Marseilles (Mediterranean Sea)	
		Length [b]	# Sequences			# Sequences	
		Eunicella/Leptogorgia	*E. verrucosa*	*E. gazella*	*lusitanica*	*E. verrucosa*	*sarmentosa*
EF1EC1	5' end	164/--- bp	7	1	-	7	-
	3' end	376/--- bp	5	1	-	9	-
i56		812/485 bp	3	-	7	4	6
Total			14 [a]	1 [a]	7	15 [a]	6

[a] Total number of sequences considering the 5' and 3' end fragments matches of EF1EC1 as a single sequence
[b] Including indels

analyzed. Under concerted evolution, the homogenization of the different copies of ITS present across the genome is expected to occur (Arnheim et al. 1980; Elder and Turner 1995; Liao 2000). However, because of various organismal processes such as hybridization, one cannot assume that homogenization and the presence of a single ITS sequence type holds true for every species analyzed (Álvarez and Wendell 2003). In fact, concerted homogenization of ITS seems to affect species differently (e.g. *Eunicella cavolini*: Calderón et al. 2006; *Corallium rubrum*: Constantini et al. 2007a; in contrast to *Pseudopeterogorgia elisabethae*: Gutiérrez-Rodríguez et al. 2009).

Our study included for the first time the genus *Eunicella* in a broader molecular analysis of Octocorallia. Surprisingly, *E. verrucosa* was found to group with members of the subfamily Stenogorgiinae (Plexauridae) and of family Acanthogorgiidae. Although weakly supported by bootstrap values (31%), *E. verrucosa* grouped with three plexaurid species, which were included in the Paramuriceidae clade proposed by Wirshing et al. (2005). Former taxonomic subdivisions (Bayer 1961) have placed genus *Eunicella* in family Plexauridae but not in the obsolete Paramuriceidae. The grouping obtained here, though with low support, again recalls for the need of a deep revision of family Gorgoniidae. Future insights into familial and genera relationships may be attained not only through the corroboration of molecular results (both mitochondrial and nuclear) with morphology-based taxonomy, but also by including all members presently assigned to Gorgoniidae and the closely related Plexauridae.

Low levels of mtDNA variation

The proportion of variable positions at the mitochondrial loci tested here (COI, ND2, ND6 and *msh1*) was very low both at the intraspecific (0-0.8%) and intergeneric levels (3-6.7%). Likewise, pairwise distances (uncorrected *p* distance) varied between 0 and 0.3% at the intraspecific level, and between 1.4 and 3.4% for intergeneric comparisons (*Eunicella* spp.-*L. sarmentosa*). Considering the whole data set used in the ML phylogeny (*msh1*) the pairwise distance was 12.1 %. Although few studies have tested/presented information on intraspecific variation of octocorals mitochondrial genes, similar values (publication bias can not be ruled out) have been reported. For example, France et al. (1996) and France and Hoover (2001) found little or no intraspecific sequence variation (*p's* < 0.2% and 0.9%, respectively) for the 16S rRNA, and ND3, ND4L and *msh1*, respectively; McFadden et al (2004) obtained virtually no intraspecific variation among the NADH dehydrogenase subunits 2, 3 and 6 for the soft coral families Alcyoniidae and Xeniidae; and Calderón et al. (2006) obtained identical COI sequences for distant populations of *Corallium rubrum* and *Eunicella* spp.. For comparisons among species spanning at different taxonomic levels (i.e. from genera to inter-ordinal level), sequences divergence of mitochondrial genes have been reported to range from 0 to 22.1 % (France et al. 1996; France and Hoover 2001, 2002; McFadden et al. 2004; Calderón et al. 2006).

Despite the fact of being present among other organisms (e.g. plants: Wolfe et al. 1987; sponges: Duran et al. 2004; Wörheide 2006), low levels of intraspecific and interspecific mtDNA differentiation are generalized in several anthozoan taxa, particularly octocorals (see Shearer et al. 2002 for review). Some mechanisms have been suggested to explain this low variation (e.g. introgression due to hybridization among recently diverged species, *Montastraea annularis* species complex: Medina et al. 1999; selective pressure acting on mitochondrial genes: van Open et al. 1999) but none of them have been tested (Shearer et al. 2002). In octocorals the lack of mtDNA polymorphism is frequently suggested to be associated with the presence of a mismatch repair gene (*msh1*), homolog to the *mutS* gene found in the bacterial MSHL mismatch pathway, and exclusive to octocorals mtDNA (Pont-Kingdon et al. 1995, 1998; Culligon et al. 2000; France and Hoover 2001; McFadden et al. 2006a; Ledoux et al. unpublished). Although there is no evidence of a loss of function for *msh1* (based on mutations susceptible to alter translation; McFadden et al. 2006a), eukaryotes mismatch repair pathways involve the interaction between several different protein complexes (see Eisen 1998; Malik and Henikoff 2000). For this reason, the functionality of this gene may be merely speculative since it would require the importation of other

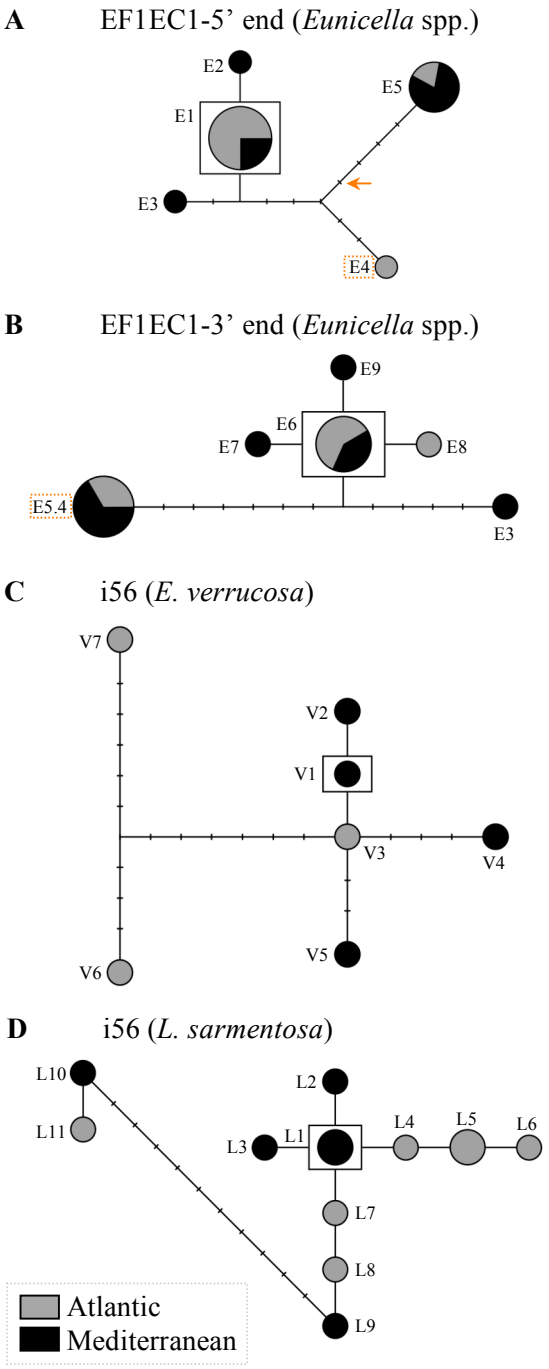

Fig. 11 Parsimony composite haplotype networks for (A) 5' end fragment of EF1EC1 – *Eunicella* spp., (B) 3' end fragment of EF1EC1 – *Eunicella* spp., (C) i56 – *E. verrucosa*, and (D) i56 – *Leptogorgia sarmentosa*. Composite haplotypes observed in Arrábida, Portugal (Atlanntic Ocean), and Maire Island, Marseilles (Mediterranean Sea) are displayed in grey and black, respectively. The size of each circle is proportional to composite haplotype frequencies, and rectangles indicate the supposedly inferred ancestral haplotype. Notches symbolize intermediate missing haplotypes. Orange arrow stands for composite haplotype E3 loop connection site. Dashed orange boxes indicate *E. gazella* composite haplotypes.

components not encoded in the mitochondrial genome of octocorals (Wolstenholme 1992 in Shearer et al. 2002).

In most cases, the mtDNA of octocorals has been shown to be adequate for solving high-level relationships (i.e., subordinal and familial level), rather than low-level taxonomy (Sánchez et al. 2003a, 2003b; Wirshing et al. 2005; McFadden et al. 2006a). However, the variability of mitochondrial genes varies among genera and might be informative at the interspecific level within some of them (e.g. McFadden et al. 2004; *Leptogorgia msh1* phylogeny of the present study). Here, the mitochondrial genes sequenced completely failed to reveal any species differentiation among *Eunicella* spp., in agreement with previous results from Calderón et al. (2006) and Mokhtar-Jamaï et al. (2009) for COI, as well as among the different geographical variants of *L. sarmentosa*. Between *Eunicella* species and considering all genes analyzed here, there were 3 mutations shared by all *E. gazella* specimens and one of *E. verrucosa* (E. verrucosa_ARR6; Fig. 10). After a reexamination of the latter specimen we recognized a possible misclassification, so these three mutations are likely to be characteristic of *E. gazella* relative to the other *Eunicella* species analyzed. Concerning *L. sarmentosa* this is the first analysis focusing on the differentiation of the geographical subspecies/variants described by Grasshoff (1992). Although only one *sarmentosa* variant was sequenced for all genes, the sequence of this specimen was shared by the *lusitanica* variants. In addition, for the *msh1* gene, where we obtained more sequences, all *sarmentosa* variants (4) grouped in a single haplotype together with five specimens of *lusitanica*. Hence, based on mitochondrial markers there is no evidence supporting the elevation of *lusitanica* and *sarmentosa* variants to the species level, and the morphological variability of color (Table 2) and branching patterns might reflect no more than phenotypic plasticity. Nevertheless this does not lead to the rejection of the hypothesis of being different species, as mt genes failed to separate the well-characterized European species of genus *Eunicella* (Calderón et al. 2006; present study).

