Universidade de Aveiro Departamento de Biologia
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciências do Mar, realizada sob a orientação científica do Doutor Henrique José de Barros Brito Queiroga, Professor Associado do Departamento de Biologia da Universidade de Aveiro, da Doutora Silvia Cocito, investigadora do ENEA, Italian National Agency for New Techoligies, Energy and Sustainable Economic Development, Marine Environment Research Centre, La Spezia, Italy e da Professora Anna Occhipinti, Full Professor of Ecology, Department of Earth and Environmental Sciences, University of Pavia, Italy

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agradecimentos

I would like to thank my supervisors, Prof. Henrique Queiroga and Dr. Silvia Cocito, for their patient guidance, encouragement and advices they have provided throughout my time as their student. I would also like to thank Prof. Ana Ochipinti for her help.

This study was supported by the Ph. D. grant from the Erasmus Mundus Marine Ecosystem Health and Conservation (MARES) doctoral program; http://www.mares-eu.org/. and the DiverseShores research project (Testing associations between genetic and community diversity in European rocky shore environments), funded by Fundação para a Ciência e Tecnologia (PTDC/BIA-BIC/114526/2009). Financial support was allocated by FCT under the COMPETE Programme, which includes components from the European Regional Development Fund and from the Ministério da Ciência, Tecnologia e Ensino Superior.

Special thanks to C. Lombardi, A. Peirano, R. Albuquerque, F. Fernandes, J. Rodrigues and E. Mancuso for their help during the fieldwork.

palavras-chave gorgônias, mortalidade, dispersão, genética, conservação

resumo

A gorgónia vermelha, *Paramuricea clavata*, é uma espécie engenheira que habita costas e recifes rochosos do Mediterrâneo e do Atlântico ao longo da costa de Portugal, a profundidades que variam entre 15 e os 80 m. Esta espécie foi severamente afetada, no Mediterrâneo NO, por eventos de mortalidade induzidos por variações climáticas recentes. O objetivo geral deste estudo foi a investigação da diversidade genética de *P. clavata* no Mar da Ligúria (Mediterrâneio NO), uma região altamente impactada por eventos de mortalidade em massa causados por temperaturas elevadas, e no Atlântico, onde mortalidade em massa nunca foi observada em consequência de temperaturas genericamente mais baixas. Foram utilizados microsatélites para o estudo da contribuição da reprodução clonal, padrões de conectividade, estrutura genética e diversidade genética. Adicionalmente, um marcador mitocondrial (Cytochrome Oxidase I) foi usado para comparar as populações do Atlântico e do Mediterrâneo. Os resultados revelaram que a propagação clonal não desempenha um papel importante em *P. clavata*, uma vez que em quatro dos nove sítios não foram detetados clones e que a máxima prevalência de clones detetada atingiu apenas 13%. No entanto, a prevalência de clones detectada no presente estudo foi maior do que o previamente relatado. O estudo não conseguiu detetar qualquer perda de diversidade genética nas populações de *P. clavata* afetadas por eventos de mortalidade em massa. Foi possível descrever o padrão de migração entre os sítios afetados pela mortalidade em massa e os não afetados. Os resultados confirmaram que a baixa capacidade de dispersão larvar na gorgónia vermelha pode ainda ser ecologicamente significante para a recolonização e persistência populacional, permitindo a migração entre populações locais. A troca de larvas foi mais comum entre recifes separados por 200-300m, mas também foi detectada entre locais separados por 20 km. Os dados indicaram ainda migrações comuns entre recifes localizados a menores profundidades, impactados por mortalidade em massa, e recifes mais profundos, não impactados, do mesmo local. A presente investigação identificou uma importante descontinuidade genética na distribuição da gorgónia vermelha, com ambos os marcadores utilizados no estudo, mtDNA e microsatélites, revelando a mesma descontinuidade entre o Mediterrâneo e o Atlântico. Foram também encontradas diferenças significativas na diversidade genética entre o Mediterrâneo e as populações do Oceano Atlântico, com a heterozigosidade e a riqueza alélica ligeiramente, mas significativamente, mais elevadas no Mediterrâneo, possivelmente como resultado da história da colonização ou isolamento dos locais do Atlântico. Finalmente, foram ainda detectadas diferenças na diversidade genética entre as populações superficiais e mais profundas. A riqueza alélica foi menor nas populações menos profundas, menos estáveis devido a eventos de mortalidade induzidos pelo aquecimento e por outros fatores, e maior nas populações mais profundas e estáveis. Estes resultados devem revelar-se particularmente valiosos para a conservação de comunidades de gorgónias e assim a biodiversidade marinha global.

keywords gorgonians, mortality, dispersal, genetics, conservation

abstract

The red gorgonian *Paramuricea clavata* is an engineering species, inhabiting rocky shore in the Mediterranean Sea and Portuguese coast of the Atlantic Ocean. The species was severely impacted by climatically induced mass mortality events in the NW Mediterranean. The general aim of the study was to investigate the genetic diversity of *P. clavata* in the Ligurian Sea (NW Mediterranean), a region highly impacted by past mass mortality events, and the Atlantic Ocean, where mass mortality was never observed due to generally lower water temperature. Microsatellites were used to study the contribution of clonal reproduction, connectivity pattern, genetic structure and diversity. Additionally one mitochondrial marker (Cytochrome Oxidase I) was used to compare the Atlantic and Mediterranean populations. The results revealed, that clonal propagation does not play an important role in *P. clavata*, since at four out of nine sites clones were not detected and the maximum prevalence of clones reached only 13%. The study failed to detect any genetic diversity loss in the *P. clavata* populations affected by mass mortality events. The migration pattern among sites affected by mass mortality and unaffected ones was described. The results confirmed that low larval dispersal capability in the red gorgonian may still be ecologically significant for population replenishment and persistence, enabling migration between local populations. This research has identified an important genetic break within the red gorgonian distribution. Both markers used in the present study, mtDNA and microsatellites, revealed the same discontinuity between the Mediterranean and Atlantic. Significant differences were found in the genetic diversity between the Mediterranean and Atlantic populations, with heterozygosity and allelic richness being slightly, but significantly, higher in the Mediterranean Sea, possibly as a result of colonization history or isolation of the Atlantic sites. The differences in genetic diversity were also detected between deep and shallow populations. Allelic richness increase with depth, being lower in the shallow, less stable populations due to past mortality events induced by warming and other interacting factors and higher in deeper, stable populations.The results should prove to be particularly valuable for the conservation of soft corals communities and thus the overall marine biodiversity.

parole chiave Gorgonie, mortalità, dispersione, genetica, conservazione

abstract

La gorgonia rossa *Paramuricea clavata* è una 'engineering species', vive sui fondali rocciosi del Mediterraneo e della costa portoghese dell'Oceano Atlantico. La specie è stata severamente impattata da eventi di mortalità di massa indotti dal CC nel Mediterraneo nord-occidentale. Lo scopo dello studio è stato di indagare la diversità genetica di *P. clavata* nel Mar Ligure (Mediterraneo nord-occidentale), una regione fortemente impattata da eventi di mortalità, e nell'Oceano Atlantico, dove eventi di mortalità non sono mai stati registrati grazie a valori di temperatura dell'acqua generalmente più bassi. Per studiare il contributo della riproduzione clonale, i pattern di connettività, la struttura e la diversità genetica sono stati usati i microsatelliti. In aggiunta, un marcatore mitocondriale (Cytochrome Oxidase I) è stato utilizzato per confrontare le popolazioni atlantiche con quelle mediterranee. I risultati hanno mostrato che la propagazione clonale non gioca un ruolo importante in *P. clavata*, in quanto in quattro siti su nove non sono stati individuati cloni e la predominanza massima di cloni ha totalizzato solo il 13%. Lo studio non ha riscontrato perdita di diversità genetica nelle popolazioni di *P. clavata* colpite da eventi di mortalità. Sono stati descritti i pattern di migrazione tra siti colpiti da mortalità e quelli non colpiti. I risultati hanno confermato che la bassa dispersione larvale nella gorgonia rossa può essere ancora ecologicamente significativa per il rifornimento e la persistenza di popolazioni, favorendo la migrazione tra popolazioni locali. Questa ricerca ha identificato un importante break genetico nella distribuzione della gorgonia rossa. Entrambi i marker usati in questo studio, mtDNA e microsatelliti, hanno rivelato la stessa discontinuità tra Mediterraneo ed Atlantico. Differenze significative sono state riscontrate nella diversità genetica tra popolazioni mediterranee e atlantiche, con eterozigosità e ricchezza allelica leggermente, ma significativamente più alte nel Mediterraneo, probabilmente come risultato della storia di colonizzazione o isolamento dei siti atlantici. Le differenze nella diversità genetica sono state riscontrate anche tra popolazioni profonde e superficiali. La ricchezza allelica aumenta con la profondità, risultando più bassa nelle popolazioni più superficiali, meno stabili a causa degli eventi di mortalità indotti dal riscaldamento e da altri fattori interagenti, e più alta nelle popolazioni più profonde e relativamente più stabili. I risultati sono di particolare interesse per la conservazione delle comunità di coralli molli e quindi nel complesso per la biodiversità marina.

Chapter 1 has been submitted to *Scientia Marina*: Pilczynska J, Boavida J, Cocito S, Lombardi C, Peirano A, Queiroga H. Low clonal propagation in Atlantic and Mediterranean populations of the red gorgonian (*Paramuricea clavata*). Scientia Marina. Submitted

Chapter 2 has beed published in *PLoS ONE* as: Pilczynska J, Cocito S, Boavida J, Serrão EA, Queiroga H (2016) Genetic Diversity and Local Connectivity in the Mediterranean Red Gorgonian Coral after Mass Mortality Events. PLoS ONE 11(3): e0150590. doi:10.1371/journal.pone.0150590

Chapter 3 has been submitted to *Marine Ecology* as: Pilczynska J, Cocito S, Boavida J, Serrão EA, Queiroga H. High genetic differentiation of the red gorgonian populations from the Atlantic Ocean and the Mediterranean Sea. Marine Ecology

Chapter 4 has been submitted to *Estuarine, Coastal and Shelf Science* as: Pilczynska J, Cocito S, Boavida J, Serrão EA, Assis JM, Queiroga H. Genetic diversity increases with depth in red gorgonian populations form the Mediterranean and Atlantic. Estuarine, Coastal and Shelf Science. Submitted

Table of contents

General introduction

Coralligenous assemblages as a highly biodiverse and endangered community

Ecological value of coralligenous communities

Coastal ecosystems are the most diverse of marine habitats and at the same time the most impacted by human activity (Halpern et al. 2008). One of the most species rich marine reservoirs is the Mediterranean Sea (Coll et al. 2010). Around 4-18% of the world's marine species can be found here, which is a relatively large proportion, since the surface area and volume of the sea represents only 0.82% and 0.32% of the world ocean, respectively. High levels of endemism, averaging more than 25% among Mediterranean species, are another reason for considering this region a biodiversity hot spot (Bianchi and Morri 2000). Coralligenous assemblages are among the most species rich communities in the Mediterranean Sea - it is assumed that only the *Posidonia oceanica* meadows harbor greater species diversity (Boudouresque 2004).

Coralligenous assemblages are calcareous formations of biogenic origin, growing in dim light conditions. These hard-bottom communities are characteristic of Mediterranean Sea (Ballesteros 2006). Irradiance is the most important environmental factor influencing the development of the coralligenous framework and the required light level must be between 0.05 and 0.3% of the surface light (Ballesteros 1992). Communities usually develop in the circalittoral zone throughout the Mediterranean Sea, except the coast of Lebanon and Israel, but most of the available data comes from studies on the western part of the sea. Coralligenous communities are mainly composed of encrusting calcareous algae belonging to the family Corallinales (Piazzi and Balata 2011). Another type of coralligenous assemblages are gorgonian forests, consisting mainly of red gorgonian *Paramuricea clavata* (Ballesteros 2006). Animal dominated communities are usually located deeper than algal assemblages, because of a low light level, insufficient for algae (Garrabou et al. 2002). Gorgonian forests similar to those from the Mediterranean are also present in the Atlantic Ocean, on the Portuguese coast (Cúrdia et al. 2013).

The great biodiversity of coralligenous communities has been emphasized by many authors (Laubier 1966; Hong 1980). It has been estimated that about 1241 invertebrate species live in this habitat (Ballesteros 2006) and there are at least 315 species of macroalgae (Boudouresque 1973). Animal dominated structures are generally more diverse and complex (Garrabou et al. 2002). Invertebrates living in coralligenous assemblages play an important role as builders. Together with macroalgae, they create a very complex structure that offers microhabitats for many other species. In food-rich sites with high concentrations of zooplankton, POC and DOC, suspension feeders often dominate the community, including gorgonians, sponges and bryozoans (Ballesteros 2006). High biodiversity has been described for assemblages dominated by the red gorgonian *Paramuricea clavata*, occurring mainly on vertical rocky walls (Gili and Ballesteros 1991).

Gorgonians form dense populations, which in turn serve as a substrate for many species, such as other cnidarians, sponges and encrusting bryozoans (Cocito et al. 2002). Many rare species are associated with coralligenous communities, although none of them is exclusive to this environment. Ballesteros (2006) listed 16 species of endangered macroalgae inhabiting the community. Some of the commercially valuable species are also present, including red coral *Corallium rubrum* (Ballesteros 2006).

The growth rate of the main framework builders is very low and well-developed assemblages may be a few thousand years old (Ballesteros 2006). Long succession time is another characteristic feature and the persistence of the animals and plants is high, especially in deep, often animal-dominated structures. Community spatial pattern complexity increases with depth and is highly mosaic and patchy (Garrabou et al. 2002). Clear annual cycles of activity are evident for such suspension feeders as gorgonian *Paramuricea clavata*, sponge *Dysidea avara* and ascidian *Halocynthia papillosa*. The respiration rate is significantly lower in the summer conditions of higher water temperatures, suggesting energy limitation during this period for benthic suspension-feeding taxa (Coma et al. 2002).

Threats to coralligenous communities

As indicated above, coralligenous communities are complex, fragile habitats characterized by low dynamics and slow development rate. These characteristics make the assemblage highly vulnerable to human induced environmental changes (Ballesteros 2006). Climate fluctuations have a great impact on both terrestrial and marine ecosystems (Stenseth et al. 2002). Human induced global warming do not only increases ocean temperature, but may also have a significant impact on water chemistry and sea currents, changing communities composition, species range, populations dynamics and migrations patterns (Harley et al. 2006).

Severe damages for coralligenous communities, called mass mortality events, have been linked with increased sea water temperature (Ballesteros 2006; Cerrano et al. 2000; Perez et al. 2000). Temperature anomalies in northwestern Mediterranean Sea became more frequent in recent years. While mean monthly winter temperature is rather constant over time, the summer temperatures at 0-20 m depth are increasing at a rate of 0.05 °C per year (Coma and Ribes, 2003). Positive temperature anomalies were reported in the Ligurian Sea (NW Mediterranean) in 1999 and 2003, when the mean temperature during the summer was 1.7–2.3°C and 0.6-1.6°C higher than in the entire study period (1997-2005) (Cocito and Sgorbini 2013). In contrast, gorgonian forests in the Atlantic Ocean were not exposed to abnormally high temperatures, due to generally colder ocean water and upwellings, commonly occurring along the Portuguese coast in the summer season (Relvas et al. 2007).

During the abnormally warm summers higher temperatures increase water masses stability: the upper warm layers do not mix with the lower, cold water masses. The thermocline develops earlier and water stratification persists for longer (Sparnocchia et al. 2006). Organisms living above the thermocline are longer exposed to summer conditions, which are characterized by high water stability and low food availability as a consequence of the lack of water mixing (Coma and Ribes 2003). Even if the high temperature is not lethal for organisms, exposure may cause physiological stress and energy shortage due to increased respiration rate (Coma et al. 2002). Direct effects on organisms' physiology were recorded for Mediterranean cnidarians. During laboratory experiments, the two corals *Corallium rubrum* and *Cladocora caespitosa* showed decrease calcification rates after exposure to temperature 6°C higher than the average ambient temperature (Rodolfo-Metalpa et al. 2006; Torrents et al. 2008). Decreased calcification rate, as well as decreased photosynthetic efficiency, but for a greater temperature increase, was obtained for the symbiotic gorgonian *Eunicella singularis*, highlighting the differences in species thermal tolerance and their response to disturbance (Ferrier-Pagès et al. 2009). Measurements of phytoplankton concentration, as an indicator of water trophic status, showed a negative correlation with sea surface temperature (Vezzulli et al. 2010), indicating that the availability of food decreased in high temperatures. Reduced resources and high metabolic activity in warmer water increase the probability of mass mortality events in coastal communities (Coma et al. 2009). As a consequence, the most affected by mass mortality events were the species exhibiting energy shortages, such as suspension feeding soft corals and sponges (Coma and Ribes 2003).

The two largest mass mortality events in the NW Mediterranean Sea were linked with seawater temperatures 3-4°C higher in the summer and fall than the average (Cerrano et al. 2000; Sparnocchia et al. 2006) and strongly affected shallow water communities (10-40 m; Ballesteros 2006). During the heat wave of 1999 a total of 30 benthic species were affected and the maximum depth of the impact was 40 m in most sites (Perez et al. 2000). The signs of mass mortality were recorded along the Ligurian coast (Italy) and Provence (France) (Cerrano et al. 2000). In 2003, another mass mortality event affected 25 species to depths of 15-30 m. This event had greater spatial range: the signs of rocky bottom species die-off were visible also in the Catalan coast, Corsica, Sardinia, and the Gulf of Naples (Garrabou et al. 2009). Impacted species belonged to hard bottom communities and several of them were key species in coralligenous assemblages. Tissue necrosis or bleaching, followed by its detachment, were observed in some gorgonian species (*Paramuricea clavata*, *Eunicella cavolinii, Eunicella singularis*), corals (*Corallium rubrum*, *Cladocora caespitosa*), sponges (*Petrosia ficiformis*, *Spongia officinalis*, *Cacospongia* spp., *Ircinia* spp.), and bryozoans (*Myriapora truncata* and *Sertella* spp.). The degree of impact increased proportionally to temperature (Garrabou et al. 2009).

During both mass mortality events, the most affected species were long-living ones, with low growth rate, recruitment and mortality (Garrabou and Harmelin 2002; Coma et al. 1995a; 2004; Linares et al. 2008). Therefore, their recovery is slow, especially because of short time periods between the mortality events (Linares et al. 2007a). Moreover, some of the affected species, such as gorgonians, erect bryozoans and large sponges are key species in coralligenous framework, providing biogenic structure for other animals. The disappearance of these engineer species may have indirect effects on the whole community, changing habitat conditions (flow regime, food availability and shelter; Soulé et al. 2003). Filter-feeding organisms remove large amounts of suspended organic matter from the water column (Coma and Ribes 2003), so their reduction may increase the amount of food available for other species, changing food web and species composition (Cerrano and Bavestrello 2008). In the future, extreme events like heat waves are predicted to occur more frequently and persist longer (Diffenbaugh et al. 2007; Déqué 2007).

Physiological stress caused by elevated temperature and food deficiency has a significant impact on organisms' fitness. Poor physiological conditions decrease the efficacy of defense, making pathogen infections more probable (Cerrano et al. 2000). As reported by Bally and Garrabou (2007) the bacterial community associated with *Paramuricea clavata* differs between healthy organisms and colonies partially damaged during mass mortality events. Infected colonies harbor *Vibrio coralliilyticus*, a temperature-dependent coral pathogen responsible for cnidarians' diseases in the Red Sea and Indian Ocean (Ben-Haim and Rosenberg 2002). Elevated temperatures trigger the pathogenic process after infection, suggesting that environmental stress fosters pathogen virulence and increases host sensitivity (Bally and Garrabou, 2007).

Physical injury caused by fishery, anchoring and diving is a serious threat in densely populated areas. Fishing activities may seriously damage large areas of coralligenous concretions. Trawling has been considered as one of the most destructive methods (Palanques et al. 2001). Trawling nets cause direct physical damage by breaking the structure and detaching algae and animals from the bottom, but also increasing turbidity and sedimentation rate, which negatively affects filter-feeding and photosynthesis of coralligenous algae. Fishing lines and other gear seriously damage gorgonians, entangling colonies and mechanically scraping the tissue under the sea currents action. After tissue detachment the skeleton is colonized by numerous epibionts, weakening the colony structure and increasing the probability of breakage due to higher weight. Damages caused by fishing are most visible in shallow water (20-30 m; Bavestrello et al. 1997).

Coralligenous communities, due to their great diversity of species and beautiful seascapes, are the most popular diving spots in the Mediterranean Sea. High levels of diving activity have negative impacts on the community. Divers often cause mechanical damage, mainly by breaking the colonies of large gorgonians and bryozoans. According to Sala et al. (1996), the fragile calcareous bryozoan *Pentapora fascialis* was significantly less abundant and its colonies were smaller in sites often visited by divers in Medes Islands, Spain. Coma et al. (2004) found three times higher

mortality rates of adult *Paramuricea clavata* colonies in recreational diving sites in the NW Mediterranean compared to unfrequented ones, mainly due to toppling and unintentional colonies breaking. Negative impacts of diving activities on gorgonians forests were also reported for the Marine Park Professor Luiz Saldanha in the Atlantic Ocean (Rodrigues 2008). Anchoring of boats in diving sites has also a negative impact on coralligenous communities, causing detachment of the bottom organisms (Ballesteros 2006).

Mechanical damage may also occur as a consequence of natural extreme events. Severe storms, like the one in 2008 in the NW Mediterranean Sea, change species richness and composition. Mainly large, fragile organisms such as calcareous algae, sponges, anthozoans, bryozoans and tunicates were impacted, showing cover losses up to 100% in the most exposed sites (Teixidó et al. 2013).

Water pollution is another issue for coralligenous communities. Although there is no recent study in this topic, it is assumed that waste waters runoff from mainland, especially in highly urbanized areas, decrease both biodiversity of coralligenous assemblages and the density of individuals (Hong 1980).

Paramuricea clavata as a study target

The present research focus on the red gorgonian *Paramuricea clavata* (Cnidaria, Anthozoa, Octocoralia). Considering the numerous studies about the species biology (Coma et al. 1995ab; Linares et al. 2007ab) and its importance for biodiversity (Ballesteros 2006), the red gorgonian may be consider as a model organism. A growing body of literature describes mass mortality events affecting *P. clavata* (e.g. Bally and Garrabou 2007; Coma et al. 2009) and examines impact of mortality on gorgonian populations (e.g. Linares et al. 2005; Cerrano and Bavestrello 2008; Cupido et al. 2008). This section summarizes the existing knowledge about *P. clavata* biology and mortality episodes.

Distribution and biology

The red gorgonian is a key species of sublittoral rocky habitats, contributing to one of the most diverse communities in the Mediterranean Sea and in the Atlantic (Ballesteros 2006). Gorgonians form dense populations, which in turn serve as a substrate for numerous species, including other cnidarians, sponges and encrusting bryozoans (Cocito et al. 2002). The species creates a structure for the coralligenous assemblage and thus the survival of the community may be dependent on the survival of the species (Gili and Coma 1998).

Corralligenous assemblages dominated by *P. clavata* are widespread in the western Mediterranean Sea (Carpine and Grasshof 1975) and in the Adriatic Sea (Kipson et al. 2014), and less common in the Aegean Sea (Öztürk et al. 2004). The species is present on the Portuguese coast (Cúrdia et al.

2013), but data from the Atlantic Ocean are limited. Personal observations confirmed the red gorgonian occurrence in the Algarve, Arrábida and Berlengas, the last location being probably the northern species range in the Atlantic. Assemblages dominated by *P. clavata* are common on vertical surfaces with low irradiance and intense water flow (Ballesteros 2006), with the highest population abundance between 15 and 35 m (Linares et al. 2008).

The red gorgonian is a passive suspension feeder with a broad and heterogeneous diet, which contains nanoeukaryotes, phytoplankton and zooplankton, as well as detritus (Ribes et al. 1999). According to Ribes et al. (1999) detrital carbon accounts for 86% of the total ingested carbon. Phillips and Gregg (2003) reported that particulate and suspended organic matter (POM and SOM) from seawater and sediment contributes 75% of the diet of *P. clavata*. The red gorgonian does not exhibit seasonality in food preference and organic matter from seawater and sediment is supposed to be the main food source in the summer and winter (Cocito et al. 2013).

P. clavata is characterized by low growth rate but long life span. Individual colonies may reach up to 1.5 m height (Linares et al. 2007a), which may correspond to an age up to 100 years (Coma et al. 2001). It is difficult to estimate the age of living gorgonian colonies because breakage of branches is relatively common and therefore colonies may be much older than they appear. Additionally, estimates of growth rates vary between different studies. Coma et al. (2001) estimated average growth rates of the Medes Islands population at 0.8 $cm \cdot yr^{-1}$ in colony height, suggesting ages of up to 50–100 years for the older colonies. The annual growth rate for La Spezia (Ligurian Sea) population was estimated at 3 $cm \cdot yr^{-1}$ for adult colonies and 4.5 for recruits (Cupido et al. 2012). *P. clavata* reach sexual maturity when colonies attain a size of 20 cm at an age of 13 years (average from Medes Islands; Coma et al. 1995a), but these values also may vary among locations. The height of fertile female colonies in the La Spezia population was 8.5 cm and the age was estimated at 3 years (Cupido et al. 2012). The demographic characteristics, such as survival, growth, and fecundity are rather more influenced by size than by age, which is typical for species with indeterminate growth.

The reproduction biology of *P. clavata* has been well studied. The species is almost exclusively dioecious (Coma et al. 1995b), since the abundance of hermaphroditic colonies does not exceed 1% (Gori et al. 2007). The sex ratio was reported to be close to 1:1 in undisturbed populations (Coma et al. 1995a). However, a skewed sex ratio was found in a population from the Cape de Palos, Spain, where male colonies were more abundant. Significant segregation of sexes at a small spatial scale was also found in the La Spezia population (Cupido et al. 2012). Synchronous spawning occurs twice a year in June, around the new and full moons. Spawning episodes last 2-3 days and are separated by several days (Linares et al. 2007a). *P. clavata* is a surface brooder. Females secrete a mucous, which adheres the eggs to the surface of the mother colony, while male gametes are released into the water (Coma et al. 1995b). This mode of reproduction seems to provide high fertilization rate by avoiding both sperm limitation and polyspermy (Lasker 2006).

Fertilization, embryogenesis and maturation of the planula larvae take place on the surface of the mother colony (Linares et al. 2007a). However, strong currents may detach the mucus material from the colony, in which case larval development takes place away from the mother colony. During field observations, the first mature planulae appeared 48 hours after the spawning event (Coma et al. 1995b). In laboratory experiments, embryos reached the blastula stage after 24 hours and embryogenesis ended after 48-72 hours, when the pear-shaped planulae appeared (Linares et al. 2007a). The larvae have vitelline reserves that comprised their sole food source during their motile stage (Coma et al. 1995b). Planulae can swim actively or crawl on the sea bottom (Linares et al. 2007a). *In situ* observations indicate that the dispersive stage of the larvae is short because of negative phototaxis and negative buoyancy – the larvae settle a few minutes after hatching, near the mother colony (Coma et al. 1995b). The short larval phase does not favor dispersal, but possibly decreases larval mortality and wastage, contributing to replenishment of local populations (Linares et al. 2007a).

A percentage of mature colonies and a proportion of fertile polyps in the colony increase rapidly, proportionally to colony size (Cupido et al. 2012). Proportion of fertile polyps and a number of gonads per polyp is the highest in the first order (apical) branches. Since first order branches make up the largest share of colony biomass, they produce 85% of all gametes (Coma et al. 1995a). Therefore, colony size is the major determinant of reproductive output. The percentage of colonies with gonad-bearing polyps increase from 70% in colonies shorter than 10 cm to 100% in colonies higher than 30 cm (Coma et al. 1995a). Young individuals (height < 10 cm) invest 0.2-2% of the carbon weight of tissue for gamets production, whereas colonies higher than 40 cm invest 84-98% of the carbon weight of tissue. Although large colonies constitute only a few percent of the population, their contribution to the reproductive output is as large as 40% production of the female gametes and 33% of the male gametes (Coma et al. 1995a).

Red gorgonians make significant investment in reproduction each year but recruitment rates are typically low (Coma et al. 1995a; 2001). In laboratory experiments, survival of the planula stage was 70%, but only 12% of individuals survived metamorphosis and turned into primary polyp. Linares et al. (2007a) reported that during 2 years of monitoring the Medes population any of the 230 settled polyps observed in the study area survived longer than 7 months.

Mass mortality events

In 1999 and 2003, two episodes of mass mortality events affected several populations of benthic suspension feeders in the northwestern Mediterranean (Linares et al. 2005; Coma et al. 2006; Cupido et al. 2008; Huete-Stauffer et al. 2011). It is generally agreed that mass mortality of Mediterranean benthic communities was caused by temperature anomalies linked to global warming (Garrabou et al. 2009). Increased metabolic activity in high temperature, together with low food availability in the summer, were the most probable factors causing gorgonians' mortality (Coma et al. 2009).

Physiological stress, caused by an increase in seawater temperature, decrease defense capacity and make organisms more susceptible to disease. The presence of virulent *Vibrio* species on damaged *P. clavata* coenchyme portions indicates that bacterial infection may be an additional factor causing gorgonian mortality (Martin et al. 2002). Bally and Garrabou (2007) identified *Vibrio coralliilyticus*, a thermodependent pathogen of tropical corals, to be virulent for *P. clavata*. The occurrence of *Vibrio* spp. in seawater is temperature dependent. *V. coralliilyticus* increase in abundance in the warm season and was recorded only in temperatures higher than 18°C (Vezzulli et al. 2010). Additionally, when temperature returned to lower levels after the mortality event, *Vibrio* concentration in healthy and recovering colonies did not differ, highlighting the role of temperature in the pathogen virulence.

Paramuricea clavata was one of the most impacted species during the mass mortality events in 1999 and 2003. As reported in Garrabou et al. (2009), the proportion of affected colonies reached 80% at some sites. Small-scale mortality events were recorded also in 2006 by Cerrano and Bavestrello (2008) and Cupido et al. (2008). The first signals of mortality appeared in late summer, when parts of *P. clavata* colonies showed necrosis of tissue, changing its color from red to grey as a result of organic parts decomposition. Dead tissue detached from the colony's axis, leaving a bare skeleton that was rapidly overgrown by epiphytic organisms (Cerrano et al. 2000; Garrabou et al. 2009). Large colonies were more affected and their density decreased dramatically, causing a clear shift towards smaller size classes (Linares et al. 2008; Cupido et al. 2008; Huete-Stauffer et al. 2011). Partial mortality affected also colony morphology, changing it from planar, fan-shaped to bushy, with several equivalent branches. This regeneration pattern may have significant impact on colony fitness, decreasing growth rate due to self-shading (Cerrano and Bavestrello 2008).

The number of colonies affected by partial mortality decreased with depth, with colonies living below 30-40 m being much less affected than those from the shallower sites (Linares et al. 2005; Huete-Stauffer et al, 2011). The clear differences between the healthy, deep populations, and the shallow ones, affected by mass mortality, were visible in population structure several years after the last mortality event. Size classes' distribution in the deep population were characteristic for the species, with large colonies predominance and low recruits density. In the shallow sites, small, unfertile colonies dominated and the density of large individuals was very low (Cerrano and Bavestrello 2008; Cupido et al. 2009). As hypothesized by Cerrano and Bavestrello (2008; 2009), deeper subpopulations may act as a reservoir, supplying larvae to shallower sites, especially because large colonies with high fecundity rates survived there.

Mortality events had also a significant impact on gorgonians reproduction. The most affected colonies (more than 33% colony injured) exhibited a decrease in fecundity one year after the event (Linares et al. 2008). The decrease in gonadal biomass and the number of fertile polyps was

proportional to the extent of the injury. This effect was more visible in female colonies (73-75% reduction in oocyte production) than in males (49-64% reduction in sperm production). A study conducted two years after the mortality event revealed similar pattern of decreased fecundity (Linares et al. 2008). In undisturbed environments the low recruitment rate observed in *P. clavata* is sufficient to ensure the persistence of local populations. However, recruitment did not compensate mortality after the mass mortality events (Linares et al. 2005). Mass mortality had greater effect on large, fertile colonies and therefore population recovery after the disturbances may be restricted.

The impact of mass mortality events on *P. clavata* population is still felt several years after the event and the regeneration of the population started after a substantial period, lasting 3-4 years (Linares and Doak, 2010). Partially damaged colonies can survive the loss of living tissue and regrow it, but regeneration depends on the extent of injuries and is often fairly low (Linares et al. 2005). Regeneration of the tissue on the exposed skeleton of partially damaged colonies was estimated at 0.15 ± 0.2 cm day^{-1} (Bavestrello et al. 1997). Colonies density increase in the damaged sites was slow due to low recruitment rate. Bavestrello et al. (1997) observed only 2 recruits on the experimental plot from which 23 colonies were removed four years earlier. However, in the years following the mortality event (2004-2008) increased population densities in the Gulf of La Spezia were detected. The number of recruits reached 24 % of colonies and their density increased from 2.6 recruits per m^2 in 1998 (before mortality) to around 6 in 2007 and 2008. Recruitment rate in this site became two fold greater than before the mass mortality event (Cupido et al. 2009).

Research gaps

The Mediterranean coralligenous assemblages have been widely studied. However, the response of coralligenous assemblages to past mass mortality events is not fully understood. Mass mortality events may have a wide impact on populations. The most apparent consequences are density and abundance decreases. Less evident are changes in population structure, size classes' distribution and reproductive output. One of the least studied consequences of mass mortality is the impact of decreased population abundance on genetic diversity. To date, the effect of climatically induced mass mortality events on genetic diversity of affected populations has not been studied in the Mediterranean Sea.

The mechanisms of population recovery after mass mortality events are still poorly understood. Increased reproduction or recruitment rate may be among the factors, which allow to increase the population density. The reproduction of sessile invertebrates, in particular the red gorgonian, has been studied extensively, but much uncertainty still exists about the larval phase duration and propagation distance. Additionally, little is known about the role of asexual reproduction in temperate gorgonians, despite the fact that clonal propagation may led to rapid increase in population densities and therefore enable population recovery after mass mortality.

Population recovery may be supported by migrants from neighboring, non-impacted sites. Little is known about short distance larval migration, which may support population recovery. The estimates of connectivity pattern among Marine Protected Areas and larval migration from protected populations to damaged ones, are essential for effective protection plans, especially important for fragile, engineering species. This study use the red gorgonian *P. clavata* as a model organism to obtain data which will help to address these research gaps.

Objectives and structure of the thesis

My research is being performed after two events of mass mortalities of benthic organisms in the Mediterranean Sea, in 1999 and 2003. The general aim of the study is to investigate the genetic diversity of *Paramuricea clavata* in the Ligurian Sea (NW Mediterranean), a region highly impacted by past mass mortality events, and the Atlantic Ocean, where mass mortality was never observed due to generally lower water temperature.

First chapter focuses on reproduction mode of the red gorgonian populations from the Ligurian Sea (NW Mediterranean), severely affected by climatically- induced mortality events in 1999, 2003 and 2006 and from the Atlantic Ocean, where mass mortality was never observed. The aim of the study was to evaluate the contribution of asexual reproduction and to investigate if clonal propagation plays an important role in *P. clavata* reproduction at sites that have been affected by mass mortality in the recent past.

Second chapter describes the genetic structure of *P. clavata* populations in a region impacted by mass mortality events, the Ligurian Sea (NW Mediterranean). The aim of the study was to investigate a possible bottleneck effect of past mortality events on genetic diversity in a region where some populations were highly affected by mass mortality events and others were not. The second objective was to assess a connectivity pattern and migration at the local scale (10s of km).

The aim of the third chapter was to compare the genetic diversity between *P. clavata* populations from the Ligurian Sea and Atlantic Ocean. Highly variable microsatellite markers were used together with mitochondrial DNA COI gene. The differences in genetic composition and diversity between populations from two basins were discussed in terms of species phylogeography.

In the fourth chapter, the differences in genetic diversity in the red gorgonian coral from populations inhabiting different depths in the Atlantic and Mediterranean were examined. We hypothesize that genetic diversity may change with depth, being lower in the shallow populations due to past mortality events, and higher in deep, more stable environments

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Chapter 1. Low clonal propagation in Atlantic and Mediterranean populations of the red gorgonian (*Paramuricea clavata*)

1.1. Abstract

Clonal propagation is a common feature of benthic marine organisms. In the present study, we investigated the contribution of clonal reproduction in the red gorgonian *Paramuricea clavata*. Mediterranean populations of *P. clavata* were severely affected by mortality events in 1999 and 2003, caused by increased water temperature. The populations are characterized by slow growth and episodic recruitment, however, after the observed mortalities, an unexpected high recovery rate was observed in the severely affected populations from the Ligurian Sea, NW Mediterranean. Ten years after the last mortality event, we investigated the contribution of clonal propagation in populations from the Ligurian Sea, where some populations were highly affected by mass mortality events, and from the Atlantic, where mortality was never observed. All individuals were genotyped for 10 microsatellite loci. The contribution of clonal reproduction varied from 0 to 13% and did not differ significantly between affected and unaffected populations. We confirm by using genetic markers that clonal propagation in *P. clavata* is not common and the contribution of clones is too low to play an important role in red gorgonian reproduction and cannot contribute to population recovery in sites that have been affected by mass mortality events.

1.2. Introduction

Clonal propagation is widespread among marine invertebrates and a number of studies have attempted to explain its evolutionary importance and adaptive significance (Coffroth and Lasker 1998; McFadden 1991). Asexual reproduction does not only allow domination of the community by the most adapted genotype (Miller and Ayre 2004), but has also a significant role in the colonization of new areas, since it may allow for a faster increase in abundance compared to sexual reproduction (Dybdahl and Kane 2005; Mergeay et al. 2006).

Asexual reproduction may play an important role in corals, supporting high population growth rates (Lasker 1988). In species with frequent vegetative propagation and low recruitment of larvae produced through sexual reproduction, a few successful clones may dominate the population (McFadden 1991, 1997). Additionally, observations of skewed sex ratio, frequently reported in octocorals (Kahng et al. 2011), may be generated by asexual reproduction, such as in the Caribbean gorgonian *Plexuara* sp. populations (Brazeau and Lasker 1989). This species reveals extremely low males contribution, but at the same time reproductive output is high, suggesting that eggs develop parthenogenetically. If this is the case, *Plexuara* sp. clones, spread locally by fragmentation (Lasker 1984) and between reefs by the dispersal of parthenogenetic eggs, may reach wide geographic distributions. Chen et al. (2002) found noticeably high contribution of clonal reproduction in a local population of the gorgonian coral *Junceella fragilis* from Taiwan. The

population was dominated by only two distinct genotypes, probably as a result of multiple clonal reproduction events following colonization by two founder individuals. Numerous cnidarian species may change their reproductive mode and increase or decrease the contribution of clonal reproduction to recruitment in response to environmental changes. As reported in Coffroth and Lasker (1998), the lowest genotypic diversity, meaning the highest contribution of clones, may be related to wave action, such as in the gorgonian *Plexuara kuna* populations from the Caribbean (Lasker et al. 1998). Wave action promotes the detachment of the colony branches, but fragments need calm periods to reattach to the substratum and become established, therefore the highest contribution of clones are found at sites with intermediate wave impact. Changes in reproductive mode may occur seasonally. The soft coral *Alcyonium* spp. from North-western Pacific exclusively uses sexual reproduction during the summer and thus clonal reproduction becomes more important in winter, when animals do not spend energy on sexual propagation (McFadden 1991). Clonal reproduction may be also promoted by human activities, i.e. anchoring and fishing gear, causing colonies detachment (Harmelin and Marinopoulos 1994). Detached coral fragments may reattach to substratum and create a new colony.

The red gorgonian (*Paramuricea clavata*, Risso 1826) is widely distributed in the western Mediterranean Sea (Carpine and Grasshof 1975) and along the Portuguese coast of the Atlantic (Boavida et al. 2015). Assemblages dominated by *P. clavata* are common on vertical surfaces with low irradiance and intense water flow (Ballesteros 2006), with the highest population abundance between 15 and 35 m (Linares et al. 2008). The species is known to reproduce almost exclusively by sexual propagation (Coma et al. 1995ab). In the Mediterranean Sea synchronous spawning occurs twice a year in June, around the new and the full moon. Fertilization, embryogenesis and maturation of the planula larvae take place on the surface of the mother colony (Linares et al. 2007). The reproductive effort of *P. clavata* increases with colony size (Coma et al. 1995a; Cupido et al. 2012). Coma et al. (1995a) reported that large colonies (height >40 cm) are generally scarce in the population from the Medes Islands (NW Mediterranean), constituting less than 3% of colonies, but their contribution to the production of gametes was on the order of 40% of female gametes and 33% of male gametes. In contrast, the recruitment rates are considered to be low. Linares et al. (2007) reported that during 2 years of monitoring the population from Medes Islands none of the settled polyps in the study area survived longer than 7 months.

P. clavata may also reproduce asexually by fragmentation of the colony or stolonization. Colonies originated from fragments differ in appearance from the typical fan-shaped colonies. They are attached to the substratum at several points and have several parallel branches growing up from a branch lying on the substrate (Coma et al. 1995b). This morphology can not only be a result of asexual reproduction, but also an adaptation to hydrodynamics, i.e. to turbulent current regime or a result of partial colony mortality in the past (Cerrano and Bavestrello 2008). Colonies originating from stolons are connected to the mother colony until they reach around 15 cm in height, but the connection breaks up with time (Coma et al. 1995b). Based on colony morphology, Coma et al.