Phenotypic plasticity is common among plants (Schlichting 1986; Sultan 2000) as well as among several marine organisms including octocorals (e.g. sponges: Palumbi 1984, Hill and Hill 2002; barnacles: Marchinko 2003, Li and Denny 2004; hexacorals: Bruno and Edmunds 1997, Muko et al. 2000; octocorals: West et al. 1993, Kim et al. 2004, Sánchez et al. 2007, Prada et al. 2008; among others). However, in some cases, the apparently phenotypic plasticity can mean morphological polymorphism, i.e., genetically determined morphological differences as observed, for instance, among humans (e.g. Romualdi et al. 2002). Genetic variation can therefore account for plasticity in nature (Pigliucci 2005) and species should not be viewed as fixed entities, although the level of morphological variability a species should or should not encompass deserves cautious evaluation. The *Eunicella*

species used in this study are a good example of such a problem, since three well-established species (Grasshoff 1992), showed virtually no genetic mtDNA variation.

Genus *Leptogorgia*: transatlantic divergence/ speciation or different lineages?

Despite of not being part of our primary goals, the Octocorallia ML phylogeny obtained here, revealed interesting biogeographical patterns between *Leptogorgia* species that deserve preliminary comments. Similarly to previous molecular analyses that found the genus *Leptogorgia sensu lato* to be polyphyletic (*msh1*: Lepard 2003 in Sánchez 2007) or paraphyletic (ITS2: Aguilar and Sánchez, after the reassignment of 5 East African *Leptogorgia* species to *Filigorgia*: see Sánchez 2007) with other gorgoniids, our best-fit ML tree (Figs. 8, 9) and an alternative tree with monophyly reinforced ($p < 0.001$; Table) also rejected the monophyly of this genus. Instead, two well supported clades were identified, one containing all species occurring at the East Atlantic Ocean (say Atl, 100%), which included the four *L. sarmentosa* haplotypes and four species from the East African coast; and a second clade of intermingled species from East Pacific and West Atlantic (say Pac-Atl, 99%). Transisthmian divergence resulting from the closure of the Isthmus of Panama, was not evident between *Leptogorgia* species occurring in the East Pacific and West Atlantic, and alternative topologies with these two regions constrained to be monophyletic (limited to *Leptogorgia* spp.), significantly decreased the likelihood score of our best-fit tree (Table 3). Although the topological position of the Atl clade relative to the Pac-Atl and remaining gorgoniids that grouped monophyletically could vary (bootstrap value = 51%; Fig. 8), two alternative conjectures may be pointed: 1) that we are in the presence of a transatlantic divergence/speciation event; and 2) that the two *Leptogorgia sensu lato* clades are well separated lineages with convergent morphologies.

Fukami et al. (2004) tested the inter-oceanic monophyly of members from two scleractinian coral families through mitochondrial and nuclear markers, and discovered that conventional taxonomy was obscuring a deep divergence between Atlantic and Pacific lineages. Some Atlantic species assigned to different families (Faviidae and Mussidae), were more closely related to each other then to their respective "congeners" in the Pacific. Our results can represent a similar situation but at the generic level, i.e., among *Leptogorgia sensu lato*. For instance, the grouping of the Indo-Pacific *Pseudopterogorgia* spp. (Fig. 8) with the Atl clade of *Leptogorgia* found here, tough weakly supported (49%), is in agreement with the morphological similarities described by Williams and Lindo (1997) for some *Leptogorgia* (e.g. the endemic to southern Africa *L. capensis*) and the Indo-Pacific *Pseudopterogorgia* spp.. Likewise, in case of representing a single lineage that because of methodological limitations appeared to be so divergent, the differentiation between the Atl and the Atl-Pac clades of *Leptogorgia* can represent a case of allopatric speciation (vicariance). The full evaluation of a biogeographic pattern for *Leptogorgia* would require: 1) the determination of accurate biogeographic distributions of all species, which in this case was based on somehow limited occurrence registers (see Appendix 2); and 2) the inclusion of all species from the geographical areas/clades identified here. Additionally, the calibration with a molecular clock would be useful, but unfortunately, fossil records, the only direct evidence/calibration point for historical biogeography (Williams and Reid 2004) are lacking for octocorals (Bayer 1973; McFadden et al. 2006a).

Nuclear markers: utility for species boundaries delimitation and phylogeography

Nuclear introns (EF1EC1, EF1EC4 and i56) have been amplified and sequenced with the goal of analyzing the degree of connectivity between Atlantic and Mediterranean populations of *L. sarmentosa* and *E. verrucosa*. Both EF1EC1 and i56 required cloning procedures for almost all individuals because of length variant heterozygosity (LVH), which in the former case did not solve the problem anyway. The loss of signal after the first ~ 345 bp (longest sequences) made the EF1EC1 sequences unreadable upward even for cloned sequences. Moreover, among the cloned sequences (sometimes 2 clones/individual for the 3' end fragment) it was impossible to determine phase and match to the 5' end fragment obtained by direct sequencing in most cases. EF1EC1 and EF1EC4 failed to amplify for *L. sarmentosa*, situation that may indicate genus-specific sequence variation at the primer site. Concerning i56, there was a large amount of genetic variability and almost all clones sequenced corresponded to a different sequence/haplotype. Because of all these laboratorial constraints, the number of individuals cloned and sequenced (never exceeding 2 clones/individual) was limited, so we did not apply any method to resolve LVH heterozygotes (e.g. Sousa-Santos et al. 2005).

The number of sequences obtained for each marker was too small to infer any phylogeographical pattern among populations of *L. sarmentosa* and *E. verrucosa*. Moreover the genetic data is not adequate since it does not represent all or the true alleles, particularly for EF1EC1. EF1EC1 fragments displayed contrasting genetic groupings for *E. gazella* (Fig. 11 A, B), which either grouped with the *E. verrucosa* composite haplotypes E5.4 (3' end fragment) or constitute the single composite haplotype E4 (5' end fragment). Nevertheless, the determination of phase sequences might reveal diagnostic alleles differentiating *E. verucosa* and *E. gazella*. I56 seems to be the most promising marker presented here, particularly for *L. sarmentosa*, because of the level of polymorphism it displayed and the straightforward sequencing after cloning. Obviously, obtaining more sequences is a

prerequisite, in order to fully evaluate any differentiation pattern between populations, for instance through allelic frequencies. Like the mitochondrial genes used here, i56 did not indicate a clear separation between *lusitanica* (Atlantic) and *sarmentosa* (Mediterranean) variants of *L. sarmentosa* (Fig. 11D), but these results are not definitive or the proof that they represent a single species. For i56 we found no shared haplotypes between the Atlantic Ocean and Mediterranean Sea for both *E. verrucosa* and *L. sarmentosa*. However, future analysis with more sequences and frequencies analyses (e.g. AMOVA) are likely to refine this last question.

Overall, we present here two nuclear introns that may shed light on the phylogeography of *E. verrucosa* and *L. sarmentosa* across the Atlantic-Mediterranean transition, as well as on the controversy concerning *L. sarmentosa* subspecies/variants. Introns are becoming more and more widely used in phylogeography, population genetics and species boundaries inference, and have produced some interesting results in the past years, particularly among corals (e.g. Hatta et al. 1999; van Open et al. 2000, 2001; Vollmer and Palumbi 2002; Conception et al. 2008; Mokhtar-Jamaï et al. 2009). The need of markers other than mitochondrial for these organisms (among others; see Galtier et al. 2009), the high level of introns polymorphism relative to mtDNA, and the ease of primer designing in the flanking regions (e.g. EPIC) (Zhang and Hewitt 2003), will probably make these markers an interesting tool for octocoral population biologists.

Conclusion

In the last years it has became evident that octocorals morphology-based taxonomy does not reflect the phylogenetic relationships between taxa and are in need of profound revision (Sánchez et al. 2003a, 2003b; Wirshing et al. 2005; McFadden et al. 2006a; Aguilar and Sánchez 2007; Sánchez 2007; present study). Our results reinforce the acknowledged lack of polymorphism displayed by mitochondrial genes for resolving species boundaries, particularly at controverse species subdivisions such as *L. sarmentosa*, but its suitability for high-level taxonomic relationships. Moreover, mtDNA can be useful for clarifying trans-oceanic boundaries among octocoral genera as outlined by our *msh1* phylogeny preliminary results on *Leptogorgia*, and by Wirshing et al. (2005). Finally, we present two alternative nuclear markers that are likely to improve our understanding of the differentiation and connectivity patterns of *E. verrucosa* and *L. sarmentosa* populations across the Atlantic-Mediterranean transition, as well as on the phylogenetic relationships within the *Eunicella* species, poorly defined at the morphological level. Both species boundaries delimitation, and estimation of gene flow between populations are essential steps for biodiversity management and conservation. Future studies following the tools provided here can play an important role in the conservation of European gorgoniids.

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Appendix 1

Cloning protocol applied to the nuclear markers studied. Adapted from Promega technical manual.

PCR product precipitation

1. Mix the PCR product with 0.1 V of 3 M sodium acetate (pH 7) and 2.5 V of cold 100% ethanol (EtOH) in a 1.5 ml microcentrifuge tube
2. Incubate at -80 ºC for 30 min
3. Centrifuge for 10 min at 13000 *g*
4. Discard the supernatant and add 100 µl of cold 70% EtOH. Mix by pipetting
5. Centrifuge for 10 min at 13000 *g*
6. Discard the supernatant and let the samples dry at room temperature
7. Resuspend the pellet in 12 µl of ultra pure water, quantify the DNA and dilute to 40 ng/µl

Ligation

Appendix 1 (Contd.)