(1995b) evaluated the frequency of colonies originated from asexual reproduction to be 0.3% by fragmentation and 2% by stolonization. However, these estimates on the prevalence of clonal reproduction have never been validated with genetic markers. Only in the study of Mokhtar-Jamaï et al. (2013) 4 out of 104 colonies sharing the same genotype were found, but the authors excluded repeated genotypes since their paper did not investigate clonal propagation.

The impact of climate-induced mortality events on *P. clavata* reproduction mode has not been studied so far, despite the fact that the species has experienced severe damages in the Mediterranean Sea in the past. Two mass mortality events in the summers of 1999 and 2003 reduced *P. clavata* colony density by 78% in the Ligurian Sea (NW Mediterranean), affecting mainly the large, most fertile individuals (Cupido et al. 2008). These events affected a wide variety of species and taxa of hard-bottom communities and were observed in the entire NW Mediterranean region, affecting several thousand kilometers of coastline (Garrabou et al. 2009; Perez et al. 2000). The mortality was caused by unusually high sea water temperatures with an enhanced stratification, causing thermal stress and food limitation due to lack of water mixing (Coma et al. 2009). In 2003, the temperature down to the thermocline was between 1 and 3 °C above the mean monthly temperature in the NW Mediterranean (Garrabou et al. 2009). Damage intensity decreased with depth and community dwelling below the thermocline (25-30m) was significantly less affected than the shallow one (Linares et al. 2005). Red gorgonian populations from the Atlantic Ocean were never monitored, although we may suspect that lower water temperatures in the Atlantic has prevented mass mortality events. The Portuguese coast is influenced by strong and persistent upwelling events during spring and summer (Relvas et al. 2007), which decrease surface temperature and mix the water column, preventing the formation of a strong thermocline. In the Mediterranean, temperature related mortality events impacted the reproductive output from sexual propagation by decreasing not only colony density, but also fecundity (Linares et al. 2008). The recovery of impacted assemblages may be delayed because of low growth rate (0.8 cm yr-1 in colony height; Coma et al. 2001) and the late age of first reproduction (7-13 year; Coma et al. 1995a). However, an unexpected high recovery rate was observed in the La Spezia population (Ligurian Sea, NW Mediterranean) in the years following the 2003 event, caused by an unusually high recruitment rate. The density of recruits increased from 2.6 recruits per m2 in 1998 (before mortality) to around 6 in 2007 and 2008 (Cupido et al. 2009).

The present study used microsatellite markers to investigate for the first time the contribution of asexual reproduction in the red gorgonian populations from the Atlantic and the Mediterranean Sea. We also investigated if clonal propagation plays an important role in *P. clavata* reproduction at sites that have been affected by mass mortality in the recent past.

1.3. Methods

1.3.1. Sampling

In order to compare the contribution of clonal reproduction according to mortality history, we analyzed populations from two regions. Samples were taken by scuba divers from three sites in the Mediterranean Sea (Pilczynska et al. 2016), which were affected by past mass mortality events, and from two sites in the Atlantic Ocean, where *P. clavata* mass mortality was never reported. The distance between the two sites in the Atlantic was approximately 280 km, whereas, in the Mediterranean, sites were separated by distances of 20 to 60 km (Fig. 1.1.). At each site, two different reefs, separated by 200-500 m, were chosen (Fig. 1.1., Table 1.1.). Three plots separated by at least 5 m were randomly chosen at every reef. At each plot we randomly sampled up to 10 different ramets (discrete, spatially isolated colonies) within a circle of 1.0 m radius. Around 3-4 cm of colony branch tip was taken and stored in individual plastic tube under the water. Samples were placed on ice during transport and preserved in ethanol after arrival to the laboratory, no later than 3 hours after collection. In the following text, each reef will be referred to by its code (see Table 1.1.). Fieldwork was carried out in 2013 and 2014. An exception to this sampling scheme was Site 2 (Sagres, Atlantic Ocean), where just 2 plots (17 colonies) were sampled at Reef 3 and only 2 colonies at Reef 4, because of low gorgonian abundance.

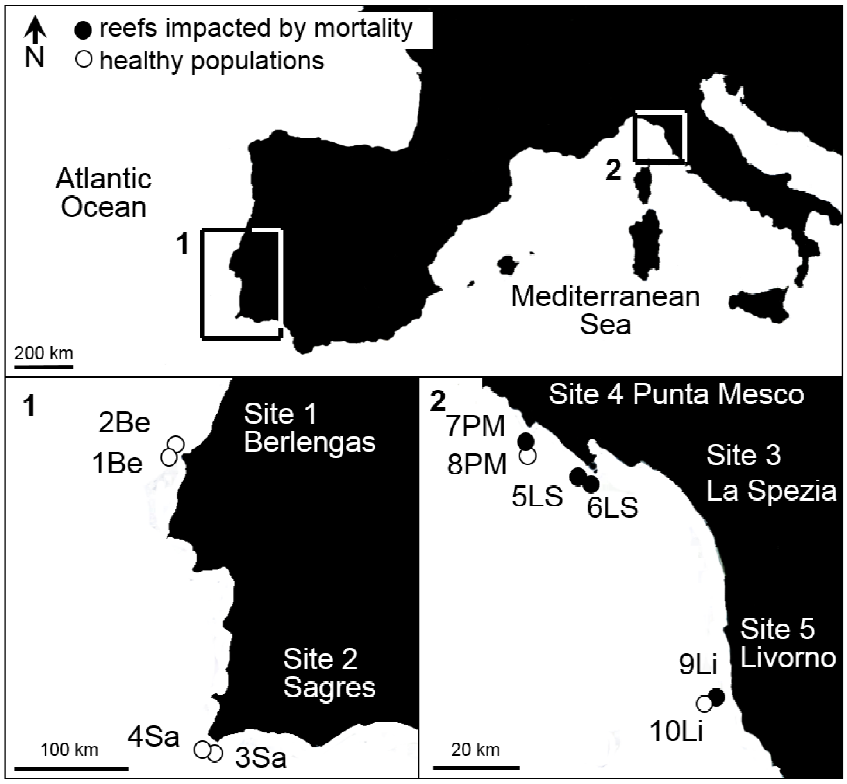

Fig. 1.1. Sampling sites in the Atlantic and the Mediterranean, showing *P. clavata* reefs impacted by mass mortality events (black circles) and healthy populations (white circles).

Table 1.1. Sampling site characteristics: depth range of sampled reefs, year of past mass mortality events and number of the *P. clavata* colonies sampled at each reef.

Site	*Reef code*	*Depth [m]*	*Past mass mortality events*	*Nr of colonies sampled*
Berlengas	1Be	19 - 24	No	30
	2Be	8 - 12	No	30
Sagres	3Sa	11 - 12	No	17
	4Sa	21 - 22	No	2
La Spezia	5LS	18 - 22	1999 and 2003	29
	6LS	19 - 20	1999 and 2003	30
Punta Mesco	7PM	21 - 23	1999 and 2003	30
	8PM	28 - 29	No	30
Livorno	9Li	23 - 25	2006	25
	10Li	30 - 31	No	30

Sampling for *P. clavata* poses several logistic challenges, because of the sparse distribution of populations over very large geographical areas, and depth that limits sampling by conventional SCUBA diving. Additionally, the impacted populations in the Mediterranean are located at shallower depths (< 25 to 30 m) than the non-impacted populations (> 30m; Huete-Stauffer et al. 2011; Linares et al. 2005). This has prevented the development of a balanced sampling design that could account for the effects of geographical region, mortality history and depth. Accordingly, in the present study only two non-impacted and four impacted reefs could be sampled in the Mediterranean, whereas four healthy reefs could be sampled in the Atlantic.

1.3.2. Molecular methods

Coral DNA was extracted using E.Z.N.A. Mollusc DNA Kit according to the manufacturer supplied handbook. We analyzed 10 microsatellites, developed by Agell et al. (2009) and Molecular Ecology Resources Primer Development Consortium (2010), following the protocols published by the authors. Loci Par_a, Par_b, Par_d, Par_f and Par_m were amplified in 10 µl solution of dNTPs (0.25 mM each), selected primers (0.25 µM each), 4 mM of $MgCl_2$, 1x manufacturer-supplied buffer (pH 8.8, 0.1% Tween 20, 25 mM $MgCl_2$) and 0.25 u DFS-Taq DNA Polymerase (Bioron). The PCR program was: 2 min 94°C, (10 sec 94°C, 20 sec annealing temperature, 1 min 72°C)x30, 5 min 72°C. Annealing temperature for particular loci was: Par_a: 59°C, Par_b: 47°C, Par_d: 51°C, Par_f, Par_m: 52°C. To amplify loci Parcla_9, Parcla_10, Parcla_12, Parcla_14 and Parcla_17, a total

genomic DNA was dissolved in 10 µl solution of dNTPs (125 µM each), selected primers (0.5 µM each), 0.25 u GoTaq® DNA Polymerase (Promega) and 1x manufacturer-supplied PCR buffer (pH 8.5, 7.5 mM $MgCl_2$). The PCR program was: 3 min 94°C, (1 min 94°C, 1 min 60°C, 1 min 72°C)x30, 5 min 72°C. The length of amplified fragments was analyzed on an ABI 3730XL Genetic Analyzer using an internal size standard (GeneScan 500 LIZ).

1.3.3. Detection of clonal reproduction

The analysis of DNA fragment lengths was performed with STRand (Toonen and Hughes 2001). Scored microsatellite fragment sizes were then visualized in R environment using the MsatAllele_1.02 package to track and reanalyze scoring errors. MICRO-CHECKER v.2.2.3 (Van Oosterhout et al. 2004) was used to estimate null allele frequency and to check for scoring errors owing to stutters and large allele dropout. Linkage disequilibrium among all pairs of loci was tested in GENEPOP 4.2. (Raymond and Rousset 1995; Rousset 2008) with significance levels determined by the Markov chain method (dememorization = 5000, batches = 500, iterations = 10 000). GIMLET 1.3.3 (Valiére 2002) was used to identify matching multi-locus genotypes indicating clonal origin of the colonies. The probability of identity (*PI*) was calculated in GIMLET using the allele frequencies to quantify the ability of the microsatellite markers to discriminate between two individuals. Two *PI* approaches were used: biased, for randomly mating individuals ($PI_{theoric}$) and unbiased, correcting for small sample sizes ($PI_{unbiased}$). In order to determine if parent/offspring pairs or siblings may have the same observed genotype, specific probability values ($P_{par\text{-}off}$ and P_{sib}) were calculated for each genotype. Matching genotypes were classified as clones when the *PI* and *P* values mentioned above were less than 0.05. Genotypic richness was calculated as N_g/N in order to estimate the maximum contribution of sexual reproduction to local recruitment (Coffroth and Lasker 1998). N_g is the number of unique multi- locus genotypes at each reef and N is the number of colonies sampled at each reef. This index varies between 1 when all individuals have unique genotype, and 0 when one genotype is shared between all ramets. Observed genotypic diversity (G_o) (Stoddart and Taylor 1988) was calculated as: $G_o=1/\Sigma g_i^2$, where g_i is the frequency of ith genotype. Genotypic evenness (G_o/N_g) (Coffroth and Lasker 1998) represents the number of colonies per genet and varies from 1 when individuals are distributed evenly among the clones to 0 when one clone dominates the population. Genotypic diversity, calculated as G_o/G_e, measures the relative contribution of clonal and sexual propagation in a population (Baums et al. 2006). G_e is the expected genotypic diversity and equals the total number of individuals sampled per reef.

In order to evaluate the association between the occurrence of past mass mortality events and frequency of clonal propagation we used log-linear analysis of frequency tables (implemented in Statistica 10). This analysis was restricted to Mediterranean populations because there are no records of past mass mortality events in the Atlantic.

1.4. Results

Among 253 colonies sampled, 250 were successfully genotyped to identify colonies with identical multi-locus genotypes, i.e. clones (2 colonies were lost during the dive and 1 did not amplify at any loci). All loci amplified and were polymorphic, except Par-b, which was monomorphic in both populations from La Spezia, according to the 0.95 frequency criterium. No large allele dropout nor scoring errors was detected by MICRO-CHECKER at any locus. The mean null allele frequency across all reefs varied from 0 for Parcla_10, Parcla_17 and Par_f to 0.18 for Par_m. No significant linkage disequilibrium was observed between any pair of loci (all p>0.05 after FDR correction), thus all loci were considered as genetically independent. Mean number of alleles per locus equalled 14. The probability of identity for pooled samples for all loci was $1.93 \cdot 10^{-12}$ ($PI_{theoric}$) and $1.28 \cdot 10^{-12}$ ($PI_{unbiased}$), both values indicating a low probability of misidentifying clones. An exception was Site 2 (Sagres), where a high percentage of PCR failure, reaching 45%, was observed, affecting mostly Par_a, Par_b, Parcla_9 and Parcla_10. Therefore we may have to low power to detect identical multi locus genotypes. $P_{par\text{-}off}$ and P_{sib} values for all individuals from Reef 3 with the same genotype were higher than 0.05, indicating a high probability that these colonies did not originate from clonal propagation, but were siblings or parents/offspring. Therefore, all colonies from Sagres were assumed to be individual genets.

The contribution of clones was low, although colonies generated from clonal reproduction were detected at all sites. Out of the 250 colonies genotyped, we obtained 236 unique multi-locus genotypes (UMG) and 7 genotypes that appeared more than once (identical multi-locus genotypes – IMG). In the Atlantic, 2 IMG were found at Reef 1Be (in total 4 colonies), whereas in the Mediterranean Sea, 1 IMG was found at 6LS, 2 at 7PM, 1 at 8PM and 1 at 9Li (in total 10 colonies). Clones were not detected at 2Be, 5LS and 10Li. None of the multi-locus genotypes were shared between different plots.

The contribution of sexual reproduction to local recruitment (genotypic richness) varied between 0.93 and 1. Genotypic richness (N_g/N) was the lowest at Reef 1Br and Reef 7PM and the highest at reefs 2Be, 3Sa, 5LS and 10Li, where no clonal propagation was observed. Genotypic evenness (G_o/N_g) and genotypic diversity (G_o/G_e) revealed the same pattern (Table 1.2.). Unique genets were never shared by more than two colonies.

Table 1.2. Genotypic diversity in *P. clavata* based on 10 microsatellite loci; reef 4 (Sagres) was excluded from analysis due to small number of samples.

Site	*Berlengas*		*Sagres*	*La Spezia*		*Punta Mesco*		*Livorno*		*Mean (SD)*
Reef	*1Be*	*2Be*	*3Sa*	*5LS*	*6LS*	*7PM*	*8PM*	*9Li*	*10Li*	
N	30	28	16	29	30	30	30	25	30	27.6 (±4.6)
N_g	28	28	16	29	29	28	29	24	30	26.3 (±5.6)
N_g/N	0.93	1	1	1	0.97	0.93	0.97	0.96	1	0.95 (±0.08)
G_o	26.47	28.00	16.00	29.00	28.13	26.47	28.13	23.15	30.00	25.3 (±6.6)
G_o/N_g	0.95	1	1	1	0.97	0.95	0.97	0.96	1	0.95 (±0.09)
G_e	30	28	16	29	30	30	30	25	30	27.6 (±4.6)
G_o/G_e	0.88	1	1	1	0.94	0.88	0.94	0.93	1	0.90 (±0.15)

N - number of colonies in population; N_g - number of genets; N_g/N - genotypic richness; G_o - observed genotypic diversity; G_o/N_g – genotypic evenness (number of ramets per genet); G_e - expected genotypic diversity; G_o/G_e - genotypic diversity.

Log-linear analysis of frequencies indicated no overall differences in contribution of clonal reproduction between reefs impacted by mass mortality and healthy populations in the Mediterranean (χ^2=0.74, df=1, p=0.39).

1.5. Discussion

1.5.1. Clonal propagation

This study examined for the first time the contribution of asexual reproduction in the red gorgonian *Paramuricea clavata* using molecular markers. Our findings corroborates previous indications that clonal propagation is not common for this key species. Additionally, our results indicate that past mortality history of Mediterranean populations does not affect the level of asexual reproduction.

Coma et al. (1995b) reported that colonies originating from clonal reproduction constitute around 2% of the red gorgonian population, but that study was based on morphological characters, such as colony shape and presence of stolons connecting mother and daughter colonies. The employment of genetic markers allowed for a much more reliable results. In our study, 14 colonies (5.6% of all examined colonies) had genotypes that appeared more than once (identical multi-locus genotypes – IMG). At two reefs 13.3% of the colonies (4 out of 30) shared multi-locus genotype. At four reefs, however, all sampled colonies had unique multi-locus genotypes, suggesting that in

these populations clonal reproduction does not take place or is infrequent. Previous genetic studies on *P. clavata* did not report occurrence of clonal propagation. In Mokhtar-Jamaï et al. (2011) the distance between sampled colonies was not mentioned, and thus we cannot exclude the possibility that the distance was too large to detect clones. Mokhtar-Jamaï et al. (2013) however, found 4 pairs of colonies sharing the same multi-locus genotype. In this case colonies were separated by less than 5 cm and the probability that they share identical genotype by chance, through sexual reproduction, was very low. This four colonies constitute 3.8% of investigated population, which value is in the same order of magnitude of that reported here. The study of Mokhtar-Jamaï et al. (2013) was not focused on clonal propagation and therefore the authors excluded repeated genotypes from further analysis and did not discuss this phenomenon. The genotypic richness and diversity values in our study were high at all reefs (N_g/N>0.93; G_o/G_e>0.88) indicating that all populations rely on sexual reproduction as the dominant mode of propagation. Previous studies have reported that clonal propagation in octocorals is most common among tropical soft coral species in the families form the Alcyoniina suborder, including Alcyoniidae, Nephtheidae, and Xeniidae and in Clavulariidae from the Stolonifera suborder (Simpson 2009). However, other tropical gorgonians are also known to propagate asexually. The gorgonian *Plexuara kuna* is able to dominate the local community with small number of clones, reaching high colony densities probably faster than via sexual reproduction (Coffroth and Lasker 1998). The gorgonian coral *Junceella juncea* from Taiwan relies on clonal propagation to maintain established populations, which was confirmed by the low values of genotypic diversity (G_o/G_e between 0.217 and 0.650) (Liu et al. 2005).

The low number of clones found in our study may be also a result of sampling error. Colonies grow in dense aggregations and it may be difficult to distinguish separate ramets. However, during our fieldwork we paid attention to the base of sampled colonies to be sure they are separated. Additionally, sampling was conducted always by a team of two divers, therefore one person could constantly monitor which colonies are being sampled. Also in the study of Mokhtar-Jamaï et al. (2013) clones were detected, therefore we may expect that clonal reproduction in *P. clavata* may occur.

Asexual propagation in the *P. clavata* population from La Spezia (reefs 5LS and 6LS) cannot be responsible for the high number of recruits present in this site after the mortality event, as recorded by Cupido et al. (2012). The results of the present study indicate that other factors, such as increased reproductive output and/or recruitment rate after the mortality event, or decreased competition because of a larger area of available substratum, rather than clonal propagation, enables disturbed populations to recover after being affected by climatic events. Similarly, the red coral *Corallium rubrum*, impacted by mass mortality events in the Mediterranean Sea, has a limited capability for clonal propagation, thus the only way to recover from disturbance is via sexual propagation (Garrabou et al. 2001). This reproductive feature of habitat forming species has an

important meaning for the conservation of coralligenous assemblages - one of the most species rich communities in the Mediterranean Sea (Ballesteros 2006).

Man-induced sources of the red gorgonian detachment (anchors, fishing apparatus, involuntary handling by divers; Harmelin and Marinopoulos 1994) may produce colony fragments increasing clonal reproduction frequency in sites subjected to human activities. However it seems to not be a case here, since the highest number of clones was found in Punta Mesco, located in the Cinque Terre Marine Protected Area where fishing, anchoring and diving is prohibited.

In the Sagres population, high percentage of PCR failure was observed, possibly indicating incompatibility of primers. Colonies from Sagres differed from all other investigated populations, being bright yellow, not purple. Yellow colonies are reported to be rare in the Mediterranean, whereas purple colonies with yellow apical branches are more common (Carpine and Grasshoff 1975). Further studies are necessary to determine if the yellow type is a separate species, or a phenotype of the same species.

The balance between sexual reproduction and clonal propagation affects the transmission of genetic variation and therefore may greatly influence genetic diversity (Les 1991; Piquot et al. 1996) and ultimately the evolutionary potential of populations (Williams 1975). Although genetic diversity, measured as heterozygosity and allelic richness, does not decrease in populations with prevalence of asexual reproduction (Balloux et al. 2003), the diversity of genotypes is being reduced, possibly decreasing adaptive potential of population. Indeed, clonal propagation is more frequent in rare and endangered species, as shown by Silvertown (2008) for terrestrial plants. Rare asexual reproduction in *P. clavata* and high diversity of genotypes may be an advantage for the species in a changing environment, possibly allowing to adapt more easily to temperature anomalies.