1. Briefly centrifuge the pGEM®-T Easy Vector, ligation buffer, DNA ligase and DNA tubes in order to collect its contents at the bottom of the tubes
2. Place the DNA ligase in the freezer and the remaining tubes on ice
3. Set up the ligation reactions as described below:

	Volume (µl)
Ultra Pure Water	1
2X Rapid Ligation Buffer (Promega)	5
25 ng/ml pGEM®-T Easy Vector (Promega)	1
T4 DNA Ligase (Promega)	1
PCR Product (40 ng/µl)	2
Total	10

4. Mix the reactions by pipetting and incubate during 1h 30 min at room temperature

Transformation

1. Place the DH5α *E. coli* Competent Cells (Invitrogen) in an ice bath until just thawed (10-15 min). Mix the cells by gently flicking the tube.
2. Centrifuge the tubes containing the ligation reactions. Add 3 µl of each ligation product and 25 µl of Competent Cells to a new 2.0 ml microcentrifuge tube. Avoid excessive pipetting, as the Competent Cells are extremely fragile
3. Place the tubes on ice for 20 minutes
4. Heat-shock the cells for 20 seconds in a bain-marie at exactly 42 ºC
5. Immediately return the tubes to ice for 2 min
6. Add 100 µl of LB broth to the tubes containing the transformed cells and incubate for 1 h 30 min at 37 ºC with gently shake
7. Plate each transformation culture (approximately 130 µl) onto LB/ampicillin/X-Gal plates (final concentrations of 100 µg/ml for ampicillin and 80 µg/ml for X-Gal)
8. Incubate the plates overnight at 37 ºC (switch the plates upside-down after 30 min)

Appendix 2

List of octocoral specimens used in the *msh1* phylogenetic reconstruction. Sub-ordinal groups are indicated in uppercase boldface and square brackets, family in lowercase boldface. Dates correspond to the sampling date; DNA sequences were acquired from GenBank. *, collection locality according to the geographic distribution of the species, since there wasno available information on sample origin (Following Grasshoff 1992; Breedy and Guzman 2005; and the Ocean Biogeographic Information System (http://www.iobis.org/); †, Ledoux et al. (unpublished); and §, direct submission to GenBank. Adapted from McFadden et al. (2006).

Family, [subfamily] and species	Collection locality	Date	Accession no.
Order ALCYONACEA			
[STOLONIFERA]			
Clavulariidae			
[Clavulariinae]			
Clavularia inflata Schenk, 1896	Great Barrier Reef, Qld, AUS	1991	DQ302799

Appendix 2 (Contd.)

Family, [subfamily] and species	Collection locality	Date	Accession no.
Rhodelinda sp.	Kermadec Islands, New Zealand		DQ302800
[Telestinae]			
Telestula sp.	Tasman Sea, AUS	2003	DQ302803
[ALCYONIINA]			
Alcyoniidae			
[Alcyoniinae]			
Discophyton rudyi (Verseveldt & van Ofwegen, 1992)	Tatoosh Island, WA, USA	1991	DQ302808
Sarcophyton glaucum (Quoy & Gaimard, 1833)	Rowley Shoals, WA, AUS	1987	DQ280525
Sinularia maxima Verseveldt, 1971	Piti Bay, Guam	1998	DQ302813
[Anthomastinae]			
Anthomastus ritteri Nutting, 1909	Pebble Beach, CA, USA	1998	DQ302816
Nephtheidae			
Gersemia sp.	Balsfjorden, Norway	1978	DQ302819
Umbellulifera sp.	Gulf of Carpentaria, NT, AUS	1990	DQ302827
Nidaliidae			
[Nidallinae]			
Nidalia sp.	West Channel, Palau	2005	DQ302828
[Siphonogorgiinae]			
Siphonogorgia sp.	Rowley Shoals, WA, AUS	1987	DQ302832
Paralcyoniidae			
Paralcyonium spinulosum Delle Chiaje, 1822	Sesimbra, Portugal	2001	DQ302833
Xeniidae			
Xenia sp.	Ngederak, Palau	2000	DQ302842
[SCLERAXONIA]			
Briareidae			
Briareum polyanthes Duchassaing & Michelotti, 1860	Caribbean sea *	-	AY533653
Anthothelidae			
[Anthothelinae]			
Erythropodium caribaeorum (Duchassaing & Michelotti, 1860)	Bocas del Toro, Panama	2003	DQ302843
[Semperininae]			
Iciligorgia sp.	Tasman Sea, AUS	2003	DQ302844
Coralliidae			
Corallium ducale Bayer, 1955	Cross Seamount, Hawaii	1993	DQ297416
Corallium niobe Bayer, 1964	Kelvin Seamount, Atlantic	-	EF060051
Corallium rubrum (Linnaeus, 1758)	Marseilles, France	2005	†
[HOLAXONIA]			
Acanthogorgiidae			
Acanthogorgia angustiflora Kükenthal & Gorzawsky, 1908	Bear Seamount	2000	DQ297418
Acanthogorgia sp.	Bishop Seamount, Hawaii	1993	AY268461
Anthogorgia sp.	Tasman Sea, AUS	2003	DQ302849
Calcigorgia sp.	Bobrof Islands, Alaska, USA	2003	DQ297419
Gorgoniidae			
Gorgonia flabellum Linnaeus, 1758	San Salvador, Bahamas	1999	AY126427
Gorgonia ventalina Linnaeus, 1758	Cayo Lobo, Puerto Rico	2000	AY126425
Guaiagorgia sp.	Gulf of Carpentaria, NT, AUS	2003	DQ302851
Leptogorgia alba Duchassaing & Michelotti, 1864	E Pacific *	-	AY268452
Leptogorgia chilensis Verrill, 1868	E Pacific *	-	AY268460
Leptogorgia cuspidata Verrill, 1865	Panama & Mexico, E Pacific *	-	AY268450
Leptogorgia dichotoma Verrill, 1870	Guine, NE Atlantic *	-	AY268445
Leptogorgia gracilis (quoted by LePard & France, unpublished) §	-	-	AY268454

Appendix 2 (contd.)

Family, [subfamily] and species	Collection locality	Date	Accession no.
Leptogorgia hebes Verrill, 1869	Miami & Mexico Gulf, NW Atlantic *	-	AY268459
Leptogorgia labiata Verrill, 1870	Panama & Mexico, E Pacific *	-	AY268447
Leptogorgia piccola Grasshoff, 1988	Senegal, NE Atlantic *	-	AY268444
Leptogorgia pulcherrima Bielschowsky, 1918	Angola, SE Atlantic *	-	AY268443
Leptogorgia punicea (M. Edwards & Haime, 1857)	Brazil, W Atlantic *	-	AY268449
Leptogorgia ramulus (Valenciennes, 1855)	E Pacific *	-	AY268451
Leptogorgia styx Bayer, 2000	NE Pacific *	-	AY268453
Leptogorgia violacea (Pallas, 1766)	W Atlantic & AUS *	-	AY268448
Leptogorgia violetta Grasshoff, 1988	Dakar, Angola & Senegal, E Atlantic *	-	AY268446
Leptogorgia virgulata (Lamarck, 1815)	North Carolina, USA	2000	AY126418
Pacifigorgia elegans (M. Edwards & Haime, 1857)	Trinidad	1977	AY126419
Pacifigorgia stenobrochis (Valenciennes, 1846)	E Pacific	1996	AY126420
Phyllogorgia dilatata (Esper, 1806)	Rio de Janeiro, Brazil	2000	AY126428
Pseudopterogorgia acerosa (Pallas, 1766)	Lee Stocking Island., Bahamas	2000	AY126421
Pseudopterogorgia americana (Gmelin, 1791)	San Salvador, Bahamas	1999	AY126423
Pseudopterogorgia australiensis (Ridley, 1884)	NE Indian	-	AY268442
Pseudopterogorgia bipinnata (Verrill, 1864)	Bahamas	-	DQ640646
Pseudopterogorgia sp.	Gulf of Carpentaria, NT, AUS	2003	DQ302852
Pterogorgia anceps (Pallas, 1766)	Florida, USA	2000	AY126403
Pterogorgia citrina (Esper, 1792)	Lee Stocking Island., Bahamas	2000	AY126402
Keroeididae			
Keroeides gracilis Whitelegge, 1897	Tasman Sea, AUS	2003	DQ302853
Plexauridae			
[Plexaurinae]			
Alaskagorgia aleutiana Sánchez & Cairns, 2004	Aleutian Islands, Alaska, USA	1994	DQ297433
Eunicea fusca Duchassaing & Michelotti, 1860	Lee Stocking Island., Bahamas	2000	AY126407
Euplexaura sp.	Gulf of Carpentaria, NT, AUS	2003	DQ302854
Muricea muricata (Pallas, 1766)	Lee Stocking Island., Bahamas	2000	AY126408
Plexaurella nutans (Duchassaing & Michelotti, 1860)	Lee Stocking Island., Bahamas	2000	AY126415
Pseudoplexaura crucis Bayer, 1961	Lee Stocking Island., Bahamas	2000	AY126401
[Stenogorgiinae]			
Astrogorgia sp.	Tasman Sea, AUS	2003	DQ302856
Menella sp.	Gulf of Carpentaria, NT, AUS	2003	DQ302858
Muriceides sp.	Tasman Sea, AUS	2003	DQ302859
Paramuricea sp.	Muir Seamount	2003	DQ297420
Paraplexaura sp.	Gulf of Carpentaria, NT, AUS	2003	DQ302861
Swiftia sp.	Tasman Sea, AUS	2003	DQ302862
[CALCAXONIA]			
Chrysogorgiidae			
Metallogorgia melanotrichos (Wright & Studer, 1889)	Manning Seamount	2003	DQ297423
Isididae			
[Keratoisidinae]			
Lepidisis olapa Muzik, 1978	Cross Seamount, Hawaii, USA	1993	DQ297426
Primnoidae			
Thouarella sp.	Oceanographer Cyn, NW Atlantic	2001	DQ297430
Order PENNATULACEA			
[SESSILIFLORAE]			
Kophobelemnidae			
Kophobelemnon macrospinum (quoted by McFadden *et al.,* 2006)	Tasman Sea, AUS	2003	DQ302865
Protoptilidae			

Appenxix 2 (Contd.)