1.5.2. Conclusions

Clonal propagation does not play an important role in *P. clavata*. Although asexual reproduction is not a sporadic phenomenon, as indicated by previous assessments, it was not the dominant factor accounting for population recovery in sites that have been affected by past mass mortality events because i) maximum prevalence of clones was *ca.* 13% and ii) there were no differences in clone prevalence between impacted and non-impacted sites. Infrequent clonal propagation, in addition to sporadic recruitment and low larval dispersal, makes recovery a difficult and time-consuming process. There is, therefore, a definite need to develop conservation plans to protect local populations and already existing colonies by controlling anthropogenic stressors, such as harboring, trawling and diving.

1.6. References

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Chapter 2. Genetic diversity and local connectivity in the Mediterranean red gorgonian coral after mass mortality events

2.1. Abstract

Estimating the patterns of connectivity in marine taxa with planktonic dispersive stages is a challenging but crucial task because of its conservation implications. The red gorgonian *Paramuricea clavata* is a habitat forming species, characterized by short larval dispersal and high reproductive output, but low recruitment. In the recent past, the species was impacted by mass mortality events caused by increased water temperatures in summer. In the present study, we used 9 microsatellites to investigate the genetic structure and connectivity in the highly threatened populations from the Ligurian Sea (NW Mediterranean). No evidence for a recent bottleneck neither decreased genetic diversity in sites impacted by mass mortality events were found. Significant IBD pattern and high global F_{ST} confirmed low larval dispersal capability in the red gorgonian. The maximum dispersal distance was estimated at 20-60 km. Larval exchange between sites separated by hundreds of meters and between different depths was detected at each site, supporting the hypothesis that deeper subpopulations unaffected by surface warming peaks may provide larvae for shallower ones, enabling recovery after climatically induced mortality events.

2.2. Introduction

Extreme weather events, including floods, heat waves and droughts, are currently emerging as one of the most important facets of climate change, and a growing body of literature is focused on extreme events (Jentsch et al. 2007). Anomalous and extreme events due to global warming have increased considerably during recent decades in temperate regions such as the Mediterranean Sea and an increase in the frequency of heat wave extremes of 200-500 % is predicted at the end of the twenty-first century (Giorgi and Lionello 2008). Extreme events, together with other sources of mortality caused by human impact, such as overfishing and environmental pollution, may cause significant impacts on genetic diversity as a result of population size decrease (Pujolar et al. 2011a), as has been observed after mass mortality events (e.g. Arnaud-Haond et al. 2009; Pujolar et al. 2011b).

If natural populations consist of reduced numbers of individuals, loss of genetic variability may dramatically influence the populations themselves, since genetically depauperate populations might fail to adapt to future environmental changes, eventually causing their disappearance. Due to the predicted intensification of weather extremes, many species may not have sufficient genetic potential for the evolution of strategies able to mitigate their impact. Nevertheless, genetic diversity reduction after mass mortality events is not a rule in the marine realm (e.g. Pujolar et al. 2011a; Colson and Huges 2004). There is an urgent need to carry out dedicated research to acquire more extensive, sound data across a range of life-history and demographic features, with the ultimate

aim of formulating better predictions about the role of catastrophic disturbances in determining genetic structure and genetic diversity.

Connectivity patterns and gene exchange among populations are major research topics in marine ecology and are essential for the planning of marine reserves (Palumbi 2003), to function as interconnected networks that can supply recruits to sites that undergo population bottlenecks or local extinctions. Genetic recovery from events of mass mortality, either natural or human-induced, may be dependent on the possibility of dispersal from external sources or from small local populations at refugial pockets (Underwood et al. 2007; Hughes 2007; Maggs et al. 2008). This can result in much slower recovery of genetic diversity than population density, because demographic recovery is possible from a few founder or bottleneck survivors but genetic recovery requires extensive levels of connectivity or a long period of time (Arnaud-Haond et al. 2009). However, inferring levels of connectivity is particularly challenging in the marine environment, where many species disperse exclusively by means of planktonic propagules, such as larval or spore stages, in a 3-dimensional fluid medium. Direct estimation of dispersal by tracking marine propagules is often not feasible in most instances, because of their small size and the unbounded nature of the marine environment (Bullock et al. 2006; Levin 2006), so indirect methods, such as the use of neutral genetic markers (Gilg and Hilbish 2003; Planes et al. 2009), are commonly applied.

The red gorgonian *Paramuricea clavata* (Risso, 1826) is a key species of sublittoral rocky habitats (Ballesteros 2006), widespread in the western Mediterranean Sea and in the Adriatic Sea (Carpine and Grasshof 1975) and less common in the Aegean Sea (Öztürk et al. 2004). The species is a surface brooder with a short larval dispersal phase (Linares et al. 2007a; Coma et al. 1995a). In situ observations indicate that the dispersive stage of the larvae may last only a few minutes and thus the larvae are expected to settle near the mother colony (Linares et al. 2007a). This mechanism does not favor dispersal, but possibly decreases larval mortality and wastage, contributing to the replenishment of local populations (Linares et al. 2007b). However, metamorphosis may be delayed in the laboratory for up to 25 days after egg collection, suggesting high dispersal capacity, at least under certain conditions (Linares et al. 2007a). In 1999 and 2003, two episodes of mass mortality, connected with increased water temperature reaching 24°C (Romano et al. 2000), affected several populations of benthic suspension feeders in the northwestern Mediterranean, causing a drastic decrease of *P. clavata* colony density (Linares et al. 2005; Cerrano et al. 2005; Cupido et al. 2008). The number of colonies affected by partial mortality decreased with depth (Cerrano et al. 2005; Huete-Stauffer et al. 2011), because mortality affected the community from the surface to approximately 20 m, the approximate depth of the thermocline. Hereafter we designate as deep the sites with colonies living below the thermocline, which were not exposed to abnormally long lasting summer conditions (Coma et al. 2009). Therefore, the clear differences in the number of damaged colonies and the extent of injury were visible between shallow populations (dwelling above the thermocline) and the deep ones, even when the depth difference was only of a few meters (Linares et al. 2005; Huete-Stauffer et al. 2011). As

hypothesized by Cerrano and Bavestrello (2008; 2009), the deep dwelling subpopulations may act as a reservoir, supplying larvae to shallower sites, especially because large colonies with high fecundity rates survived there. However, little is known about short distance dispersal of the species. Surveys based on microsatellite loci in the Mediterranean Sea, showed that *P. clavata* exhibits a high level of genetic differentiation at the small and large spatial scales, which is consistent with the short larval dispersal displayed by the species (Mokhtar-Jamaï et al. 2011; 2013; Arizmendi-Mejía et al. 2015). Connectivity among populations of *P. clavata* separated by less than 14 km was weak in the study of (Arizmendi-Mejía et al. 2015), since the mean immigration rate was below 9% and most of the immigrants came from neighboring populations located only hundreds of meters away.

In the present study we used microsatellite markers to study the genetic structure of *P. clavata* populations in an area impacted by mass mortality events, in the Ligurian Sea, Mediterranean. Our objectives were twofold. The first objective was to investigate a possible bottleneck effect of past mortality events on genetic diversity in a region where some populations were highly affected by mass mortality events and others were not. In this case we are expecting to detect the decrease of genetic diversity at sites impacted by mortality. Our second objective was to understand the connectivity patterns and migration at the scale of a few tens of km since up to date very few studies have examined short distance migration in the species. We expect to detect migrations between non-impacted reefs and impacted ones, supporting recovery of damaged populations. Our research contributes to better understand the mechanisms that enable recovery of threatened populations by providing data about larval migrations between impacted and healthy populations of the red gorgonian. The results should prove to be particularly valuable for the conservation of soft corals communities and thus the overall marine biodiversity.

2.3. Material and methods

2.3.1. Sampling

P. clavata colonies were sampled by scuba divers following a hierarchical sampling design. Samples were taken from three sites, Punta Mesco, La Spezia and Livorno, in the Ligurian Sea (NW Mediterranean). At each site two different reefs were chosen (Fig. 2.1., Table 2.1.). In the remaining of the text, each reef will be referred to by its code from Table 2.1. At Punta Mesco and Livorno reefs were sampled at different depths. At these two sites shallow reefs (<25 m depth) were impacted by mass mortality events, whereas colonies dwelling below 25 m (referred as deep reefs in the remaining of the text) remained non-impacted (Peirano et al. 2009; Di Fiore M., pers com). At La Spezia both reefs were sampled at the same depth, since the rocky cliff ends on a muddy bottom at 24-25 m, and therefore both reefs were damaged during the mass mortality (Table 2.1). Populations impacted by mass mortality recovered significantly. Colonies density in La Spezia population declined from over 35 to nearly 8 colonies m^{-2} shortly after the mass mortality

and increased to 20 colonies m^{-2} four years later (Santangelo et al. 2015). In Portofino (Ligurian sea), located near Punta Mesco, colonies density decreased after the mortality event from nearly 20 to 5 colonies m^{-2}, but 3 years after the event the density recovered to pre-mortality levels (Cerrano et al. 2005). We do not have data from Punta Mesco and Livorno but we may expect that the recovery pattern was similar and, therefore, all impacted populations consist of survivors and new colonies that settled after the mortality. Sites were separated by distances ranging from 20 to 60 km (Fig. 2.1.) whereas reefs within each site were separated by 200 to 300 m. Site 1, Punta Mesco, is located in the Cinque Terre Marine Protected Area. A small portion of colony branch (around 3-4 cm of branch tip) was taken from up to 30 different colonies randomly chosen from each reef. The branch tip from each colony was stored individually in a plastic tube underwater. Samples were placed on ice during transport and preserved in ethanol after arrival to the laboratory, no later than 3 hours after collection.

The Cinque Terre Marine Protected Area authorized ENEA to conduct fieldwork and sampling at Punta Mesco (reef 1 and 2). La Spezia Islands (reef 3 and 4) are included in a recently established marine conservation area where ENEA is authorized by the Regional Natural Park of Porto Venere to conduct fieldwork and sampling. No specific permission was required for sampling at Livorno. *P. clavata* is not endangered nor protected.

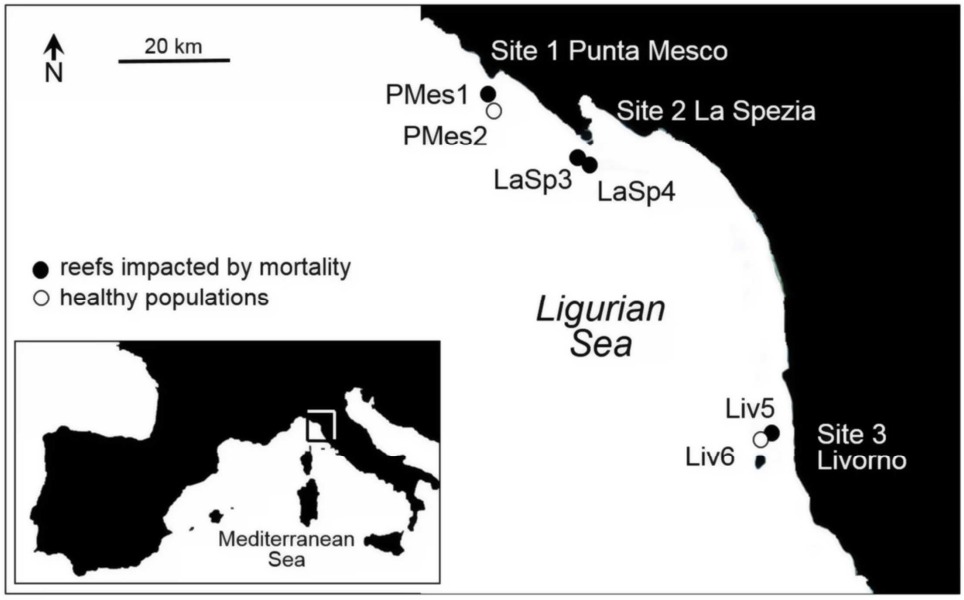

Fig. 2.1. Sampling sites in the Mediterranean. Reefs impacted by mass mortality events (in black) and healthy reefs (in white).

Table 2.1. Sampling sites characteristics.

Site	*Reef code*	*Depth [m]*	*Past mass mortality events*	*N*	Reef coordinates
1 Punta Mesco	PMes1	21 - 23	1999 and 2003 (Peirano et al. 2009)	30	44°7'59''N 9°38'7''E
	PMes2	28 - 29	No (Peirano et al. 2009)	30	44°7'59''N 9°38'9''E
2 La Spezia	LaSp3	18 - 22	1999 and 2003 (Cerrano et al. 2000; Cupido et al. 2008)	29	44°1'25''N 9°51'2''E
	LaSp4	19 - 20	1999 and 2003 (Cerrano et al. 2000; Cupido et al. 2008)	30	44°1'22''N 9°51'4''E
3 Livorno	Liv5	23 - 25	2006 (Di Fiore M, pers com)	25	43°27'50''N 10°19'48''E
	Liv6	30 - 31	No (Di Fiore M, pers com)	30	43°28'5''N 10°19'49''E

The depth range of sampled reefs, the year of past mass mortality events, N - the number of colonies sampled at each reef and the geographic coordinates.

2.3.2. *Microsatellite analysis*

Coral DNA was extracted using E.Z.N.A. Mollusc DNA Kit according to the manufacturer handbook. We analyzed 10 microsatellite, developed by Agell et al. (2009) and Molecular Ecology Resources Primer Development Consortium (2010) following the protocols published by the authors. Loci Par_a, Par_b, Par_d, Par_f and Par_m were amplified from total genomic DNA in 10 µl solution of dNTPs (0.25 mM each), selected primers (0.25 µM each), 4 mM of $MgCl_2$, 1x manufacturer-supplied buffer (pH 8.8, 0.1% Tween 20, 25 mM $MgCl_2$) and 0.25 u DFS-Taq DNA Polymerase (Bioron). The PCR program was: 2 min 94°C, (10 sec 94°C, 20 sec AT, 1 min 72°C)x30, 5 min 72°C. Annealing temperatures (AT): Par_a: 59°C, Par_b: 47°C, Par_d: 51°C, Par_f, Par_m: 52°C. To amplify loci Parcla_9, Parcla_10, Parcla_12, Parcla_14 and Parcla_17, total genomic DNA was dissolved in 10 µl solution of dNTPs (125 µM each), selected primers (0.5 µM each), 0.25 u GoTaq® DNA Polymerase (Promega) and 1x manufacturer-supplied PCR buffer (pH 8.5, 7.5 mM $MgCl_2$). The PCR program was: 3 min 94°C, (1 min 94°C, 1 min 60°C, 1 min 72°C)x30, 5 min 72°C. The length of amplified fragments was analyzed on an ABI 3730XL Genetic Analyzer using an internal size standard (GeneScan 500 LIZ). The analysis of DNA fragment length was performed with STRand (Toonen and Hughes 2001). Scored microsatellite fragment sizes were then visualized in R environment using the MsatAllele_1.02 package to track and reanalyze scoring errors.

2.3.3. *Genetic diversity*

MICRO-CHECKER v.2.2.3 (Van Oosterhout et al. 2004) was used to estimate null allele frequency and to check for scoring errors owing to stutters and large allele dropout. Linkage disequilibrium

among all pairs of loci was tested in GENEPOP 4.2. (Raymond and Rousset 1995; Rousset 2008) with significance levels determined by the Markov chain method (dememorization = 5000, batches = 500, iterations = 10 000).

Observed (H_o) and Nei's (1973) unbiased expected heterozygosity (H_e) were computed in GENETIX v.4.05 (Belkhiret al. 2004). The rarefaction procedure implemented in HP-RARE software (Kalinowski 2005) was used to estimate allelic richness (A_r) and private allelic richness (A_p). The minimum number of genes was set to 25 (the minimum sample size). Differences in heterozygosity and allelic richness between impacted and healthy reefs were tested using Kruskal-Wallis. The power of the test and minimum sample size to achieve 90% power were calculated in PASS 14 (2015). Single and multi-locus Weir and Cockerham's (1984) *f* estimator of F_{IS} were calculated using GENEPOP 4.2. Departures from Hardy–Weinberg (HW) equilibrium within sample for each locus and over all loci were tested in GENEPOP 4.2. The level of significance was determined by the Markov chain method using the default parameters (dememorization = 1000, batches = 100, iterations = 1000).

Populations that experienced a recent mass mortality are predicted to lose allelic diversity faster than heterozygosity (Luikart and Cornuet 1998) and thus exhibit a heterozygosity excess relative to the heterozygosity expected from the observed number of alleles. To establish whether there is a heterozygosity excess or deficit, the BOTTLENECK software (Piry et al. 1999) computes a distribution of the expected heterozygosity under the assumption of mutation-drift equilibrium, calculated from the observed number of alleles, and compares it to the heterozygosity expected under Hardy-Weinberg equilibrium. The presence of a possible bottleneck effect was tested using 9999 simulations under the Two-Phase Model (TPM), since it generally fits microsatellite evolution better than either pure stepwise or infinite allele models (Luikart and Cornuet 1998; Piry et al. 1999). TPM assumes that the majority of mutations are single steps, when alleles increase or decrease by one repeat unit. The mutation sizes for remaining mutations are drawn from a geometric distribution and larger mutations are rare, but do occur. The frequency of step mutations was set to 0.9 (ps), the variance of mutations to 12 and the Wilcoxon test was used to test the null hypothesis of no significant heterozygosity excess.

2.3.4. Population differentiation and connectivity

Global and pairwise Weir and Cockerham's (1984) estimator of F_{ST} was estimated in GENEPOP. The genotypic differentiation between all pairs of reefs was tested in GENEPOP with default parameters.

The isolation by distance pattern was tested in GENEPOP. The shortest possible distance over sea between each reef pair was measured using Goggle Earth 7.1.2.2041. The relationship between genetic distance [$F_{ST}/(1-F_{ST})$] and the spatial distance [km] was tested using a Mantel test (n = 2000).

In order to quantify genetic variation within reefs, among reefs within a site and among sites a hierarchical analysis of molecular variance (AMOVA) was performed in ARLEQUIN 3.5 (Excoffieret al. 2005). The significance of these variance components was tested using 50000 permutations.

The Bayesian approach implemented in STRUCTURE v.2.2 was used to investigate population structure. The recessive allele option was used to deal with null alleles (Falush et al.2007). The number of clusters (K) in the data set was evaluated under the admixture model with correlated allele frequencies. First run of 10 iterations, burnin of 10000 and MCMC = 50000 was computed for K from 1 to 10. The value of K that captures the major structure in the data was selected based on the plot of logarithm of the likelihood of observing the data [LnP(D)] as a function of K (Pritchard et al. 2007). STRUCTURE was then run 30 times for K values from 2 to 6. The results were merged in CLUMPP (Jakobsson and Rosenberg 2007) and graphically displayed in DISTRUCT (Rosenberg 2004). The analysis was then repeated in the groups defined in the first run of STRUCTURE, when K=3, to search for substructure within the groups.

A Bayesian assignment method (Rannala and Mountain 1997) implemented in GENECLASS2 (Piry et al. 2004) was used to detect putative first generation migrants (F0). A Monte Carlo resampling method, as described in Paetkau et al. (2004), was performed to evaluate each individual's probability of belonging to a population from each reef.

Whenever multiple tests were conducted (linkage disequilibrium, HW equilibrium, genetic differentiation), the level of significance was adjusted using a false discovery rate (FDR; Benjamini et al. 1995).

2.4. Results

2.4.1. Genetic diversity

No large allele dropout was detected by MICRO-CHECKER at any locus, but evidence of scoring errors due to stuttering was found in Parcla_12 and this locus was excluded from further analysis. The mean null allele frequency across all reefs varied from 0 for Parcla_10, Parcla_17 and Par_f to 0.18 for Par_m. No significant linkage disequilibrium was observed between any pair of loci (all $p>0.05$ after FDR correction), thus all loci were considered as genetically independent.

Eight of the loci were polymorphic at all sites, whereas one locus, Par_b, was monomorphic at LaSp3 and LaSp4 according to the 0.95 frequency criterium. The total number of alleles ranged from 4 for Par_b, Par_d and Par_f to 27 for Par_m. Unbiased expected heterozygosity (H_e) varied between 0.57 at PMes1 to 0.66 at Liv6, with a mean value of 0.62 (0.03). H_o ranged from 0.42 for LaSp4 to 0.59 at Liv5, with a mean value of 0.51 (0.07) (Table 2.2.). The lowest allelic richness (A_r) was found at PMes1 (4.77) whereas the highest value was found at PMes2 (5.84). Private allelic richness (A_p) varied from 0.34 at PMes1 to 1.17 at PMes2 (Table 2.2.). There was no evidence that reefs affected by mass mortality events had lower genetic diversity than healthy ones (Kruskal-

Wallis, all p>0.05). The power of the test was low for He (20%) and Ar (20%), and moderate for Ho (70%) and Ap (66%). In our case, to achieve test power reaching 90% for the 0.05 confidence level, sample size would need to be at least 166 colonies per reef. Multilocus F_{IS} ranged from 0.08 at Liv5 to 0.31 at LaSp4 (Table 2.2.). When all loci were examined separately, the lowest value was -0.26, for Par_b, and the highest one, 0.70, for Par_a.

Table 2.2. Measures of genetic diversity.

	He (± SD)	Ho (± SD)	Ar	Ap	F_{IS}
PMes1	0.57 (± 0.28)	0.46 (± 0.27)	4.77	0.34	**0.20**
PMes2	0.61 (± 0.27)	0.48 (± 0.23)	5.84	1.17	**0.20**
LaSp3	0.60 (± 0.25)	0.52 (± 0.27)	4.92	0.55	**0.13**
LaSp4	0.61 (± 0.25)	0.42 (± 0.27)	4.93	0.39	**0.31**
Liv5	0.64 (± 0.18)	0.59 (± 0.23)	5.40	0.57	**0.08**
Liv6	0.66 (± 0.14)	0.57 (± 0.15)	5.18	0.47	**0.13**
mean	*0.62 (± 0.03)*	*0.51 (± 0.07)*	*5.17 (± 0.40)*	*0.58 (± 0.30)*	*0.17 (± 0.08)*

Measures of genetic diversity (mean ± SD) in 6 reefs of Paramuricea clavata at 9 microsatellite loci. H_e – Nei's (1973) unbiased expected heterozygosity; H_o - observed heterozygosity; Ar and Ap - allelic and private allelic richness, respectively (with rarefaction size of 25 genes); F_{IS} - Weir and Cockerham's (1984) f estimator of F_{IS} with significant values in bold (0.05 threshold after FDR correction).

Significant heterozygote deficiency was detected at all reefs (Table 2.2.). However, the departures from HW equilibrium were not evident for all loci at all sites. Populations from all reefs revealed a departure from HW equilibrium at locus Par_m, but the highest frequency of null alleles was found in this locus and evidence for null alleles was also detected at all reefs for this locus. When Par_m was excluded from the analyses, heterozygote deficiency was still significant at all reefs except Liv5 after FDR correction.

No evidence for a recent genetic bottleneck was detected for any of the investigated reefs. A heterozygote excess (an indicator of recent bottleneck) was not found at any reef (Wilcoxon, all p>0.58) neither was a heterozygote deficit (i.e., a sign of expansion) (all p>0.08).

2.4.2. Population differentiation and connectivity

Global F_{ST} value was 0.118, whereas pairwise comparisons between all pair of reefs varied from -0.003 between Liv5 and Liv6 to 0.189 between PMes1 and Liv6 (Table 2.3.). All comparisons were significant (p<0.05), except Liv5 and Liv6, which revealed also no statistical differences in genotypic composition (Chi2=18.5, df=18, p=0.42).

Table 2.3. Pairwise F_{ST} values

	PMes2	LaSp3	LaSp4	Liv5	Liv6
PMes1	0.00411	0.08499	0.07483	0.18168	0.18917
PMes2		0.08048	0.06603	0.17626	0.18165
LaSp3			0.00483	0.17214	0.17452
LaSp4				0.16189	0.16454
Liv5					-0.00332

Global and pairwise Weir and Cockerham's (1984) estimator of F_{ST} between all pairs of P. clavata reefs. All but one (Liv 5 and Liv6) comparisons were significant.

The correlation between $F_{ST}/(1-F_{ST})$ and distance was significant (p=0.01), supporting an isolation by distance model of gene flow in *P. clavata* at the local scale (Fig. 2.2).

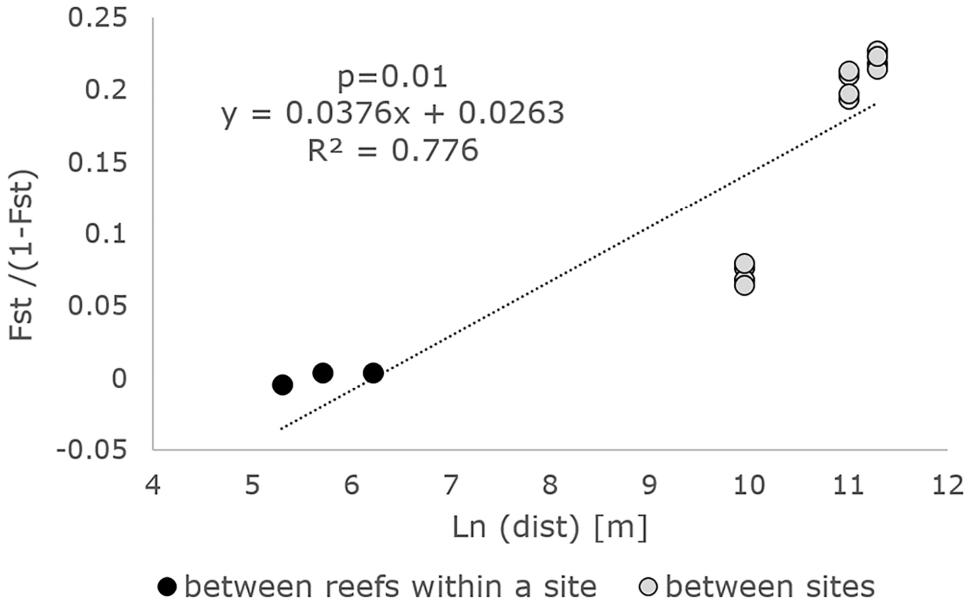

Fig. 2.2. The isolation by distance pattern for *P. clavata*. Linear regression of the genetic distance measured as $F_{ST}/(1 - F_{ST})$ over the geographic distance (m).

The AMOVA (Table 2.4.) revealed that a highly significant percentage (11.19%) of the total genetic variation occurred among sites, whereas a smaller, but still significant, percentage of variation was explained by differences among reefs within sites (1.17%). Indeed, most of the variance was explained by differences within reefs (87.64%) and this was highly significant.

Table 2.4. AMOVA

Source of variation	df	Sum of squares	Variance components	% of variance	*p*-value
Among sites	2	101.88	0.39 Va	11.19	<0.0001
Among reefs within sites	3	16.36	0.04 Vb	1.17	<0.05
Within reefs	342	1050.82	3.07 Vc	87.64	<0.0001
Total	347	1169.05	3.51		

Analysis of molecular variance (AMOVA) among P. clavata reefs.

In the STRUCTURE analysis the plot of LnP(D) as a function of K revealed a plateau for K≥3. Samples were divided into 3 clearly separated clusters, each of them grouping the two reefs from the same site (Fig. 2.3.). Samples from Livorno (Liv5 and Liv6) displayed a high coefficient of population membership while reefs from Punta Mesco (PMes1 and PMes2) and La Spezia (LaSp3 and LaSp4) showed a low level of admixture. A second run of STRUCTURE did not reveal any genetic structure within the groups (data not shown). When K was set to 2, reefs from Livorno were separated from the others, whereas a K value over 3 did not reveal any additional structure.

According to GENECLASS2, 37.9% of the colonies were first generation migrants (F0). Most of them (31.6%) were exchanged between reefs separated by 200-300 m within the same site. The contribution of F0 migrants from the same site varied from 23.3 to 31.0% for PMes1 and LaSp3, respectively (Table 2.5.). Between-sites migration was detected only between La Spezia and Punta Mesco, separated by 20km. The data suggest that migration occurred in both senses, with 10.0% of the colonies from PMes (percentage from both reefs pooled together) assumed as migrants from LaSp and 8.5% of the colonies form LaSp contributed from PMes. Migrants exchanged between Liv5 and Liv6 were not evaluated because these sites do not differ significantly (F_{ST}=-0.00332).

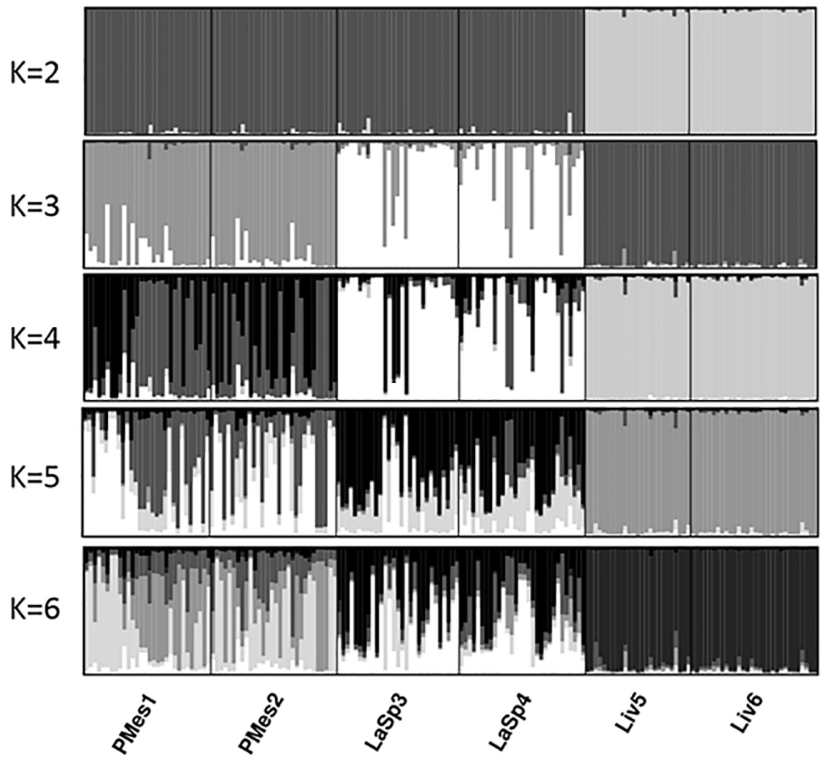

Fig. 2.3. Clustering analysis. Population structure revealed by the first run of clustering analyses in STRUCTURE. Each individual is represented by a vertical line, divided into segments representing the proportion of the genome of the individual that is assigned to each cluster. The number of clusters was set to 2-6. Reefs are separated by a black vertical line.

Table 2.5. First generation migrants.

	Source of migrants					
	PMes1	PMes2	LaSp3	LaSp4	Liv5	Liv6
PMes1	-	23.3	3.3	6.7	0.0	0.0
PMes2	30.0	-	3.3	6.7	0.0	0.0
LaSp3	3.4	0.0	-	31.0	0.0	0.0
LaSp4	3.3	10.0	26.7	-	0.0	0.0
Liv5	0.0	0.0	0.0	0.0	-	-
Liv6	0.0	0.0	0.0	0.0	-	-

The percentage of P. clavata colonies assumed as first generation migrants (F0) from each of the investigated reefs.

2.5. Discussion

2.5.1. Genetic diversity

Our results did not support the hypothesis of genetic effects of past mass mortality events in *P. clavata* from the Ligurian Sea. No differences in genetic diversity between reefs affected by mass mortality events and healthy ones were detected. Additionally, the levels of genetic diversity found in *P. clavata* from the Ligurian Sea were not much lower than the range of values reported previously for the species (Mokhtar-Jamaï et al. 2011) and other Mediterranean (Costantini et al. 2007a) and tropical corals (Gutiérrez-Rodríguez et al. 2004; Maier et al. 2005; Magalon et al. 2005). Numerous gorgonian populations in the Mediterranean were affected by mass mortality in the recent past (Linares et al. 2005; Cerrano et al. 2005; Cupido et al. 2008) and there is the concern that these may have gone through genetic bottlenecks as a result of decreased population densities. However, confounding effects may obscure bottleneck results. For example, in *Corallium rubrum* (Linnaeus, 1758), 16 of 40 Mediterranean shallow populations showed a sign of recent expansion after a bottleneck (Ledoux et al. 2010), although a Wahlund effect could not be discarded. In a study of *P. clavata* from Ibiza (Balearic Islands, Spain), partial mortality, measured as the proportion of colony tissue damaged, was negatively correlated with effective population size, mean number of alleles per population and proportion of recent migration rates (Arizmendi-Mejía et al. 2015). These results indicated that populations with colonies that are partially affected by mortality are less diverse, undergo a larger effect of drift and receive less immigrants than healthy gorgonian populations. Additionally, in our study, allelic richness was slightly higher in populations non-impacted by mass mortality, which is in agreement with published results (Arizmendi-Mejía et al. 2015). Yet, in spite of the extensive density reductions that mass mortality events had on the Ligurian red gorgonian (ca. four-fold; see methods section), our results show no difference in genetic diversity between healthy and impacted sites. This may suggest that the adaptive potential of surviving populations was not reduced. Nonetheless, this interpretation should be cautious given that our sampling design was limited to six reefs, among which only two were not impacted by mass mortality. Consequently the test presents low to moderate power (20-70%) to detect differences between genetic diversity of healthy and impacted sites (high probability of making a type II error, i.e., failing to detect existing differences). The need to increase power in such tests with higher number of samples and loci has been highlighted in previous studies (e.g., Peery et al. 2012). To achieve a power of 90%, we would need to sample 166 colonies per reef (ca. 1000 colonies in total), which is unrealistic, because of the logistic effort and the limited number of colonies at some reefs. It is also noteworthy that heterozygosity excess tests have been shown to have a limited power to detect mild-bottlenecks of 10-1000-fold population declines (Girod et al. 2011). Even if past mass mortality events have affected genetic diversity, it may not be detected by our study, because of the lack of power of the statistical tests and other confounding factors such as recent expansion or Wahlund effect.

The departures from Hardy-Weinberg equilibrium found in the present research, indicated by high and significant F_{IS} values, are typical for species exhibiting low larval dispersal, and were already reported in *P. clavata* populations [29]. Heterozygote deficit as a result of inbreeding was previously reported in coral species characterized by having a short larval dispersal (Gutiérrez-Rodríguez et al. 2004; Magalon et al. 2005; Costantini et al. 2007b). Our findings confirm previous studies and are in accordance with the reproductive biology and larval ecology of *P. clavata*. Planulae exhibit negative phototaxis and negative buoyancy and settle near the mother colony (Linares et al. 2007a), increasing the probability of subsequent mating with closely related individuals, associated with reduced dispersal of the male gametes also. High F_{IS} values may also result from a high number of F0 migrants, analogously to Wahlund effect, since migrants come from genetically distinct population. The presence of null alleles may also partially explain the result, but heterozygote deficit was still prevalent in the absence of the main locus suspected to have null alleles.

2.5.2. Genetic structure and connectivity

The high value of global F_{ST} indicates strong genetic differentiation among reefs and additionally supports the low dispersal capability of the larvae. The value obtained in the present study was nearly equal to the one previously found in the species (0.118 in the present study versus 0.116; Mokhtar-Jamaï et al. 2011). Our results indicated that the majority of variance in the population can be explained by the within reefs variation, indicating large variability among individuals. Although the variation among reefs within sites is smaller, reefs differ significantly in relation to the variability present at their respective site. Finally, variation among sites is high and significant as well. Therefore, the high diversity within each population should not be interpreted to conclude that populations differ only slightly. Higher differences between sites than between reefs within sites are consistent with a significant IBD pattern. Distance clearly acts as a barrier to gene flow in *P. clavata*, which is typical for species with short larval dispersal (Santangelo et al. 2005). Moreover, isolation by distance is a phenomenon that occurs at local scales (Selkoe ad Toonen 2011) and therefore it is evident among the closely located populations studied here. At larger spatial scales (hundreds to thousands of km), *P. clavata* also displayed a significant IBD pattern, similarly to another Mediterranean coral, *C. rubrum* (Ledoux et al. 2014). However, at a fine spatial scale (cm to m), red gorgonian colonies did not show significant IBD, in contrast to *C. rubrum* (Ledoux et al. 2014).

Short distance migration (hundreds of meters) is likely to be the dominant scale of dispersal in *P. clavata*, but our results also suggest that migration from close undisturbed sites may be a significant source of recruits for disturbed areas to recover, since the maximum larval dispersal was between 20 to 60 km, with migrants detected in reefs separated by 20 km, but not by 60 km. This strengthens evidence from studies showing that coral population recovery after catastrophic mortality may be mainly supported by local migration from undamaged sites. A study of genetic

connectivity in coral populations recovering after catastrophic bleaching revealed that the majority of detected migrants originated from the only site that was not decimated by a recent mortality event. Most of these immigrants were received by the site which reached pre-bleaching diversity, highlighting their role in population recovery (Underwood et al. 2007). Our results demonstrate that larvae may disperse from the Cinque Terre Marine Protected Area (Punta Mesco) to the adjacent populations, supporting their recovery after the disturbance. Additionally, the larval transport from deep, healthy reefs to the shallow ones, impacted by past mass mortality events, may have great importance for recovery after climatically induced population collapses.

Our findings indicate higher larval dispersal potential than reported in (Coma et al. 1995a), which observed that larvae settle immediately on the substrate surrounding their mother colony. We found that transport of the larvae was not only common over distances of hundreds of meters, but also for tens of kilometers. The maximum migration distance detected in the present study reached 20 km, i.e. the distance that separates the two sites of La Spezia and Punta Mesco. Ten percent of the colonies from Punta Mesco were estimated as migrants from La Spezia. In this case, the larval dispersal was consistent with predominant currents, with the large scale Ligurian circulation being characterized by a cyclonic, east-to-west flow, active all year round and modulated by seasonality and wind forcing (Astraldi and Gasparini 1992). High contribution of migrants from Punta Mesco (8.5%) found in La Spezia may be caused by the predominantly southwesterly summer wind (Libeccio), which occasionally reverses coastal current direction (Haza et al. 2010). In contrast to the previous two sites, Livorno was genetically homogeneous. This site is separated from La Spezia by 60 km of sandy bottom, unsuitable for the red gorgonian. The nearest known population to the south is located around 25 km from Livorno, at the deep rocky shoal below 40-50 m. It seems that both distance and depth differences may isolate Livorno reefs from other populations.

Theoretically, the high number of migrants exchanged between Punta Mesco and La Spezia should lead to panmixia in a few generations (Slatkin 1985), but the genetic structure between these sites remains relatively high. These observations may be explained by an increased recruitment rate after the mortality. In stable populations the number of recruits is low, but after mass mortality the amount of available substrate increased and was occupied by new settlers, including migrants. When colonies recruited after the mass mortality events reached maturity and started reproducing, most of the available substrate was already occupied and only a minor proportion of their offspring could settle and survive, effectively reducing the opportunity for successful out-crossing. Additionally, *P. clavata* reaches maturity when colonies are from 3 years old (data from La Spezia population, Cupido et al. 2012) to 7 years old (data from Medes Islands, Spain, Coma et al. 1995b) and therefore only a few generations may have appeared after the mortality.

Our dispersal estimate (IBD regression slope of 0.037, R^2=0.78) was similar to the one reported previously for populations from the whole Mediterranean, when IBD slope was 0.033 (R^2=0.51; Mokhtar-Jamaï et al. 2011). However, when comparing our results with a study that investigated a

small spatial scale (IBD slope of 0.012, R^2=0.85; Arizmendi-Mejía et al. 2015), the isolation by distance appears stronger in our study, implying a lower dispersal. Variability in dispersal estimates may arise form larval responses to environmental conditions, modifying planktonic larval duration (PLD), or from differential establishment success after dispersal. Indeed, PLD of up to 25 days, estimated from laboratory experiments (Linares et al. 2007a), indicate that larvae can delay their metamorphosis when lacking the necessary settlement stimuli (Selkoe and Toonen 2011) and, therefore, may be capable of long distance dispersal. The demographic state of the local receiver populations may strongly condition the success of establishment of long distance migrants. In mature populations, the rare arrival of few migrants may pass unnoticed, lost among the high recruitment mortality bottleneck that is typical of most populations with overlapping generations. Therefore large-scale dispersal is rarely detected as effective gene flow. However, where mass mortality has occurred, the opportunity of expansion in freely available habitat magnifies the probabilities of success of long distance dispersers. Moreover, the study of Arizmendi-Mejía et al. (2015) examined not only populations that experienced mortality, but also one population that was recently founded. These processes, which have been named density-barrier effects or prior colonization effects, are most strikingly demonstrated for species which have been able to rapidly expand for thousands of km along novel available habitat (e.g., in invasive expansions or postglacial recolonizations) whereas in the ancient native ranges they remain highly structured even across a few tens of km (e.g. De Meester et al. 2002; Neiva et al. 2012). Therefore, inferring probability of connectivity from genetic data alone may be misleading, where the demographic conditions of the populations may prevail over dispersal capabilities in determining the possibilities of population recovery by migration in a metapopulation.