Family, [subfamily] and species	Collection locality	Date	Accession no.
Distichoptilum gracile Verrill, 1882	Tasman Sea, AUS	2003	DQ302866
Umbellulidae			
Umbellula sp.	Tasman Sea, AUS	2003	DQ302867
[SUBSESSILIFLORAE]			
Pennatulidae			
Pennatula sp.	Tasman Sea, AUS	2003	DQ302870
Order HELIOPORACEA			
Helioporidae			
Heliopora coerulea (Pallas, 1766)	Blue Corner East, Palau	2005	DQ302872

References

Aguilar C, Sánchez JA (2007) Phylogenetic hypotheses of gorgoniid octocorals according to ITS2 and their predicted RNA secondary structures. Mol Phylogenet Evol 43: 774-786.

Alberto F, Massa S, Manent P, Diaz-Almela E, Arnaud-Haond S, Duarte CM, Serrão EA (2008) Genetic differentiation and secondary contact zone in the seagrass *Cymodocea nodosa* across the Mediterranean–Atlantic transition region. J Biogeogr 35: 1279-1294.

Alderslade P, McFadden CS (2007) Pinnule-less polyps: a new genus and new species of Indo-Pacific Clavulariidae and validation of the soft coral genus *Acrossota* and the family Acrossotidae (Coelenterata: Octocorallia). Zootaxa 1400:27-44.

Álvarez I, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. Mol Phylogenet Evol 29: 417-434.

Arnheim N, Krystal M, Schmickel R, Golder W, Oliver R, Zimmer E (1980) Molecular evidence for genetic exchanges among ribosomal genes on non-homologous chromosomes in man and apes. Proc Natl Acad Sci USA-Biol Sci 77: 7323-7327.

Avise JC (1994) Molecular markers, natural history and evolution. Chapman & Hall, New York.

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge.

Bally M, Garrabou J (2007) Thermodependent bacterial pathogens and mass mortalities in temperate benthic communities: a new case of emerging disease linked to climate change. Global Change Biol 13: 2078–2088.

Bargelloni L, Alarcon JA, Alvarez MC, Penzo E, Magoulas A, Reis C, Patarnello T (2003) Discord in the family Sparidae (Teleostei): divergent phylogeographical patterns across the Atlantic-Mediterranean divide. J Evol Biol 16: 1149-1158.

Bargelloni L, Alarcon JA, Alvarez MC, Penzo E, Magoulas A, Palma J, Patarnello T (2005) The Atlantic-Mediterranean transition: discordant genetic patterns in two seabream species, *Diplodus puntazzo* (Cetti) and *Diplodus sargus* (L.). Mol Phylogenet Evol 36: 523-535.

Bastidas C, Benzie JAH, Fabricius KE (2002) Genetic differentiation among populations of the brooding soft coral *Clavularia koellikeri* on the Great Barrier Reef. Coral Reefs 21: 233-241.

Baus E, Darrock DJ, Bruford MW (2005) Gene-flow patterns in Atlantic and Mediterranean populations of the Lusitanian sea star *Asterina gibbosa*. Mol Ecol 14: 3373-3382.

Bayer FM (1953) Zoogeography and evolution in the octocorallian family Gorgoniidae. Bull Mar Sci Gulf Caribb 3: 100-119.

Bayer FM (1961) The shallow water Octocorallia of the West Indian region: a manual for marine biologists. Stud Fauna Curacao Other Caribb Isl 12: 1-373.

Bayer FM (1973) Colonial organization in octocorals. In: Boardman RS, Cheetham AH, Oliver WA Jr (eds) Animal colonies. Dowden, Hutchinson and Ross, Inc., Stroudsburg, Pennsylvania, pp 69-93.

Bayer FM (1981a) Key to the genera of Octocorallia exclusive of Pennatulacea (Coelenterata: Anthozoa), with diagnosis of new taxa. Proc Biol Soc Wash 94: 902-947.

Bayer FM (1981b) Status of knowledge of octocorals of world seas. Seminarios de Biologia Marinha. Academia Brasileira de Ciencias, Rio de Janeiro, pp. 3-11.

Bayer FM, Muzik KM (1976) A new solitary octocoral, *Taiaroa tauhou* gen. et sp. nov. (Coelenterata: Protoalcyonaria) from New Zealand. J R Soc N Z 6: 499-515.

Bayer FM, Muzik KM (1977) An Atlantic Helioporan coral (Coelenterata: Octocorallia). Proc Biol Soc Wash 90: 975-984.

Bayer FM, Grasshoff M, Verseveldt J (1983) Illustrated Trilingual Glossary of Morphological and Anatomical Terms Applied to Octocorallia. E J Brill, Leiden, The Netherlands.

Beaton MJ, Roger AJ, Cavalier-Smith T (1998) Sequence analysis of the mitochondrial genome of *Sarcophyton glaucum*: conserved gene order among octocorals. J Mol Evol 47: 697-708.

BeebeeTJ, Rowe G (2008) An introduction to molecular ecology. 2nd edition. Oxford University Press, New York.

Beiring EA, Lasker HR (2000) Egg production by colonies of a gorgonian coral. Mar Ecol Prog Ser 196: 169-177.

Benayahu Y (1991) Reproduction and developmental pathways of Red Sea Xeniidae (Octocorallia, Alcyonacea). Hydrobiologia 216/217: 125-130.

Benayahu, Y., Loya, Y. (1986). Sexual reproduction of a soft coral: Synchronous and brief annual spawning of *Sarcophyton glaucum* (Quoy and Gaimard). Biol Bull 170: 32-42.

Ben-Yosef Z, Benayahu Y (1999) The gorgonian coral *Acabaria biserialis*: life history of a successful colonizer of artificial substrata. Mar Biol 135: 473-481.

Berntson EA, France SC, Mullineaux LS (1999) Phylogenetic relationships within the Class Anthozoa (Phylum Cnidaria) based on nuclear 18S rDNA sequences. Mol Phylogenet