The larval exchange between deep and shallow reefs, such as that inferred in Punta Mesco and Livorno, has significant importance for the species' conservation. Colonies dwelling below 25-30 m were not affected by mass mortality events, in contrast to shallow subpopulations (Linares et al. 2005; Huete-Stauffer et al. 2011). Our research supports the hypothesis of Cerrano and Bavestrello (2008; 2009) that deeper subpopulations may supply larvae to shallower sites. However, depth differences in the present study were relatively small, aimed at representing nearby areas above and below the thermocline warming effects. Further studies on larval dispersal, including deeper subpopulations, are needed to develop a more complete picture of larval exchange between different depths. Deeper reefs may have an even greater larval contribution for the shallower and other deep reefs than what we estimated here, especially after disturbances. In the STRUCTURE analysis, some of the migrants exchanged between Punta Mesco and La Spezia were grouped into a separate cluster when k=4. They might have originated from a different population, possibly from a greater depth at Punta Mesco, where *P. clavata* occurs down to 60 m. The evidence for longer larval dispersal, between Punta Mesco and La Spezia, suggests that damaged reefs may benefit from external larval sources. Unexpectedly high numbers of recruits in the La Spezia population were observed shortly after the mass mortality event (Cupido et al. 2008).

In the subsequent years recruits density was five times higher than in the pre mortality period (Santangelo et al. 2015). Our findings indicate that not only increased reproductive output might be responsible for the recovery of the damaged population, but also larval migration.

2.5.3. Conclusions

Our study failed to detect any genetic diversity loss in the *P. clavata* populations affected by mass mortality events. This may be due to the lack of test power and other confounding factors, including recent expansion or Wahlund effects. Our research confirmed low larval dispersal capability in the red gorgonian, since the maximum dispersal distance inferred from our data was between 20 and 60 km. However, this reduced ability for dispersal during the larval phase may still be ecologically significant for population replenishment and persistence, enabling migration between local populations. Population recovery after mortality events may be dependent on the possibility of propagule immigration from external sources, such as Protected Areas. Additionally, migration between reefs located at different depths implies that deeper refugia may provide larvae for shallow subpopulation recovery after climatically-induced mortality events affecting mostly shallow sites.

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Chapter 3. High genetic differentiation of the red gorgonian populations from the Atlantic Ocean and the Mediterranean Sea

3.1. Abstract

Patterns of genetic variation within a species range may be used to study the evolutionary history of marine species. In the present study we used microsatellites and mitochondrial Cytochrome Oxydase I gene (COI) to compare genetic diversity of the red gorgonian *Paramuricea clavata* in the Atlantic Ocean and the Mediterranean Sea. Populations from two basins revealed distinct genetic composition and diversity. Higher heterozygosity, allelic richness and private allelic richness were found in the Mediterranean Sea with the use of microsatellites, possibly caused by the isolation of Atlantic populations or by a founder effect. Colonization of the Atlantic region from the Mediterranean may explain lower genetic diversity reported here for Atlantic reefs, as it is expected in peripheral populations. Additionally, a clear difference was obtained from the mtDNA COI gene, since sequences from Atlantic and Mediterranean samples diverged by 1%, which is a high value for soft corals.

3.2. Introduction

Genetic variability along species distribution range may be highly complex as a result of thousands of years of cumulative historical events. Historical processes, including population division, expansion and colonization, may be responsible for producing specific patterns in the allele distribution (Templeton *et al.* 1995). Therefore we can infer about historical events based on patterns of genetic variation. Spatial genetic structure may also result from past or present barriers to gene flow (e.g. Nei and Takahata 1993; Wakeley and Hey 1997). The Gibraltar Strait, separating the Mediterranean Sea from the Atlantic, is one of the well-known examples of a barrier to gene flow for a number of species (Patarnello *et al.* 2007). However, even closely related species may exhibit contrasting patterns of genetic structure in the Gibraltar Strait. The survey on the family Sparidae, for example, revealed three fish species with a sharp Atlantic–Mediterranean separation and other two without any population structure (Bargelloni *et al*. 2003). The study of edible Atlanto-Mediterranean sea urchin *Paracentrotus lividus* revealed large degree of gene flow between populations within the Mediterranean and Atlantic, but significant genetic differentiation between these two basins, due to restricted gene flow across the Strait of Gibraltar (Duran *et al*. 2004a). Although species population structure are expected to correlate with its dispersal potential, the meta-analysis of Patarnello *et al.* (2007) did not detect any relationship between divergence of the Mediterranean and Atlantic populations and life history, reproduction, ecological niche or other biological traits.

The present research examines the genetic structure of the red gorgonian *Paramuricea clavata* (Risso, 1826) from two regions located on both sides of the Gibraltar Strait. The species is widespread in the western Mediterranean Sea and in the Adriatic Sea (Carpine and Grasshof 1975) and less common in the Aegean and Marmara Seas (Öztürk *et al*. 2004; Topçu and Öztürk 2015). Data from Atlantic Ocean are limited, but *P. clavata* is present in the Portuguese coast (Boavida *et al.* 2015). Personal observations confirmed its occurrence in the Algarve, Arrábida and Berlengas, the last being probably the northern species range in the Atlantic. The species has been also reported by fishermen in the past along the Atlantic coast of Morocco (Harmelin and Marinopoulos 1994). The red gorgonian is a surface brooder with a short larval dispersal (Coma *et al.* 1995; Linares *et al*. 2007a). This mechanism does not favor dispersal, but possibly decreases larval mortality and wastage, contributing to replenishment of local populations (Linares *et al*. 2007b). Surveys based on microsatellite loci (Mokhtar-Jamaï *et al.* 2011; 2013; Arizmendi-Mejía *et al.* 2015; Pilczynska *et al.* 2016) on *P. clavata* sampled in the Mediterranean Sea, evidenced a high level of genetic differentiation even at short distances, confirming short larval dispersal potential. In contrast to highly variable microsatellites, mitochondrial DNA (mtDNA) markers, commonly used for phylogeography investigations, did not reveal variability in *P. clavata*. Mitochondrial Cytochrome Oxydase I gene (COI) sequences did not differ between geographically isolated colonies from Mediterranean Sea (Calderon *et al*. 2006), although only 3 colonies were investigated in this study, one from Marseilles (France) and two from Medes islands (Spain).

Temperature-driven mass mortality events in the Mediterranean populations (Cerrano *et al*. 2005; Huete-Stauffer *et al*. 2011) raise concerns about conservation of the species in a rapidly warming ocean, which call for a better understanding of the species dispersal and colonization history. The aim of the present study was to compare the genetic structure and diversity of *P. clavata* populations from the Mediterranean Sea and the Atlantic Ocean, using microsatellite markers and Cytochrome Oxydase I gene (COI). Genetic structure of the populations from the Atlantic Ocean and Mediterranean Sea were not compared to date. The COI gene sequence was examined for the first time in the Atlantic, allowing us to test the mtDNA diversity in both regions.

3.3. Material and methods

3.3.1. Sampling

P. clavata colonies were sampled by scuba divers from three sites in the Ligurian Sea (Mediterranean) and from two sites in the Atlantic Ocean. At each site, two different reefs were chosen (Figure 3.1.). In the remaining of the text, each reef will be referred to by its code from Figure 3.1. The two sites in the Atlantic were separated by approximately 280 km, whereas Mediterranean sites were separated by distances ranging from 20 to 60 km. Reefs within each site were separated by 200 to 300 m. A branch tip of around 3-4 cm was taken from up to 30 different individuals from each reef. An exception was Site 2 (Sagres, Atlantic Ocean), where 17 colonies

were sampled at Sag3 and only 2 individuals at Sag4, because of low gorgonian abundance. Branch tips from each colony were stored in individual tubes. Samples were placed on ice during transport and preserved in 96% ethanol after arrival to the laboratory.

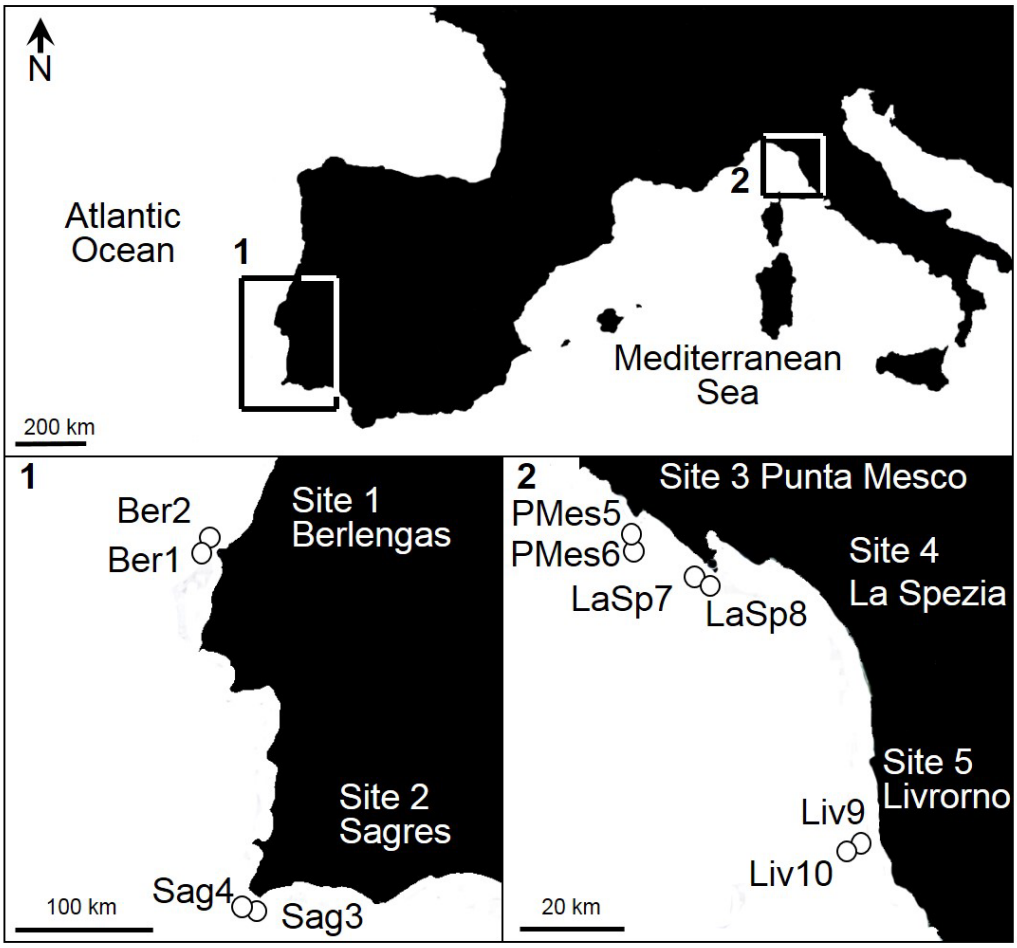

Fig 3.1. Sampling sites in the Atlantic and Mediterranean Sea. Reefs are indicated by white circle.

3.3.2. Microsatellite analysis

Coral DNA was extracted using E.Z.N.A. Mollusc DNA Kit according to the manufacturer's handbook. We analyzed 9 microsatellites, developed by Agell et al. (2009) and Molecular Ecology Resources Primer Development Consortium (2010) following the protocols published by the authors. Loci Par_a, Par_b, Par_d, Par_f and Par_m were amplified from total genomic DNA in 10 µl solution of dNTPs (0.25 mM each), selected primers (0.25 µM each), 4 mM of $MgCl_2$, 1x manufacturer-supplied buffer (pH 8.8, 0.1% Tween 20, 25 mM $MgCl_2$) and 0.25 u DFS-Taq DNA Polymerase (Bioron). The PCR program was: 2 min 94°C, (10 sec 94°C, 20 sec Annealing temperature (AT), 1 min 72°C) x 30 cycles, and 5 min 72°C. AT: Par_a: 59°C, Par_b: 47°C, Par_d: 51°C, Par_f, Par_m: 52°C. To amplify loci Parcla_9, Parcla_10, Parcla_12, Parcla_14 and

Parcla_17, total genomic DNA was dissolved in 10 µl solution of dNTPs (125 µM each), selected primers (0.5 µM each), 0.25 u GoTaq® DNA Polymerase (Promega) and 1x manufacturer-supplied PCR buffer (pH 8.5, 7.5 mM $MgCl_2$). The PCR program was: 3 min 94°C, (1 min 94°C, 1 min 60°C, 1 min 72°C) x 30 cycles, and 5 min 72°C. The length of amplified fragments was analyzed on an ABI 3730XL Genetic Analyzer using an internal size standard (GeneScan 500 LIZ). The analysis of DNA fragment length was performed with STRand (Toonen and Hughes 2001). Scored microsatellite fragment sizes were then visualized in R environment version 3.1.1 (R Foundation for Statistical Computing, 2014) using the MsatAllele_1.02 package (Alberto 2009) to track and reanalyze scoring errors.

MICRO-CHECKER v.2.2.3 (Van Oosterhout *et al*. 2004) was used to estimate the null allele frequency and to check for scoring errors owing to stutters and large allele dropout. The linkage disequilibrium among all pairs of loci was tested in GENEPOP 4.2. (Raymond and Rousset 1995; Rousset 2008) with significance levels determined by the Markov chain method (dememorization = 5000, batches = 500, iterations = 10 000).

Observed (H_o) and Nei's (1973) unbiased expected heterozygosity (H_e) were computed in GENETIX v.4.05 (Belkhir *et al*. 2004). The rarefaction procedure implemented in HP-RARE software (Kalinowski 2005) was used to estimate allelic richness (A_r) and private allelic richness (A_p). The minimum number of genes was set to 25 (minimum sample size). Sag3 was excluded from this analysis, because of numerous gaps in the data due to amplification failure, which led to overestimate the number of alleles and private alleles. The significance of differences in heterozygosity and allelic richness between Mediterranean and Atlantic population were evaluated using a Kruskal-Wallis test. Global and pairwise Weir and Cockerham's (1984) estimator of F_{ST} was evaluated in GENETIX. The genotypic differentiation between all pairs of populations was tested in GENEPOP software with default parameters.

Sag4 was excluded from most analysis because of the small number of colonies sampled. When multiple tests were conducted, the level of significance was adjusted using a false discovery rate (FDR) (Benjamini and Hochberg 1995).

3.3.3. Cytochrome Oxydase I

We analyzed Cytochrome Oxydase I gene (mtCOI) in the samples from two sites in the Atlantic Ocean (Berlengas and Sagres) and two sites in the Mediterranean Sea (Livorno and La Spezia). Five individuals were randomly chosen from each site. The previously extracted DNA was used for COI gene amplification with primers designed by Calderon et al. (2006):

COI Cni F 5′-GGY ACT YTA TAT TTA CTA TTT GG-3′

COI Cni R 5′-CCS GCA GGA TCA AAG AAW GTT G-3′

For the PCR reaction, the total genomic DNA was dissolved in 10 µl solution of dNTPs (125 µM each), primers (0.5 µM each), 0.25 u GoTaq® DNA Polymerase (Promega) and manufacturer-supplied PCR buffer. The PCR program was as follow: 3 min 94°C, (1 min 94°C, 1 min 60°C, 1 min 72°C) x 30 cycles, and 5 min 72°C. The PCR product was directly sequenced in both directions with the same primers.

Sequences were aligned by eye using MEGA version 6 (Tamura et al. 2013) and saved on FASTA format. To identify the origin of analyzed sequences they were checked in Bold Systems gene bank. The composition of nucleotides and genetic distance matrix were also computed in MEGA. Distances were calculated with Kimura 2-parameter model (for phylogenetic analyses; Kimura, 1980), which accounts for differences in transitions and transversion rates.

Sequences from Atlantic samples were (will be) deposited in GeneBank under accession number XXX.

3.4. Results

3.4.1. Microsatellites

No allele dropout and evidence of scoring errors were detected by MICRO-CHECKER at any locus. The mean null allele frequency across all populations varied from 0 for Parcla_10, Parcla_17 and Par_f to 0.23 for Par_m. Null allele frequency was not estimated for Sag3 because of insufficient amount of data due to amplification failure. No significant linkage disequilibrium was observed between any pair of loci (all $p>0.05$ after FDR correction), thus all loci were considered as genetically independent.

Eight of the loci were polymorphic at all sites, whereas one locus, Par_b, was monomorphic at Ber2, Sag3, LaSp5 and LaSp6 according to the 0.95 frequency criteria. Unbiased expected heterozygosity (H_e) varied between 0.39 at Sag3 to 0.66 at Liv10, with a mean value of 0.58. H_o ranged from 0.30 for Sag3 to 0.59 at Liv9, with a mean value of 0.48 (Table 3.1.). The highest allelic richness (A_r) was found at PMes8 (5.84) whereas the lowest value was found at Ber2 (3.88). Private allelic richness (A_p) varied from 0.96 at Pmes8 to 0.20 at Liv10 (Table 3.1.). Samples from the Atlantic revealed slightly lower genetic diversity when compared with those from the Mediterranean Sea. Expected heterozygosity and allelic richness were significantly higher in the Mediterranean (Kruskal-Wallis, $p=0.038$ and $p=0.045$, respectively), but the differences were not significant for H_0 and A_p.

Table 3.1. Measures of genetic diversity in populations of *Paramuricea clavata* at 9 microsatellite loci.

	He (SD)	Ho (SD)	Ar(25)	Ap(25)
Ber1	0.58 (0.24)	0.46 (0.26)	4.61	0.63
Ber2	0.55 (0.27)	0.48 (0.25)	3.88	0.27
Sag3	0.39 (0.24)	0.30 (0.34)	-	-
LaSp5	0.60 (0.25)	0.52 (0.27)	4.92	0.26
LaSp6	0.61 (0.25)	0.42 (0.27)	4.93	0.29
PMes7	0.57 (0.28)	0.46 (0.27)	4.77	0.27
PaMes8	0.61 (0.27)	0.48 (0.23)	5.84	0.96
Liv9	0.64 (0.18)	0.59 (0.23)	5.40	0.37
Liv10	0.66 (0.14)	0.57 (0.15)	5.18	0.20
mean	*0.58 (0.08)*	*0.48 (0.09)*	*4.94*	*0.41*

H_e – Nei's (1973) unbiased expected heterozygosity; H_o - observed heterozygosity; Ar and Ap - allelic and private allelic richness, respectively (with rarefaction size of 25 genes). SD = standard deviation

Overall F_{ST} value was 0.193, whereas pairwise comparison between all pairs of populations varied form -0.003 between Liv9 and Liv10 to 0.388 between Sag3 and PMes7 (Table 3.2.). Among 36 comparisons between populations, 35 were significant after FDR correction. One pair of populations belonging to the same site, Liv9 and Liv10, revealed no statistical differences in genotypic composition (Chi^2=18.5, df=18, p=0.42). Pairwise comparison between all Atlantic populations and all Mediterranean populations pooled together, was 0.147.

Table 3.2. Pairwise Weir and Cockerham's (1984) estimator of F_{ST} between all pairs of *P. clavata* reefs. All comparisons but one (Liv9 and Liv10) were significant.

Reef	Ber1	Ber2	Sag3	LaSp5	LaSp6	PMes7	PMes8	Liv9
Ber2	0.1152							
Sag3	0.3772	0.3752						
LaSp5	0.2391	0.2717	0.3987					
LaSp6	0.2270	0.2631	0.3772	0.0047				
PMes7	0.2073	0.2322	0.4030	0.0712	0.0643			
PMes8	0.1981	0.2350	0.4000	0.0741	0.0610	0.0043		
Liv9	0.2237	0.2449	0.3204	0.1737	0.1626	0.1794	0.1768	
Liv10	0.2332	0.2438	0.3148	0.1759	0.1652	0.1852	0.1826	-0.0035

3.4.2. Cytochrome Oxydase I

The amplified COI region was 581 bp long. All sequences obtained from the Atlantic samples were identical. Individuals from the Mediterranean Sea also shared one single genotype. Genetic differences between individuals from the Atlantic and the Mediterranean was 1%.

3.5. Discussion

Understanding the effects of barriers in the environment on genetic structure of natural populations allows analyzing essential biological processes, including speciation and species' distribution changes. Several studies have documented the role of the Gibraltar Strait in shaping diversity of algae, sessile and pelagic invertebrates and fishes, within an Atlantic–Mediterranean distribution, highlighting the reduction of gene flow between the two basins (e.g. Zane *et al.* 2000; Duran *et al.* 2004a; Lo Brutto *et al.* 2004; Roman and Palumbi 2004, Patarnello *et al*. 2007). Our results revealed the same pattern of genetic differentiation in the red gorgonian. The Gibraltar Strait may represent a major barrier to gene flow in *Paramuricea clavata*, causing the reduction of the genetic diversity in the Atlantic and the differentiation of the COI region between the two basins.