Evol 13: 417-433.
Berntson EA, Bayer FM, McArthur AG, France SC (2001) Phylogenetic relationships within the Octocorallia (Cnidaria: Anthozoa) based on nuclear 18S rRNA sequences. Mar Biol 138: 235-246.
Bérubé M, Aguilar A, Dendanto D, Larsen F, Di Sciara GN, Sears R, Sigurjónsson J, Urban-R J, Palsbøll PJ (1998) Population genetic structure of North Atlantic, Mediterranean Sea and Sea of Cortez fin whales, *Balaenoptera physalus* (Linnaeus 1758): analysis of mitochondrial and nuclear loci. Mol Ecol 7: 585-599.
Borsa P, Naciri M, Bahri L, Chikhi L, Garcia de Leon FJ, Kotoulas G, Bonhome F (1997) Zoogéographie infra-spécifique de la Mer Mediterranée: analyse des donées génétiques populationnelles sur seize espèces atlanto-méditerranéennes (poissins et invertebrates). Vie Milieu 47: 295-305.
Bramanti L, Magagnini G, De Maio L, Santangelo G (2005) Recruitment, early survival and growth of the Mediterranean red coral *Corallium rubrum* (L 1758), a 4-year study. J Exp Mar Biol Ecol 314: 69-78.
Brazeau DA, Lasker HR (1989) The reproductive cycle and spawning in a Caribbean gorgonian. Biol Bull 176: 1-7.
Brazeau DA, Lasker HR (1990) Sexual reproduction and external brooding by the Caribbean gorgonian *Briareum asbestinum*. Mar Biol 104: 465-474.
Bridge D, Cunningham CW, Desalle R, Buss LW (1995) Class-level relationships in the phylum Cnidaria - molecular and morphological evidence. Mol Biol Evol 12: 679-689.
Brown WM, George M , Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76: 1967-1971.
Bruckner AW (2002) Life-saving products from coral reefs. Issues Sci Technol 18: 39-44.
Bruno JF, Edmunds PJ (1997) Clonal variation for phenotypic plasticity in the coral *Madracis mirabilis*. Ecology 78: 2177-2190.
Buhl-Mortensen L, Mortensen PB (2004) Crustaceans associated with the deep-water gorgonian corals *Paragorgia arborea* (L., 1758) and *Primnoa resedaeformis* (Gunn., 1763). J Nat Hist 38: 1233-1247.
Buhl-Mortensen L, Mortensen PB (2005) Distribution and diversity of species associated with deep-sea gorgonian corals off Atlantic Canada. In: Freiwald A, Roberts JM (eds) Cold-water corals and ecosystems, Springer, Berlin Heidelberg, pp. 849-879.
Calderón I, Garrabou J, Aurelle D (2006) Evaluation of the utility of COI and ITS markers as tools for population genetic studies of temperate gorgonians. J Exp Mar Biol Ecol 336: 184-197.
Calderón I, Giribet G, Turon X (2008) Two markers and one history: phylogeography of the edible common sea urchin *Paracentrotus lividus* in the Lusitanian region. Mar Biol 154: 137-151.
Carpine C, Grasshoff M (1975) Les Gorgonaires de la Méditerranée. Bull Inst Océanograph Monaco.71: 1-140.
Charrier G, Chenel T, Durand JD, Girard M, Quiniou L, Laroche J (2006) Discrepancies in phylogeographical patterns of two European anglerfishes (*Lophius budegassa* and *Lophius piscatorius*). Mol Phylogenet Evol 38: 742-754.
Chen CA, Odorico DM, ten Lohuis M, Veron JEN, Miller DJ (1995) Systematic relationships within the Anthozoa (Cnidaria: Anthozoa) using the 5'-end of the 28S rDNA. Mol Phylogenet Evol 4: 175-183.
Claverie J-M, Grzela R, Lartigue A, Bernadac A, Nitsche S, Vacelet J, Ogata H, Abergel C (2009) Mimivirus and Mimiviridae: Giant viruses with an increasing number of potential hosts, including corals and sponges. J Invertebr Pathol 101: 172-180.
Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9: 1657-1659.
Coffroth MA (1984) Ingestion and incorporation of mucus aggregates by a gorgonian soft coral. Mar Ecol Prog Ser 17: 193-199.
Co□roth MA, Lasker HR (1998) Population structure of clonal gorgonian coral: the interplay between clonal reproduction and disturbance. Evolution 52: 379-393.
Coll JC (1992) The chemistry and chemical ecology of octocorals (Coelenterata, Anthozoa, Octocorallia). Chem Rev 92: 613-631.
Collins AG (2002) Phylogeny of Medusozoa and the evolution of cnidarian life cycles. J Evol Biol 15: 418-432.
Collins AG, Sshuchert P, Marques Ac, Jankowski T, Medina M, Schierwater B (2006) Medusozoan Phylogeny and Character Evolution Clarified by New Large and Small Subunit rDNA Data and an Assessment of the Utility of Phylogenetic Mixture Models. Syst Biol 55: 97-115.
Coma R, Gili J, Zabala M, Riera T (1994) Feeding and prey capture cycles in the aposymbiontic gorgonian *Paramuricea clavata*. Mar Ecol Prog Ser 115: 257-270.
Coma R, Ribes M, Zabala M, Gili J (1995) Reproduction and cycle of gonadal development in the Mediterranean gorgonian *Paramuricea clavata*. Mar Ecol Prog Ser 117: 173-183.
Concepcion GT, Crepeau MW, Wagner D, Kahng SE, Toonen RJ (2008) An alternative to ITS, a hypervariable, single-copy nuclear intron in corals, and its use in detecting cryptic species within the octocoral genus *Carijoa*. Coral Reefs 27: 323-336.
Conradi M, Megina C, López-González PJ (2004) Sibbling species of copepods in association with Mediterranean gorgonians. Scientia Marina 68: 85-96.
Constantini F, Fauvelot C, Abbiati M (2007a) Genetic structuring of the temperate gorgonian coral (*Corallium rubrum*) across the western Mediterranean Sea revealed by microsatellites and nuclear sequences. Mol Ecol 16: 5168-5182.
Constantini F, Fauvelot C, Abbiati M (2007b) Fine scale genetic structuring in *Corallium rubrum*: evidence of inbreeding and limited effective larval dispersal. Mar Ecol Prog Ser 340: 109-119.
Coyer JA, Diekmann OE, Serrao EA, Procaccini G, Milchakova N, Pearson GA, Stam WT, Olsen L (2004) Population genetics of dwarf eelgrass *Zostera noltii* throughout its biogeographic range. Mar Ecol Prog Ser 281: 51-62.
Crandall KA, Templeton AR (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. Genetics 134: 959-969.
Creer S, Malhotra A, Thorpe RS, Pook CE (2005) Targeting optimal Introns for phylogenetic analysis in non-model taxa: experimental results in Asian pitvipers. Cladistics 21: 390-395.
Culligan KM, Meyer-Gauen G, Lyons-Weiler J, Hays JB (2000) Evolutionary origin, diversification and specialization of eukaryotic MutS homolog mismatch repair proteins. Nucleic Acids Res 28: 463-471.
Cunha RL, Tenorio MJ, Afonso C, Castilho R, Zardoya R (2008) Replaying the tape: recurring biogeographical patterns in Cape Verde *Conus* after 12 million years. Mol

Ecol 17: 885-901.
Daemen E, Cross T, Ollevier F, Volckaert FAM (2001) Analysis of the genetic structure of European eel (*Anguilla anguilla*) using microsatellite DNA and mtDNA markers. Mar Biol 139: 755-764.
Dahan M, Benayahu Y (1997) Reproduction of *Dendronephthya hemprichi* (Cnidaria: Octocorallia): year-round spawning in an azooxanthellate soft coral. Mar Biol 129: 573-579.
Daly M, Brugler MR, Cartwright P, Collins AG, Dawson MN, Fautin DG, France SC, McFadden CS, Opresko DM, Rodriguez E, Romano SL, Stake JL (2007) The phylum Cnidaria: A review of phylogenetic patterns and diversity 300 years after Linnaeus. In: Zhang Z-Q, Shear WA (eds) Linnaeus Tercentenary: Progress in Invertebrate Taxonomy. Zootaxa 1668: 127-182.
Dinesen ZD (1983) Patterns in the distribution of soft corals across the central Great Barrier Reef. Coral Reefs 1: 229-236.
Drummond AJ, Ashton B, Cheung M, Heled J, Kearse M, Moir R, Stones-Havas S, Thierer T, Wilson A (2009) Geneious v3.5.6, Available from http://www.geneious.com/
Duran S, Pascual M, Turon X (2003) Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge *Crambe crambe* (Poecilosclerida). Mar Biol 144: 31-35.
Duran S, Giribet G, Turon X (2004a) Phylogeographical history of the sponge *Crambe crambe* (Porifera, Poecilosclerida): range expansion and recent invasion of the Macaronesian islands from the Mediterranean Sea. Mol Ecol 13: 109-122.
Duran S, Pascual M, Estoup A, Turon X (2004b) Strong population structure in the marine sponge *Crambe crambe* (Poecilosclerida) as revealed by microsatellite markers. Mol Ecol 13: 511-522.
Eisen JA (1998) A phylogenomic study of the MutS family of proteins. Nucleic Acids Res 26: 4291-4300.
Elder JF, Turner BJ (1995) Concerted evolution of repetitive DNA sequences in eukaryotes. Quart Rev Biol 70: 297-320.
Encalada SE, Lahanas PN, Bjorndal KA, Bolten Ab, Miyamoto MM, Bowens BW (1996) Phylogeography and population structure of the Atlantic and Mediterranean green turtle *Chelonia mydas*: a mitochondrial DNA control region sequence assessment. Mol Ecol 5: 473-483.
Encalada SE, Bjorndal KA, Bolten AB, Zurita JC, Schroeder B, Possardt E, Sears CJ, Bowen BW (1998) Population structure of loggerhead turtle (*Caretta caretta*) nesting colonies in the Atlantic and Mediterranean as inferred from mitochondrial DNA control region sequences. Mar Biol 130: 567-575.
Epifanio RA, Maia LF, Pawlik JR, Fenical W (2007) Antipredatory secosterols from the octocoral *Pseudopterogorgia Americana*. Mar Ecol Prog Ser 329: 307-310.
Eytan RI, Hayes M, Arbour-Reiley P, Miller M, Hellberg ME (2009) Nuclear sequences reveal mid-range isolation of an imperilled deep-water coral population. Mol Ecol 18: 2375-2389.
Fabricius KE (1997) Soft coral abundance on the central Great Barrier Reef: effects of *Acanthaster planci,* space availability, and aspects of the physical environment. Coral Reefs 16: 159-167.
Fabricius KE, Benayahu Y, Genin A (1995a) Herbivory in asymbiotic soft corals. Science 268: 90-92.
Fabricius KE, Genin A, Benayahu Y (1995b) Flow-dependent herbivory and growth in zooxanthellae-free soft corals. Limnol Oceanogr 40: 1290-1301.
Fabricius KE, Dommisse M (2000) Depletion of suspended particulate matter over coastal reef communities dominated by zooxanthellate soft corals. Mar Ecol Prog Ser 196: 157-167.
Fabricius K, Alderslade P (2001) Soft Corals and Sea Fans: A Comprehensive Guide to the Tropical Shallow-Water Genera of the Central-West Pacific, the Indian Ocean and the Red Sea. Australian Institute of Marine Science, Townsville.
Fadlallah YH (1983) Sexual reproduction, development and larval biology in scleractinian corals. Coral Reefs 2: 129-150.
Fallin D, Schork NJ (2000) Accuracy of Haplotype Frequency Estimation for Biallelic Loci, via the Expectation-Maximization Algorithm for Unphased Diploid Genotype Data. Am J Hum Genet 67: 947-959.
Farrant PA (1987) Population dynamics of the temperate Australian soft coral *Capnella gaboensis*. Mar Biol 96: 401-407.
Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.
Flot J-F, Tillier A, Samadi S, Tillier S (2006) Phase determination from direct sequencing of length-variable DNA regions. Mol Ecol Notes 6: 627-630.
Foltz DW (2007) An Ancient Repeat Sequence in the ATP Synthase β-Subunit Gene of Forcipulate Sea Stars. J Mol Evol 65: 564-573.
France SC, Rosel PE, Agenbroad JE, Mullineaux LS, Kocher TD (1996) DNA sequence variation of mitochondrial large-subunit rRNA provides support for a two-subclass organization of the Anthozoa (Cnidaria). Mol Mar Biol Biotech 5: 15-28.
France SC, Hoover LL (2001) Analysis of variation in mitochondrial DNA sequences (ND3, ND4L, MSH) among Octocorallia (=Alcyonaria) (Cnidaria: Anthozoa). Bull Biol Soc Wash 10: 110-118.
France SC, Hoover LL (2002) DNA sequences of the mitochondrial COI gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). Hydrobiol 471: 149-155.
Fukami H, Budd AF, Paulay G, Solé-Cava A, Chen CA, Iwao K, Knowlton N (2004) Conventional taxonomy obscures deep divergence between Pacific and Atlantic corals. Nature 427: 832-835.
Galtier N, Nabholz B, Glémin S, Hurst GDD (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal Mol Ecol 18: 4541-4550.
Garrabou J, Perez T, Sartoretto S, Harmelin JG (2001) Mass mortality event in red coral *Corallium rubrum* populations in the Provence region (France, NW Mediterranean). Mar Ecol Prog Ser 217: 263-272.
Geiser DM, Taylor JW, Ritchie KB, Smith GW (1998) Cause of sea fan death in the West Indies. Nature 394: 137–138.
Gerhart DJ (1986) Gregariousness in the gorgonian-eating gastropod *Cyphoma gibbosum*: tests of several possible causes. Mar Ecol Prog Ser 31: 255-263.
Gerhart DJ (1990) Fouling and gastropod predation: consequences of grazing for a tropical octocoral. Mar Ecol Prog Ser 62: 103-108.
Gerhart DJ, Rittschof DJ, Mayo SW (1988) Chemical ecology and the search for marine anti-foulants: studies of a prefator-prey symbiosis. J chem Ecol 14: 1905-1917.
Gerhart DJ, Coll JC (1993) Pukalide, a widely distributed octocoral diterpenoid, induces vomiting in fish. J Chem

Ecol 19: 2697-2704.
Gili J, Coma R (1998) Benthic suspension feeders: their paramount role in littoral marine food webs. Tree 13: 316-321.
Gili J, López-González JP, Bouillon J (2006) A new Antarctic association: the case of the hydroid *Sarsia medelae* (new sp.) associated with gorgonians. Polar Biol 29: 624-631.
Grasshoff M (1988) The genus Leptogorgia (Octocorallia: Gorgoniidae) in West Africa. Atl Rep 14: 91-147.
Grasshoff M (1992) Die Flachwasser-Gorgonarien von Europa und Westafrika (Cnidaria, Anthozoa). Courier Forschungsinstitut Senckenberg 149: 1-135.
Grasshoff M (1999) The shallow water gorgonians of New Caledonia and adjacent islands (Coelenterata: Octocorallia). Senkenber Biol 78: 1-121.
Grasshoff M (2001) Taxonomy, systematics, and octocorals: to Frederick M. Bayer, October 31st, 2001. Bull Biol Soc Wash 10: 3-14.
Guindon S, Gascuel O (2003) A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. Syst Biol 52: 696-704.
Gutiérrez-Rodríguez C, Lasker HR (2004a) Reproductive biology, development and planula behavior in the Caribbean gorgonian *Pseudopterogorgia elisabethae*. Invertebr Biol 123: 54-67.
Gutiérrez-Rodríguez C, Lasker HR (2004b) Microsatellite variation reveals high levels of genetic variability and population structure in the gorgonian coral *Pseudopterogorgia elisabethae* across the Bahamas. Mol Ecol 13: 2211-2221.
Gutiérrez-Rodríguez C, Lasker HR (2005) Microsatellite variation reveals high levels of genetic variability and population structure in the gorgonian coral *Pseudopterogorgia elisabethae* across the Bahamas. Mol Ecol 14: 4205-4206.
Gutiérrez-Rodríguez C, Barbeitos MS, Sánchez JA, Lasker HR (2009) Phylogeography and morphological variation of the branching octocoral *Pseudopterogorgia elisabethae*. Mol Phylogenet Evol 50: 1-15.
Gysels ES, Hellemans B, Pampoulie C, Volckaert FAM (2004) Phylogeography of the common goby, *Pomatoschistus microps*, with particular emphasis on the colonization of the Mediterranean and the North Sea. Mol Ecol 13: 403-417.
Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95-98.
Hall-Spencer JM, Pike P, Munn CB (2007) Diseases affect cold-water corals too: *Eunicella verrucosa* (Cnidaria: Gorgonacea) necrosis in SW England. Dis Aquat Org 76: 87-97.
Harvell CD, Fenical W, Greene CH (1988) Chemical and structural defenses of Caribbean gorgonians (*Pseudopterogorgia* spp.) I. Development of an *in situ* feeding assay. Mar Ecol Prog Ser 49: 287-294.
Harvell D, Kim K, Quirolo C, Weir J, Smith G (2001) Coral bleaching and disease: contributors to 1998 mass mortality in *Briareum asbestinum* (Octocorallia, Gorgonacea). Hydrobiologia 460: 97-104.
Hatta M, Fukami H, Wang WQ, Omori M, Shimoike K, Hayashibara T, Ina Y, Sugiyama T (1999) Reproductive and genetic evidence for a reticulate evolutionary history of mass-spawning corals. Mol Biol Evol 16: 1607-1613.
Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. Proc R Soc Lond Biol Sci B 270: 313-321.
Hebert PDN, Ratnasingham S, deWaard JR (2003b) Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc R Soc Lond B Biol Sci 270: S96-S99.
Hill MS, Hill AL (2002) Morphological Plasticity in the Tropical Sponge *Anthosigmella varians*: Responses to Predators and Wave Energy. Biol Bull 202: 86-95.
Karlson RH, Hughes TP, Karlson SR (1996) Density-dependent dynamics of soft coral aggregations: the significance of clonal growth and form. Ecology 77: 1592-1599.
Kelman D, Benayahu Y, Kashman Y (1999) Chemical defence of the soft coral *Parerythropodium fulvum fulvum* (Forskål) in the Red Sea against generalist reef fish. J Exp Mar Biol Ecol 238: 127-137.
Kim K, Harvell CD (2004) The rise and fall of a six-year coral–fungal epizootic. Am Nat 164: 52-63.
Kim E, Lasker HR, Coffroth MA, Kim K (2004) Morphological and genetic variation across reef habitats in a broadcast-spawning octocoral. Hydrobiologia 530/531: 423-432.
Kishino H, Hasegawa M (1989) Evaluation of the maximum-likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J Mol Evol 29: 170-179.
Knowlton N (1993) Sibling species in the sea. Ann Rev Ecol Syst 24: 189-216.
Knowlton N (2000) Molecular genetic analyses of species boundaries in the sea. Hydrobiol 420: 73-90.
Knowlton N, Weigt LA (1997) Species of marine invertebrates: a comparison of the biological and phylogenetic species concepts. In: Claridge MF, Dawah HA , Wilson MR (eds) Species: the Units of Biodiversity, Chapman & Hall, London, pp 199-219.
Krieger KJ, Wing B (2002) Megafauna associations with deepwater corals (*Primnoa* spp.) in the Gulf of Alaska. Hydrobiol 471: 83-90.
Kumagai NH (2008). Role of food source and predator avoidance in habitat specialization by an octocoral-associated amphipod. Oecologia 155: 739-749.
Lasker HR (1981) A comparison of the particulate feeding habilities of three species of gorgonian soft coral. Mar Ecol Prog Ser 5: 61-67.
Lasker HR (1984) Asexual reproduction, fragmentation, and skeletal morphology of a plexaurid gorgonian. Mar Ecol Prog Ser 19: 261-268.
Lasker HR (2006) High fertilization success in a surface-brooding Caribbean gorgonian. Biol Bull 210: 10-17.
Lasker HR, Coffroth MA, Fitzgerald LM (1988) Foraging patterns of *Cyphoma gibbosum* on octocorals: the roles of host choice and feeding preference. Biol Bull Mar Biol Lab, Woods Hole 174: 254-266.
Lasker HR, Brazeau DA, Calderon J, Coffroth MA, Coma R, Kim K (1996) *In situ* rates of fertilization among broadcast spawning gorgonian corals. Biol Bull 190: 45-55.
Lasker HR, Kim K, Coffroth MA (1998) Production, survival and recruitment of a plexaurid gorgonian. Mar Ecol Prog Ser 162: 111-123.
Leversee GJ (1976) Flow and feeding in fan-shaped colonies of the gorgonian coral, *Leptogorgia*. Biol Bull 151: 344-356.
Lewis JC, von Wallis E (1991) The function of surface sclerites in gorgonians (Coelenterata: Octocorallia). Biol Bull 181: 275-288.
Li NK, Denny MW (2004) Limits to Phenotypic Plasticity: Flow Effects on Barnacle Feeding Appendages. Biol Bull 206: 121-124.
Liao D (2000) Gene conversion drives within genic

sequences: converted evolution of ribosomal RNA genes in bacteria and archaea. J Mol Evol 51: 305-17.

Lin C, Osawa M, Chan T (2007) A new *Munidopsis* (Crustacea: Decapoda: Galatheidae) associated with gorgonian corals from the deep waters off Taiwan. Proc Biol Soc Wash 120: 167-174.

Linares C, Doak DF, Coma R, Díaz D, Zabala M (2007) Life history and viability of a long-lived marine invertebrate: the octocoral *Paramuricea clavata*. Ecology 88: 918-928.

Maddison DR, Maddison WP (2001) MacClade 4: analysis of phylogeny and character evolution. Version 4.08. Sunderland, MA: Sinauer Associates.