The present study found significant differences in the red gorgonian genetic diversity between the Mediterranean and Atlantic populations, with heterozygosity and allelic richness being slightly, but significantly, higher in the Mediterranean Sea. The investigated Atlantic populations are likely highly isolated from other *P. clavata* reefs in the Atlantic. Berlengas archipelago is located around 85 km north of the nearest known *P. clavata* reef, in the Arrábida coast, and there is no information in the literature about any other population to the north of the archipelago. It is not clear if the low temperatures in the northern species range are responsible for this distribution, or the lack of suitable habitat (hard bottom) to the south from Berlengas. Distance is a barrier to gene flow in the red gorgonian (Mokhtar-Jamaï *et al.* 2011; Pilczynska *et al*. 2016), likely due to the short larval phase duration. In the summer, when *P. clavata* reproduce, upwelling-favorable northerly winds prevail in the western coast of Portugal (Wooster *et al*. 1976), triggering offshore and southward transport of the water masses along the whole Iberian coast (Sánchez and Relvas 2003). This oceanographic condition may additionally hinder larval migration to the north, from Arrábida to Berlengas. The population in Sagres inhabits a narrow rocky passage with dim-light conditions, which may be a barrier to larvae migration and gene flow. The geographical distance between populations, and the number of connections between them, strongly influence the probability of successful migration and gene flow (García-Ramos and Kirkpatrick 1997; Kirkpatrick and Barton 1997). Isolated populations are more prone to genetic drift and bottlenecks because of low immigration rates, decreasing genetic variation (Karron 1987).

The lowest heterozygosity among all populations was found in Sagres. Additionally, the pairwise Weir and Cockerham's (1984) estimator of F_{ST} between Sagres and other reefs was the highest among all comparisons, reaching 0.4, while F_{ST} between Berlengas and Mediterranean reefs reached 0.27. This result may be simply explained by the isolation of the population in the cave. However, colonies from Sagres differed from all other investigated populations, being bright yellow instead of purple. Yellow colonies are reported to be rare in the Mediterranean, whereas purple colonies with yellow apical branches are more common (Carpine and Grasshoff 1975). Additionally, in the Sagres population, the highest percentage of PCR failure was observed, possibly indicating incompatibility of primers. Although the yellow Sagres colonies share the same COI genotype with the purple Berlengas *P. clavata* colonies, further studies are necessary to determine if the yellow, southern, type is undergoing speciation, or is a phenotype of the same species. It cannot be excluded that the low number of successfully amplified loci from the Sagres population may have biased our results. However, when taken in conjunction with the particular habitat of this population, the small population size, and the distinct phenotype, it supports the conclusion of a strong genetic differentiation and underlines its conservation interest.

Our results confirmed the low level of COI differentiation in *P. clavata* that has been reported previously. The sequences from the Mediterranean Sea obtained in the present study were identical to the ones obtained from Marseilles (France) and Medes islands (Spain) colonies, published by Calderon *et al*. (2006). The unity of COI sequences in colonies from distinct regions of the Mediterranean Sea, together with the divergence of 1% between the Atlantic and Mediterranean populations, is consistent with previous studies indicating low diversity of cnidarian mtDNA (Calderon *et al*. 2006; France and Hoover 2002). Doughty *et al*. (2014) studied the Extended Mitochondrial Barcode (COI+igr1+MutS) in *Paramuricea biscay*a populations from the Gulf of Mexico and their results indicated that sequences were 0.1–2.2% divergent from each other. However, the authors discuss the possibility that different haplotypes may belong to separate species. The results of France and Hoover (2002) revealed the low level of differentiation in COI sequences of soft corals from a number of seamounts from Atlantic and Pacific. The uncorrected pairwise distance between different species was lower than 10%, and no differences were detected between individuals belonging to the same species and among species belonging to the same genus.

The data obtained in the present study do not allow for strong conclusions about the colonization history of the species. The 1% difference in the COI region between Atlantic and Mediterranean is a high value for cnidarians, since even closely related species may not exhibit any differences in the investigated mtDNA region (France and Hoover 2002). Therefore we may expect, that Mediterranean and Atlantic populations differentiated in the distant past. Colonization of the Atlantic region from the Mediterranean may explain lower genetic diversity reported here for Atlantic reefs, as it is expected in peripheral populations as a result of colonization or extinctions followed by recolonization processes (Patarnello *et al*. 2007). It was previously reported that the Atlantic sites

revealed reduced allelic richness when compared with populations from the Mediterranean, for example in two species of barnacles, *Chthamalus montagui* and *C. stellatus* (Pannacciulli *et al.* 1997), the sponge *Crambe crambe* (Duran *et al*. 2004b) and the seagrass *Cymodocea nodosa* (Alberto *et al*. 2008). This difference between two basins may result from founder events during the past colonization of the Atlantic from the Mediterranean. Further research including populations sampled along the southern Spanish and French coasts would allow a better understanding of the Atlantic-Mediterranean colonization process for the red gorgonian.

This has identified an important genetic break within the red gorgonian distribution. Both markers used in the present study, mtDNA and microsatellites, revealed the same discontinuity between the Mediterranean and Atlantic. Further assessments of the red gorgonian genetic structure, preferably with the use of highly variable markers, such as microsatellites, and in the whole species distribution range, would add interesting information to the present knowledge about *P. clavata* phylogeography.

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Chapter 4. Genetic diversity increases with depth in red gorgonian populations form the Mediterranean and Atlantic

4.1. Abstract

Environmental stress gradients are known to affect species genetic diversity and adaptation. Along the first tens of meters of the ocean there are strong changes in the magnitude and variability of abiotic parameters that may expose shallow populations to greater environmental perturbations compared to deep ones. The present study examined the differences in genetic diversity in the red gorgonian coral (*Paramuricea clavata*) from populations inhabiting different depths in the Atlantic and Mediterranean. Expected and observed heterozygosity did not correlate with depth, but the number of alleles and allelic richness were slightly higher in deep populations. Private allelic richness showed strong correlation with depth, increasing in deep populations. Our results partially confirmed the hypothesis that genetic diversity increase with depth, being lower in the shallow, less stable populations due to past mortality events induced by warming and other interacting factors and higher in deeper, stable populations.

4.2. Introduction

Decreased biodiversity in stressful environments is a well-known fact and may not only affect species diversity (Frontier 1985), but also genetic diversity. Changes in genetic diversity between stressful and reference sites or along a stress gradient may have multiple grounds and species specific reactions may differ significantly.

One of the well-described examples are the trends in genetic structure of mountain plants along an altitude gradient. In the review of Ohsawa and Ide (2008) the authors described several types of genetic diversity changes with altitude, varying from decreasing diversity with altitude, as a result of genetic drift and bottlenecks occurring during range expansion, to increased diversity, caused by adaptation to more severe conditions at higher altitudes. A genetic diversity decrease with increasing elevation was also reported in the long-toed salamander (*Ambystoma macrodactulym*; Giordano et al. 2007). Salinity gradients may be another example of an environmental factor affecting genetic diversity. Laamanen et al. (2002) analyzed the variability in the 16S-23S rRNA internal transcribed spacer (ITS) sequences of the cyanobacterium *Aphanizomenon flos-aquae* populations along a salinity gradient in the Baltic Sea. They found that genetic diversity decreased with increasing salinity, since natural selection removes most of the lake genotypes from the Baltic Sea populations and therefore only one, better adapted genotype, can be found in the sea. Human activities may also influence genetic diversity of natural populations, for example by environmental pollution. Populations the brown bullhead (*Ameirus nebulosus*), a benthic fish, showed a consistent depression of haplotype diversity at polluted sites when compared with control sites, probably due to population bottlenecks (Murdoch and Hebert 1994).

In shallow marine coastal environments, even small depth changes may reflect significant environmental differences due to wind-induced turbulence, the presence of thermoclines and haloclines or light attenuation, which underpin the well-known gradients in species occurrence and community structure. However, to the best of our knowledge, the effects of the depth gradient in genetic diversity seem to have never been addressed. In the present study we tested the changes in genetic diversity with depth in the red gorgonian, *Paramuricea clavata* (Risso), within a small depth gradient. The species typically lives on shadowed rocky substrates in the Mediterranean roughly from 10 m to at least 110 m depth (Carpine and Grashoff 1975) and in the Atlantic (Boavida et al. 2015) down to 60 m (Boavida, personal observation). Environmental conditions at the shallow depths may be more stressful for *P. clavata* populations, because of more variable temperatures and warming events connected with water stratification. For instance, in the NW Mediterranean, past mortality events caused by increased water temperature, reaching 24°C (Romano et al. 2000), are well documented (e.g. Perez et al. 2000, Garrabou et al. 2009). In 2003, the temperature down to the thermocline was between 1 and 3 °C above the mean monthly temperature in the NW Mediterranean (Garrabou et al. 2009). Damage intensity decreased with depth and communities dwelling below the thermocline (25-30m) were significantly less affected than the shallow one (Linares et al. 2005; Cerrano et al. 2005). In the Atlantic, temperature-driven mortality events are presumed to be less frequent, because water is usually mixed due to summer upwellings (Relvas et al. 2007), but there are sporadic events of upwelling relaxation when temperature may persist for several days above 22°C (Relvas and Barton 2005) and one of such events may be sufficient to cause mortality in the shallow water populations. Indeed, the temperature changes in the Atlantic already led to the changes in community composition in subtidal kelp forest in NW Spain (Voerman et al. 2013) or a marine gastropod (*Patella rustica*) expansion to the north (Lima et al. 2006).

Genetic diversity influences not only the fitness of individuals, but also the viability of populations and the adaptive potential of species to environmental change (Reusch et al. 2005; Hughes et al. 2008). Consequently, the interplay between disturbance and genetic diversity may have significant ecological and evolutionary implications. In the present study we test the hypothesis that genetic diversity of *P. calvata* may change with depth, being lower in the shallow populations due to past mortality events, and higher in deep, more stable environments, and therefore shallow populations may be less resistant to future environmental changes.

4.3. Material and methods

Investigated populations were sampled by SCUBA diving in the Atlantic Ocean and Mediterranean Sea (Fig. 4.1.), from depths ranging from 12 to 60m. Genetic diversity indicators, including observed and expected heterozygosity, number of alleles, allelic richness and private allelic richness, were collected from the study of Boavida (2015) and Pilczynska et al. (2016). In order to

eliminate the differences in background genetic diversity between regions, anomalies of each indicator were calculated as the difference between the genetic diversity indicator at each site and the mean value for the region. Then, linear regressions of the anomalies in heterozygosity, allelic richness and private allelic richness on depth were separately tested.

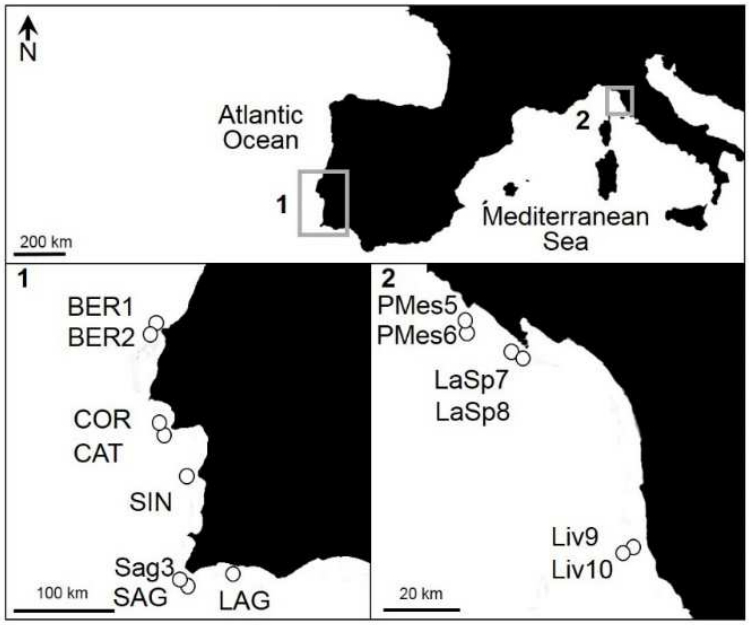


Fig. 4.1. Sites where *P. clavata* samples were taken.

In order to characterize the thermal environment and substantiate stability differences between regions and depth levels, information on ocean temperatures was compiled on a daily basis for a 20-year period (1993-2013). Surface data derived from the Operational Sea Surface Temperature and Sea Ice Analysis, a dataset that combines microwave and infrared satellite data from the Group for High-Resolution Sea Surface Temperature with in situ measurements, on a spatial resolution of ~5km (Stark et al. 2011). Temperatures at 20, 30 and 60 m depth were derived from the Hybrid Coordinate Ocean Model, a product forced by heat flux, wind speed, wind stress and precipitation on a spatial resolution of ~7km. This model is able to resolve complex oceanic processes like eddies, meandering currents, filaments and fronts (Chassignet et al. 2007) and accurately predict the temporal variation of temperatures, with low averaged bias ranging from 0.2°C to 0.3 °C depending on the regions (Kara et al. 2008, 2010). For each sampling site and depth level, the estimates of daily temperature were made by bilinear interpolation (e.g., Assis et al., 2015).

4.4. Results and discussion

Temperature variation decreases with depth and deep sites were more stable than the shallow ones (Fig. 4.2., Table 4.1.). During the 1993-2013 period temperature was typically higher in the Mediterranean Sea than in the Atlantic, frequently reaching over 25°C. However, in the Atlantic,

temperature during a summer may reach over 20°C in the upper layer for a number of days (Fig. 4.3.).

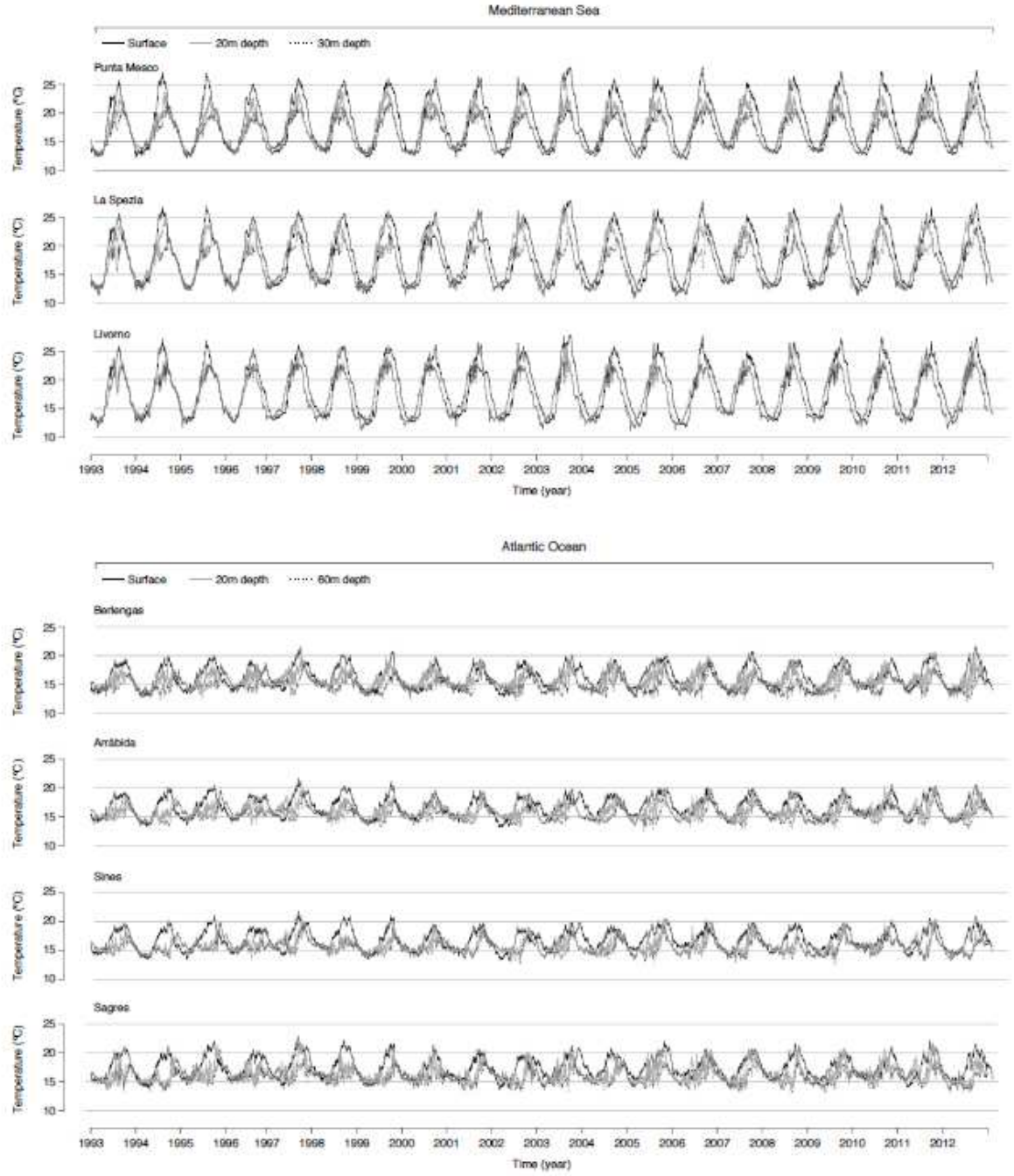


Fig. 4.2. The temperature time series at the investigated sites in the Atlantic and Mediterranean at different depths.

Table 4.1. Temperature range and variance for each site and depth level. 60m and 30m represents the maximum sampling depth in the Atlantic and Mediterranean, respectively.

	surface				20m				60m; 30 m			
Site	**SD**	**mean**	**max**	**min**	**SD**	**mean**	**max**	**min**	**SD**	**mean**	**max**	**min**
Berlengas	1.91	16.39	21.66	12.78	1.38	15.74	19.98	12.56	1.17	14.82	19.18	11.89
Arrabida	1.72	16.72	21.74	13.17	1.29	16.08	20.51	13.46	1.15	15.33	19.79	12.51
Sines	1.76	16.86	21.87	13.26	1.26	15.92	20.29	12.89	1.17	15.47	19.94	12.35
Sagres	1.91	17.46	22.89	13.55	1.50	16.47	21.76	13.05	1.17	15.64	20.59	12.91
Punta Mesco	4.38	18.41	28.03	11.83	3.13	17.23	24.85	12.28	2.45	16.50	22.51	12.27
La Spezia	4.42	18.40	27.89	11.51	3.83	17.70	26.50	10.93	2.84	16.72	23.44	10.90
Livorno	4.36	18.45	27.84	11.82	3.52	17.55	26.79	11.12	3.32	17.32	25.46	11.18

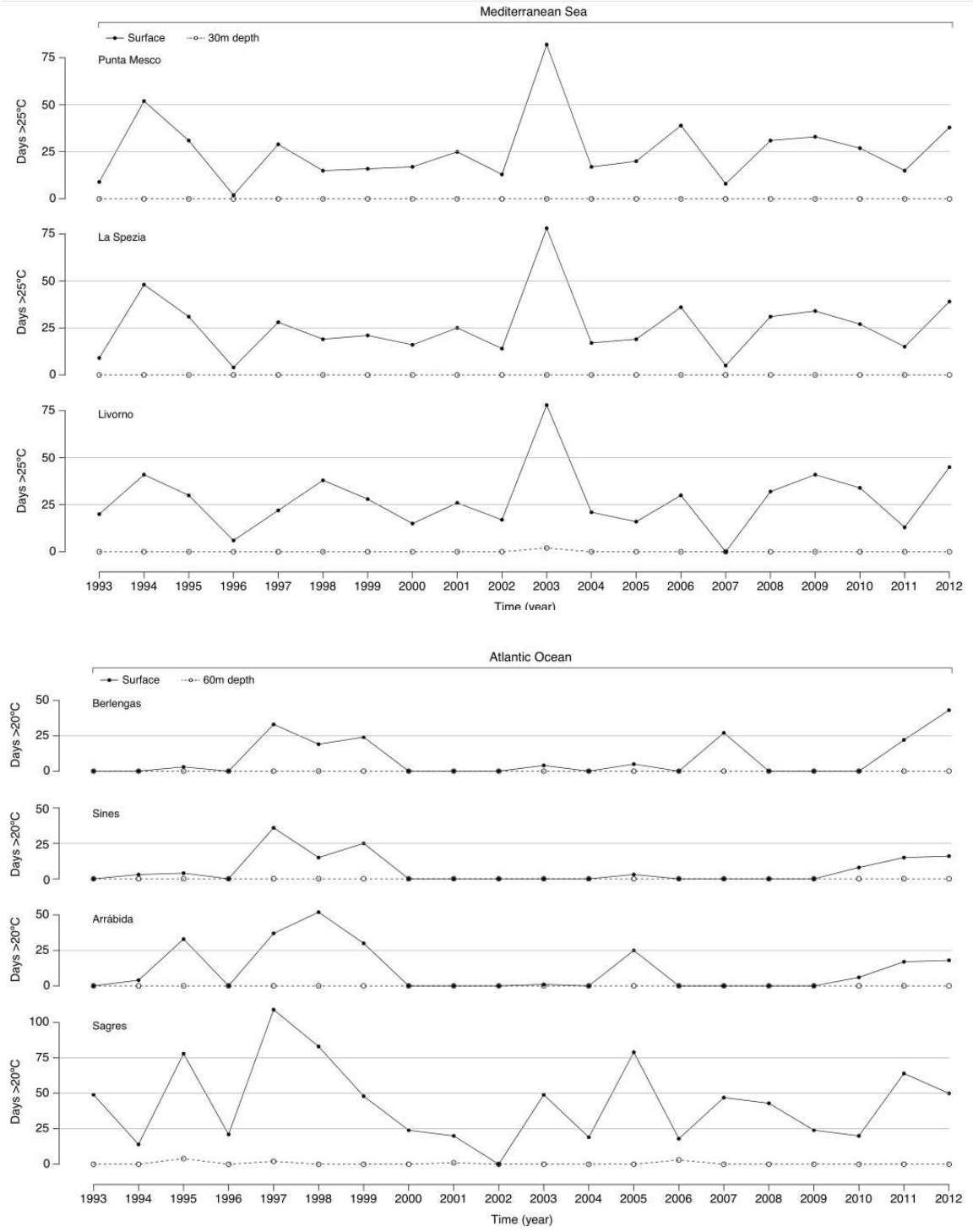


Fig. 4.3. The number of days when water temperature was higher than 25 and 20°C in the Mediterranean and Atlantic, respectively.