Mackenzie JB, Munday PL, Willis BL, Miller DJ, van Oppen MJH (2004) Unexpected patterns of genetic structuring among locations but not colour morphs in *Acropora nasuta* (Cnidaria; Scleractinia). Mol Ecol 13: 9-20.

Malik HS, Henikoff S (2000) Dual recognition–incision enzymes might be involved in mismatch repair and meiosis. Trends Biochem Sci 25: 414-418.

Marchinko KB (2003) Dramatic phenotypic plasticity in barnacle legs (*Balanus glandula* Darwin): Magnitude, age-dependence and speed of response. Evolution 57: 1281-1290.

Marschal C, Garrabou J, Harmelin JG, Pichon M (2004) A new method for measuring growth and age in the precious red coral *Corallium rubrum* (L.). Coral Reefs 23: 423-432.

Martin Y, Bonnefort JL, Chancerelle L (2002) Gorgonians mass mortality during the 1999 late summer in French Mediterranean coastal waters: the bacterial hypothesis. Water Res 36: 779-782.

Mayden RL (1997) A hierarchy of species concepts: the denouement in the saga of the species problem. In: Claridge MF, Dawah HA, Wilson MR (eds) Species: the Units of Biodiversity, Chapman & Hall, London, pp 381-424.

Mayer AM, Jacobson PB, Fenical W, Jacobs RS, Glaser KB (1998) Pharmacological characterization of the pseudopterosins: novel anti-inflammatory natural products isolated from the Caribbean soft coral, *Pseudopterogorgia elisabethae*. Life Sciences 62: 401-407.

McFadden CS, Tullis ID, Hutchinson MB, Winner K, Sohm JA (2004) Variation in coding (NADH dehydrogenase subunits 2, 3, and 6) and noncoding intergenic spacer regions of the mitochondrial genome in Octocorallia (Cnidaria: Anthozoa). Mar Biotech 6: 516-526.

McFadden CS, France SC, Sánchez JA, Alderslade PA (2006a) A molecular phylogenetic analysis of the Octocorallia (Cnidaria: Anthozoa) based on mitochondrial protein-coding sequences. Mol Phylogenet Evol 41: 513-527.

McFadden CS, Alderslade P, van Ofwegen LP, Johnsen H, Rusmevichientong A (2006b) Phylogenetic relationships within the tropical soft coral genera *Sarcophyton* and *Lobophytum* (Anthozoa, Octocorallia). Invertebr Biol 125: 288-305.

Medina M, Weil E, Szmant AM (1999) Examination of the *Montastraea annularis* species complex (Cnidaria: Scleractinia) using ITS and COI sequences. Mar Biotech 1: 89-97.

Medina M, Collins AG, Silberman JD, Sogin ML (2001) Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. Proc Natl Acad Sci USA 98: 9707-9712.

Migné A, Davoult D (2002) Experimental nutrition in the soft coral *Alcyonium digitatum* (Cnidaria: Octocorallia): removal rate of phytoplankton and zooplankton. Cah Biol Mar 43: 9-16.

Mistri M, Ceccherelli VU (1994) Growth and secondary production of the Mediterranean gorgonian *Paramuricea clavata*. Mar Ecol Prog Ser 103: 291-296.

Mitchell ND, Dardeau MR, Schroeder WW, Benke AC (1992) Secondary production of gorgonian corals in the northern Gulf of Mexico. Mar Ecol Prog Ser 87: 275-281.

Mokhtar-Jamaï K, Ledoux J-B, Garrabou J, Feral J-P, Aurelle D (2009) Interest and application of genetic markers for the study and conservation of Mediterranean sessisle invertebrates. UNEP-MAP-RAC/SPA (2009) Proceedings of the 1st Mediterranean symposium on the conservation of the coralligenus and other calcareous bio-concretions (Tabarka, 15-16 January 2009). C Pergent Martini, M Brichet (eds), RAC/SPA publ, Tunis: 269 p.

Muko S, Kawasaki K, Sakai K, Takasu F, Shigesada N (2000) Morphological plasticity in the coral *Porites sillimaniani* and its adaptive significance. Bull Mar Sci 66: 225-239.

Munro L (2004) Determining the reproductive cycle of *Eunicella verrucosa*. Ref: RR Report 07/2004 ETR 12.

Myers AA, Hall-Spencer JM (2004) A new species of amphipod crustacean, *Pleusymtes comitari* sp. nov., associated with gorgonians on deep-water coral reefs of Ireland. J Mar Biol Ass UK 84: 1029-1032.

Naciri M, Lemaire C, Borsa P, Bonhomme F (1999) Genetic study of the Atlantic/Mediterranean transition in sea bass (*Dicentrarchus labrax*). J Hered 90: 591-596.

Natoli A, Birkun A, Aguilar A, Lopez A, Hoelzel AR (2005) Habitat structure and the dispersal of male and female bottlenose dolphins (*Tursiops truncatus*). Proc R Soc Lond Biol Sci B 272: 1217-1226.

Nikula R, Väinölä R (2003) Phylogeography of *Cerastoderma glaucum* (Bivalvia: Cardiidae) across Europe: a major break in the Eastern Mediterranean. Mar Biol 143: 339-350.

Orejas C, López-González PJ, Gili JM, Teixidó N, Gutt J, Arntz WE (2002) Distribution and reproductive ecology of the Antarctic octocoral *Ainigmaptilon antarcticum* in the Weddell Sea. Mar Ecol Prog Ser 231: 101-114.

Palumbi SR (1984) Tactics of acclimation: morphological changes of sponges in an unpredictable environment. Science 225: 1478-1480.

Palumbi SR (1996) What can molecular genetics contribute to marine biogeography? An urchin's tale. J Mar Biol Ecol 203: 75-92.

Patarnello T, Volckaert FAMJ, Castilho R (2007) Pillars of Hercules: is the Atlantic–Mediterranean transition a phylogeographical break?. Mol Ecol 16: 4426-4444.

Patterson MR (1984) Patterns of whole colony prey capture in the octocoral, *Alcyonium siderium*. Biol Bull 167: 613-629.

Patterson MR (1991) Passive suspension feeding by an octocoral in plankton patches: empirical test of a mathematical model. Biol Bull 180: 81-92.

Peijnenburg KTCA, Breeuwer JAJ, Pierrot-Bults AC, Menken SBJ (2004) Phylogeography of the planktonic chaetognath *Sagitta setosa* reveals isolation in European seas. Evolution 58: 1472-1487.

Pérez-Losada M, Guerra A, Carvalho GR, Sanjuan A, Shaw PW (2002) Extensive population subdivision of the cuttlefish *Sepia officinalis* (Mollusca: Cephalopoda) around the Iberian Peninsula indicated by microsatellite DNA variation. Heredity 89: 417-424.

Pigliucci M (2005) Evolution of phenotypic plasticity: where are we going now?. Trends Ecol Evol 20: 481-486.

Pont-Kingdon G, Okada NA, Macfarlane JL, Beagley CT, Wolstenholme DR, Cavalier-Smith T, Clark-Walker GD

(1995). A coral mitochondrial MutS gene. Nature 375: 109-111.

Pont-Kingdon G, Okada NA, Macfarlane JL, Beagley CT, Watkins-Sims CD, Cavalier-Smith T, Clark-Walker GD, Wolstenholme DR (1998) Mitochondrial DNA of the coral *Sarcophyton glaucum* contains a gene for a homologue of bacterial MutS: a possible case of gene transfer from the nucleus to the mitochondrion. J Mol Evol 46: 419-431.

Posada D, Crandall KA (1998) Modeltest: Testing the model of DNAsubstitution. Bioinformatics 14: 817-818.

Prada C, Schizas NV, Yoshioka PM (2008) Phenotypic plasticity or speciation? A case from a clonal marine organism BMC Evol Biol 8, 47.

Remerie T, Bourgois T, Peelaers D, Vierstraete A, Vanfleteren J, Vanreusel A (2006) Phylogeographic patterns of the mysid *Mesopodopsis slabberi* (Crustacea, Mysida) in Western Europe: evidence for high molecular diversity and cryptic speciation Mar Biol 149: 465-481.

Ribes M, Coma R, Gili J (1998) Heterotrophic feeding by gorgonian corals with symbiotic zooxanthellae. Limnol Oceanogr 43: 1170-1179.

Ribes M, Coma R, Gili J (1999) Heterogeneous feeding in benthic suspension feeders: the natural diet and grazing rate of the temperate gorgonian *Paramuricea clavata* (Cnidaria: Octocorallia) over a year cycle. Mar Ecol Prog Ser 183: 125-137.

Ribes M, Coma R, Rossi S (2003) Natural feeding of the temperate asymbiotic octocoral-gorgonian *Leptogorgia sarmentosa* (Cnidaria: Octocorallia). Mar Ecol Prog Ser 254: 141-150.

Ribes M, Coma R, Rossi S, Micheli M (2007) Cycle of gonadal development in *Eunicella singularis* (Cnidaria: Octocorallia): trends in sexual reproduction in gorgonians. Invertebr Biol 126: 307-317.

Romualdi C, Balding D, Nasidze IS, Risch G, Robichaux M, Sherry ST, Stoneking M, Batzer MA, Barbujani G (2002) Patterns of Human Diversity, within and among Continents, Inferred from Biallelic DNA Polymorphisms. Genome Res 12: 602-612.

Ruesink JL, Harvell CD (1990) Specialist predation on the Caribbean gorgonian *Plexaurella* spp. by *Cyphoma signatum* (Gastropoda). Mar Ecol Prog Ser 65: 265-272.

Sammarco PW, Co1l JC (1992) Chemical adaptations in the Octocorallia: evolutionary considerations. Mar Ecol Prog Ser 88: 93-104.

San Martín G, Nishi E (2001) A New Species of *Alcyonosyllis* Glasby and Watson, 2001 (Polychaeta: Syllidae: Syllinae) from Shimoda, Japan, Commensal with the Gorgonian *Melithaea flabellifera*. Zool Sci 20: 371-375.

Sánchez JA (2007) A new genus of Atlantic octocorals (Octocorallia:Gorgoniidae): systematics of gorgoniids with asymmetric sclerites. J Nat Hist 41: 493-509.

Sánchez JA, Lasker HR, Taylor DJ (2003a) Phylogenetic analyses among octocorals (Cnidaria): Mitochondrial and nuclear DNA sequences (lsu-rRNA, 16S and ssu-rRNA, 18S) support two convergent clades of branching gorgonians. Mol Phylogenet Evol 29: 31-42.

Sánchez JA, McFadden CS, France SC, Lasker HR (2003b) Molecular phylogenetic analyses of shallow-water Caribbean octocorals. Mar Biol 142: 975-987.

Sánchez JA, Aguilar C, Dorado D, Manrique N (2007) Phenotypic plasticity and morphological integration in a marine modular invertebrate. BMC Evol Biol 7, 122.

Santangelo G, Carletti E, Maggi E, Bramanti L (2003) Reproduction and population sexual structure of the overexploited Mediterranean red coral *Corallium rubrum*. Mar Ecol Prog Ser 248: 99-108.

Schleyer MH, Kruger A, Benayahu Y (2004) Reproduction and the unusual condition of hermaphroditism in *Sarcophyton glaucum* (Octocorallia, Alcyoniidae) in KwaZulu-Natal, South Africa. Hydrobiologia 530/531: 399-409.

Schlichting CD (1986) The evolution of phenotypic plasticity in plants. Annu Rev Ecol Syst 17: 667-693.

Shearer TL, Van Oppen MJH, Romano SL, Wörheide G (2002) Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Mol Ecol 11: 2475-2487.

Shimodaira H (2002) An approximately unbiased test of phylogenetic tree selection. Syst Biol 51: 492-508.

Shimodaira H, Hasegawa M (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol Biol Evol 16:1114-1116.

Shimodaira H, Hasegawa M (2001) CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17: 1246-1247.

Simmons MP, Ochoterena H (2000) Gaps as Characters in Sequence-Based Phylogenetic Analyses. Syst Biol 49: 369-381.

Smith VG, Gosliner TM (2003) A New Species of *Tritonia* from Okinawa (Mollusca: Nudibranchia), and its Association with a Gorgonian Octocoral. Proc Calif Acad Sci 54: 255-278.

Sousa-Santos C, Robalo JI, Collares-Pereira MJ, Almada VC (2005) Heterozygous indels as useful tools in the reconstruction of DNA sequences and in the assessment of ploidy level and genomic constitution of hybrid organisms. DNA Sequence 16: 462-467.

Souza JRB, Rodrigues HA, Neves BM, Perez CD (2007) First report of bristleworm predator of the reef octocoral *Carijoa riisei*. Coral Reefs 26: 1033.

Sponaugle S, LaBarbera M (1991) Drag-induced deformation: a functional feeding strategy in two species of gorgonians. J Exp Mar Biol Ecol. 148: 121-134.

Stamatis C, Triantafyllidis A, Moutou KA, Mamuris Z (2004) Mitochondrial DNA variation in northeast Atlantic and Mediterranean populations of Norway lobster, *Nephrops norvegicus*. Mol Ecol 13: 1377-1390.

Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68: 978-989.

Sultan SE (2000) Phenotypic plasticity for plant development, function and life history. Trends Plant Sci 5: 537-542.

Swofford DL (2002) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods), Version 4. Sinauer Associates, Sunderland, MA.

Tahera Q (2001) Echinoderms epizoic on gorgonian corals from Karachi coast. Pak J Biol Sci 4: 1177-1179.

Targett NM, Bishop SS, McConnell, OJ, Yoder JA (1983) Antifouling agents against the benthic marine diatom *Navicula salinicola*: homarine from the gorgonian *Leptogorgia virgulata* and *L. setacea*, and analogues. J chem Ecol 9: 817-829.

Tsounis G, Rossi S, Aranguren M, Gili JM, Arntz WE (2006) Effects of spatial variability and colony size on the reproductive output and gonadal development cycle of the Mediterranean red coral (*Corallium rubrum* L.). Mar Biol 148: 513-527.

Tursch B, Tursch A (1982) The soft coral community on a sheltered reef quadrat at Laing Island (Papua New Guinea). Mar Biol 68: 321-332.

van Alstyne KL, Paul VJ (1992) Chemical and structural antipredator deterrents in the sea fan *Gorgonia ventalina*: effects against generalist and specialist predators. Coral Reefs 11: 155-160.

van Oppen MJH, Hislop NR, Hagerman PJ, Miller DJ (1999) Gene content and organization in a segment of the mitochondrial genome of the scleractinian coral *Acropora tenuis*: major differences in gene order within the anthozoan subclass Zoantharia. Mol Biol Evol 16: 1812-1815.

van Oppen MJH, Willis BL, Van Vugt H, Miller DJ (2000) Examination of species boundaries in the *Acropora cervicornis* group (Scleractinia, Cnidaria) using nuclear DNA sequence analyses. Mol Ecol 9: 1363-1373.

van Oppen MJH, McDonald BJ, Willis B, Miller DJ (2001) The evolutionary history of the coral genus *Acropora* (Scleractinia, Cnidaria) based on a mitochondrial and a nuclear marker: Reticulation, incomplete lineage sorting, or morphological convergence?. Mol Biol Evol 18: 1315-1329.

Vieira P (2008) Caracterização de espécies de gorgónias (Cnidaria:Gorgonacea) da costa Algarvia. M.Sc. Dissertation, University of Algarve.

Vollmer SV, Palumbi SR (2002) Hybridization and the Evolution of Reef Coral Diversity. Science 296: 2023-2025.

Wägele J-W (2005) Foundations of phylogenetic systematics. Verlag Dr. Friedrich Pfeil, München.

Wagner D, Kahng SE, Toonen RJ (2007) New report of nudibranch predators of the invasive octocoral *Carijoa riisei* in the Main Hawaiian Islands. Coral Reefs 26: 411.

Walker TA, Bull GD (1983) A newly discovered method of reproduction in gorgonian coral. Mar Ecol Prog Ser 12: 137-143.

Weinbauer MG, Velimirov B (1995) Biomass and secondary production of the temperate gorgonian coral *Eunicella cavolini* (Coelenterata: Octocorallia). Mar Ecol Prog Ser 121: 211-216.

Weinbauer MG, Velimirov B (1996) Population dynamics and overgrowth of the sea fan *Eunicella cavolini* (Coelenterata: Octocorallia). Estuar Coast Shelf Sci 42: 583-595.

Weinberg S, Weinberg F (1979) The life cycle of a gorgonian: *Eunicella singularis* (Esper 1794). Bijdr Dierk 48: 127-137.

Wendt PH, van Dolah RF, O'Rourke CB (1985). A comparative study of the invertebrate macrofauna associated with seven sponge and coral species collected from the South Atlantic Bight. J Elisha Mitchell Sci Soc 101: 187-203.

West JM, Harvell CD, Walls AM (1993) Morphological plasticity in a gorgonian coral (*Briareum asbestinum*) over a depth cline. Mar Ecol Prog Ser 94: 61-69.

Wilke T, Pfenninger M (2002) Separating historic events from recurrent processes in cryptic species: phylogeography of mud snails (*Hydrobia* spp.). Mol Ecol 11: 1439-1451.

Williams GC, Lindo KG (1997) A review of the Octocorallian genus *Leptogorgia* (Anthozoa: Gorgonidae) in the Indian Ocean and subantarctic, with description of a new species and comparisons with related taxa. Proc Calif Acad Sci 49: 499-521.

Williams GC, Cairns SD (2009) Systematic list of valid octocoral genera. <http://www.calacademy.org/research/izg/OCTOCLASS.htm/>.

Williams ST, Reid DG (2004) Speciation and diversity on tropical rocky shores: a global phylogeny of snails of the genus *Echinolittorina*. Evolution 58: 2227-2251.

Wirshing HH, Messing CG, Douady CJ, Reed J, Stanhope MJ, Shivji MS (2005) Molecular evidence for multiple lineages in the gorgonian family Plexauridae (Anthozoa: Octocorallia). Mar Biol 147: 497-508.

Wolfe KH, Li WH, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc Natl Acad Sci USA 84: 9054-9058.

Won JH, Rho BJ, Song JI (2001) A phylogenetic study of the Anthozoa (phylum Cnidaria) based on morphological and molecular characters. Coral Reefs 20: 39-50.

Wörheide G (2006) Low variation in partial cytochrome oxidase subunit I (COI) mitochondrial sequences in the coralline demosponge *Astrosclera willeyana* across the Indo-Pacific. Mar Biol 148: 907-912.

Yang Z (1997) PAML: A program package for phylogenetic analysis by maximum likelihood. CABIOS 13: 555-556.

Yoshioka PM, Yoshioka BB (1991) A comparison of survivorship and growth of shallow-water gorgonian species of Puerto Rico. Mar Ecol Prog Ser 69: 253-260.

Zane L, Ostellari L, Maccatrozzo L, Bargelloni L, Cuzin-Roudy J, Buchholz F, Patarnello T (2000) Genetic differentiation in a pelagic crustacean (*Meganyctiphanes norvegica*: Euphausiacea) from the North East Atlantic and the Mediterranean Sea. Mar Biol 136: 191-199.

Zhang DX, Hewitt GM (2003) Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Mol Ecol 12: 563-584.