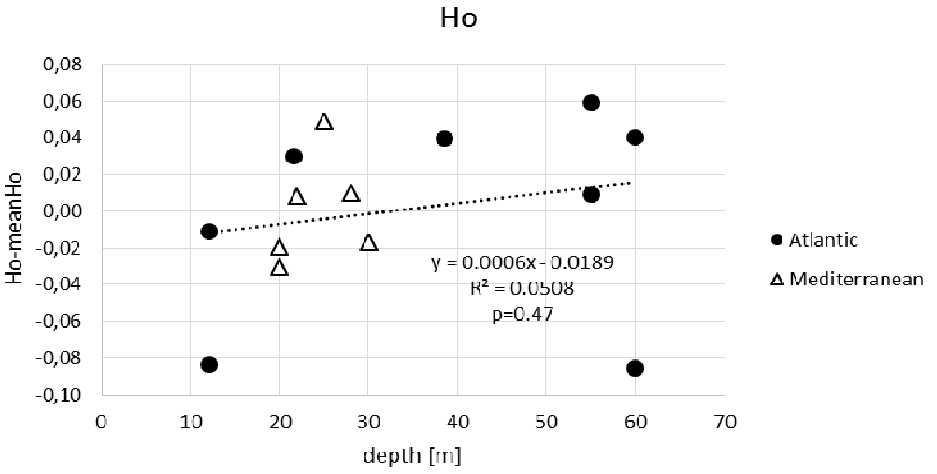
Genetic diversity measures at each site are represented in Table 4.2. Expected and observed heterozygosity, allelic richness and number of alleles across loci were not significantly related with depth. Private allelic richness increased significantly with depth (Fig. 4.4.).

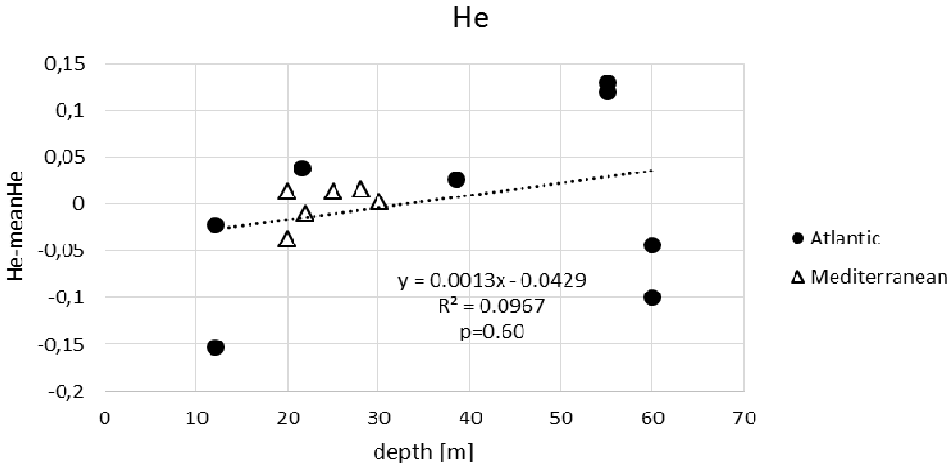
Table 4.2. Depth, the number of sampled individuals and genetic diversity measures at each sampling site.

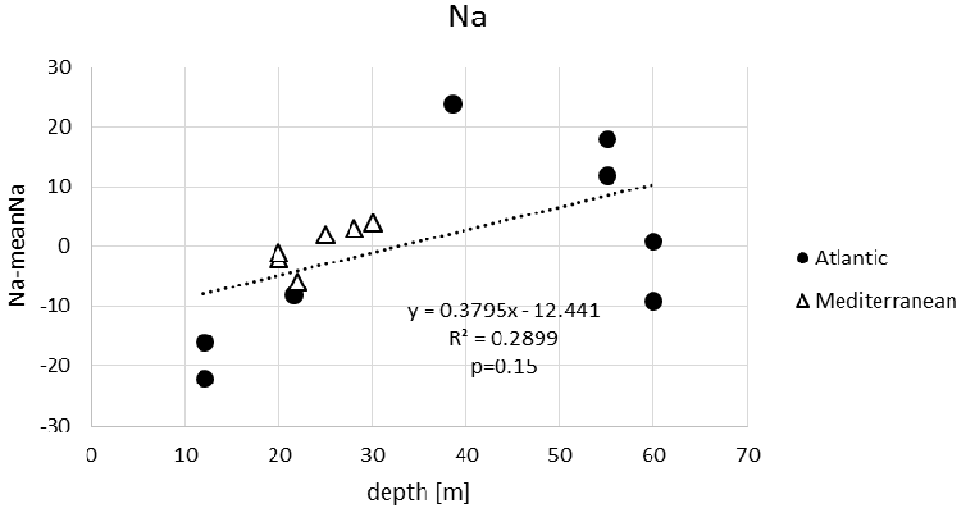
	Site	Depth (m)	n	Ho	He	Na	AvNa	Ar(20)	Ap(20)
Atlantic	COR	55	32	0.58	0.79	57	11.4	7.04	2.44
	CAT	38.5	72	0.61	0.68	63	12.6	6.05	0.90
	SIN	55	24	0.63	0.78	51	10.2	6.91	2.55
	Sag3	12	18	0.48	0.51	17	3.4		
	SAG	60	10	0.48	0.56	30	6.0	4.60	1.53
	LAG	60	34	0.61	0.61	40	8.0	4.71	1.25
	BER1	21.5	30	0.60	0.70	31	6.2	4.91	0.31
	BER2	12	28	0.55	0.64	23	4.6	4.16	0.21
Mediterranean	LaSp5	20	30	0.61	0.67	31	6.2	4.79	0.09
	LaSp6	20	30	0.60	0.72	32	6.4	5.23	0.15
	PMes7	22	30	0.63	0.69	27	5.4	4.55	0.00
	PMes8	28	30	0.64	0.72	36	7.2	5.60	0.37
	Liv9	25	30	0.68	0.72	35	7.0	5.66	0.42
	Liv10	30	30	0.61	0.71	37	7.4	5.36	0.20

n - number of individuals sampled, Ho – observed heterozygosity, He – Nei's (1973) unbiased expected heterozygosity, Na - number of alleles summed across all loci, AvNa - average Na across loci, Ar - allelic richness, rarefied for minimum sample size, Ap – private allelic richness, rarefied for minimum sample size

Deeper sites differed from the shallow ones in terms of private allelic richness, but not heterozygosity. At shallow sites environmental conditions, including temperature, are less stable and the mortality rate may be higher, removing rare and new alleles from the population. Allelic richness, rather than heterozygosity, may reflect more effectively a population's long-term evolutionary potential (Petit et al. 1998). Reduction in population size may theoretically reduce allelic richness at neutral loci, but not gene diversity, since rare/unique alleles are more exposed to genetic drift than frequent ones (Nei et al. 1975). Allelic richness was reported to be more sensitive indicator of differences in genetic diversity between the pre-bottleneck and post-bottleneck populations than multiple-locus heterozygosity (Leberg 1992; Spencer et al. 2000). Therefore the difference in private allelic richness found in the present study may reflect species history, despite the lack of differences in other genetic diversity indicators.


Ho
Ho-meanHo
depth [m]
y = 0.0006x - 0.0189
R² = 0.0508
p=0.47
Atlantic
Mediterranean


He
He-meanHe
depth [m]
y = 0.0013x - 0.0429
R² = 0.0967
p=0.60
Atlantic
Mediterranean


Na
Na-meanNa
depth [m]
y = 0.3795x - 12.441
R² = 0.2899
p=0.15
Atlantic
Mediterranean

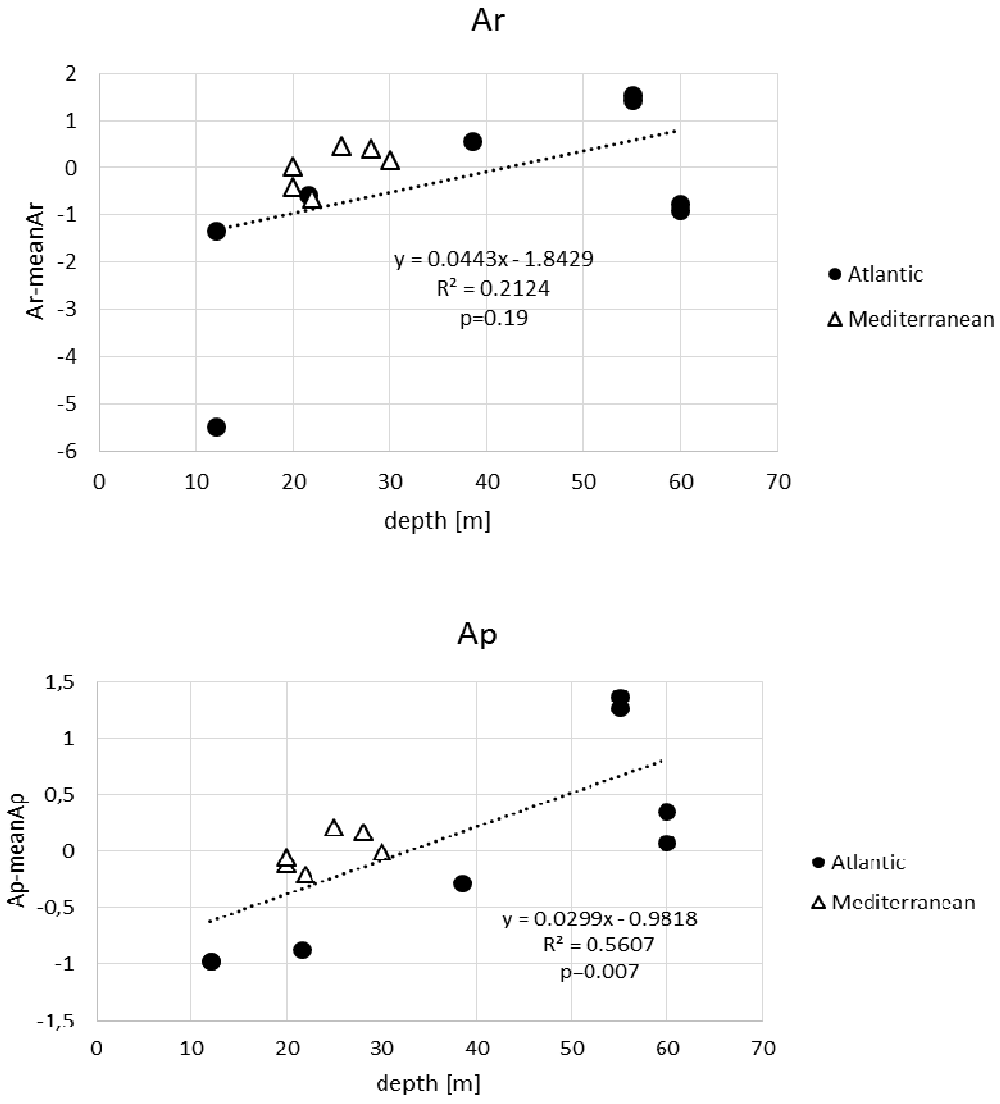


Fig. 4.4. Regression of genetic diversity indices with depth in the red gorgonian. In order to eliminate the differences in genetic diversity between regions, diversity indices were calculated as the difference between the index at each site and the respective regional mean (Mediterranean or Atlantic). Ho – observed heterozygosity, He – Nei's (1973) unbiased expected heterozygosity, Na - number of alleles summed across all loci, Ar - allelic richness, rarefied for minimum sample size, Ap – private allelic richness, rarefied for minimum sample size

Our results suggest that genetic diversity may be correlated with depth, being lower in the shallow populations and higher in deeper, more stable environments. One of the most important factors influencing the observed pattern may be the occurrence of bottlenecks caused by past mortalities in the shallow sites. The time series data clearly shows that thermal variability decreases with depth. Additionally, we can observe that in the upper water masses there were a number of days when temperature reached potentially lethal values, which we suggest to be 25 and 20°C in the

Mediterranean and Atlantic, respectively. In contrast deep populations are nearly never exposed to such high temperatures. This explanation is in line with findings from a wide diversity of species and environments (e.g. Bickham et al. 2000; Van Straalen and Timmermans 2002), which suggested bottlenecks as the cause of decreased genetic diversity in stressful environments.

However, genetic diversity changes along environmental gradients may result from a number of causes. We may not exclude genetic drift and bottlenecks occurring during range expansion of deep population to shallower sites, for example after sea level changes during the glacial maxima. Lower gene flow in the deeper sites may also decrease genetic diversity, especially allelic richness. Low dispersal ability of *P. clavata* (Linares et al. 2007) does not favor extensive connectivity and admixture between populations, nor expansion of rare alleles from deep sites. Additionally, shallow sites may be more connected to each other, exchanging larvae, and therefore the alleles are more homogenously distributed among them, with the consequence that unique alleles are less abundant.

Independently of the mechanism that generates higher diversity at deeper sites, our findings may have significant meaning in terms of *P. clavata* conservation. Populations with higher genetic diversity are less likely to suffer losses of adaptive genetic diversity associated with population bottlenecks. For example, experiments on *Zostera marina* showed, that plots with higher genotypic diversity had greater survival rate and recovery after the disturbance (Reusch et al. 2005; Hughes and Stachowicz 2004). Therefore, our results suggest that deep population have higher adaptive potential, while shallow ones may be less resistant to environmental changes.

4.5. References

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General conclusions

The impact of past mass mortality events on the coralligenous community

Historical mass mortality events have severely impacted engineering, sessile invertebrates from the Mediterranean Sea, in particular effecting density (Garrabou et al. 2009), size classes' frequency distribution (Cerrano and Bavestrello 2008; Cupido et al. 2009) and reproductive output (Linares et al. 2008a). When reproductive output is decreased, asexual reproduction may become more important for damaged populations recovery. However, we found no evidence for increased contribution of clonal propagation in the popuations affected by mass mortality evets (**Chapter 1**). Severe decline in population abundance may also decrease genetic diversity and its recovery after the disturbance requires extensive levels of connectivity or a long period of time (Arnaud-Haond et al. 2009). Therefore lower genetic diversity may be expected in populations of benthic invertebrates affected by mass mortality in the past. Contrary to expectations, this study did not find any evidence for recent bottlenecks nor significant difference in genetic diversity between impacted and healthy *P. clavata* populations (**Chapter 2**). Long generation time characteristic of the red gorgonian (Linares et al. 2007b) may act as a buffering mechanism, contributing to a reduction in the rate of genetic diversity erosion. However, genetic diversity, particulary allelic richness, increase with depth, being lower in the shallow, less stable populations and higher in deeper, stable populations (**Chapter 4**). Past mortality events induced by warming and other interacting factors may be responsible for this result.

Recovery of populations after mass mortality events

Despite the severe reduction in population density and prolonged mass mortality impact, the red gorgonian populations are recovering (Cupido et al. 2009). There are a number of mechanisms that may be responsible for observed populations' recovery, including increased reproductive output and recruitment, caused by decreased competition because of a larger area of available substratum for juveniles. Clonal propagation, although more frequent than indicated by previous assessments, may not be the dominant factor accounting for population recovery in sites that have been affected by past climatic events (**Chapter 1**).

Migrations are probably an important factor enabling population recovery after mass mortality events. Although larval phase duration is short in *P. clavata* (Coma et al. 1995), this reduced ability for larval dispersal may still be ecologically significant for population replenishment and persistence, enabling migration between local populations (**Chapter 2**). Propagules may immigrate to damaged sites from external sources, such as Protected Areas or deep reefs, which remained undisturbed during the climatically induced mortalities (Linares et al. 2005; Huete-Stauffer et al. 2011). Additionally, the lack of evidence for decreased genetic diversity in the affected populations

despite severe demographic perturbations (**Chapter 2**), may have a positive impact on population recovery, since it is generally agreed that decreased genetic diversity may reduce fitness, especially in small populations (Ellstrand and Elam 1993). However, the inability to detect bottleneck effect in the present study may be caused by the lack of test power and other confounding factors, including recent expansion or Wahlund effects. Genetic diversity decreasing with depth (**Chapter 4**) may indicate that deep population have higher adaptive potential, while shallow ones may be less resistant to environmental changes.

Phylogeography of *P. clavata*

The use of genetic markers to study the populations within a species range can provide a data about genetic diversity and connectivity, but may also be used to study evolutionary history of a species. The current study indicated, that genetic diversity of the red gorgonian differ significantly between populations from the Atlantic Ocean and Mediterranean Sea, being higher in the Mediterranean (**Chapter 3**). The differences may result from isolation of Atlantic populations and/or historical processes of colonization of new areas. In the latter case the lower genetic diversity of Atlantic populations may indicate that *P. clavata* evolved in the Mediterranean and spread into the Atlantic after opening the Gibraltar Strait. However, further research is needed to investigate the phylogeography of the species. In this study we confirmed the low diversity of mtDNA in cnidarians and its low utility for phylogeography research (**Chapter 3**).

Conservation of coralligenous assemblages

Diverse and fragile coralligenous communities are subjected to many threats linked to human activities. Some of the rare species inhabiting coralligenous assemblages are legally protected, e.g. *Savalia savaglia* and *Spongia officinalis*, but the community as a whole still remain without formal protection (Gatti et al. 2012).

The establishment of marine protected areas (MPAs) is considered as a critical step towards conservation and management of marine ecosystems (Allison et al. 1998). At present, 94 MPAs have been established in the Mediterranean Sea, most of them in the north- western part, covering 3.8% of the total surface (Abdulla et al. 2008). In the Atlantic, gorgonians forests are present in several protected areas, e.g. Reserva Natural da Berlenga (Almeida 1996) and Marine Park Professor Luiz Saldanha (Rodrigues 2008). Mediterranean MPAs create rather a network of small protected areas than a group of several large ones (Francour et al. 2001). Although it is a result of independent local decisions instead of international planning, this design is assumed to be favorable for maintain biodiversity (Allison et al. 1998), providing a series of sheltered places for commercially fished species and larval sources for other MPAs and unprotected areas (Francour et al. 2001). Protected areas in Mediterranean are widely scattered and 66% of them is separated by

more than 30 km (Francour et al. 2001). This spacing may be too wide to ensure the effectiveness of the network for species with short larval dispersal, particularly for *P. clavata*, considering low larval dispersal capability in the red gorgonian, since the maximum dispersal distance inferred from our data was 20-60 km (**Chapter 2**). Dispersal from protected area however, may contribute to a stronger resilience of adjacent non-protected populations (Palumbi, 2003). A clear understanding of mechanisms shaping distribution of the larvae and connectivity between populations is essential for an effective management plan and community conservation.

Transplantation of living colonies form healthy populations to damaged ones, may be an additional tool for conservation strategy, since the red gorgonian may reproduce clonally in the wild (**Chapter 1**). A successful transplantation of *P. clavata* was performed in Mediterranean Sea by Linares et al. (2008b) in the Medes Islands, Spain. The mortality rate among artificially attached colonies was similar to the one naturally occurring in the environment. Such a restored sites become a source of recruits for the adjacent areas. The red gorgonian populations are highly divergent genetically and the differences increase with distance (**Chapter 2 and 3**) and therefore they may be locally adapted. It might be important to use a population located near the site that will be restored as a source population to provide the most adapted genotypes for the experiment.

Protection of marine habitats by establishing protected areas or refilling damaged populations by transplants does not prevent mass mortalities related to climate changes. However, communities protected in MPAs have greater possibilities for recovery after the disturbances because of reduced and controlled anthropogenic impact. Similarly, transplantation cannot counteract the massive mortality caused by climate changes (Cerrano et al. 2000), but it may be used in small MPAs highly impacted e.g. by diving activity for rehabilitation of local exploited populations, especially when natural recovery is to slow (Epstein et al. 2001; 2005). The detailed assessment of connectivity patterns among coralligenous assemblages, connected with extended knowledge of species biology, is essential for effective protection plans.

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List of Tables

List of figures