ALMA MATER STUDIORUM
UNIVERSITA' DI BOLOGNA

FACOLTA' DI SCIENZE MATEMATICHE , FISICHE E NATURALI

Corso di laurea magistrale in *Biologia Marina*

MESOPHOTIC RED CORAL POPULATIONS: GENETIC VARIABILITY AND CONNECTIVITY

Tesi di laurea in: "Habitat marini: rischi e tutela"

Relatore:

Prof. Marco Abbiati

Presentata da:

Rosa Angela Pugliese

Correlatore:

Dott.ssa Federica Costantini

II sessione

Anno Accademico 2011/2012

INDEX

ABSTRACT

The Mediterranean red coral (*Corallium rubrum*, L. 1758) is a long living octocoral that has been commercially harvested since ancient time for its red axial calcitic skeleton. At present, shallow-water red coral populations are overexploited and consequently in decline, so deeper populations below 50m (mesophotic populations) have currently become the most commercially harvested. Unfortunately, very little is known about the biology and ecology of these mesophotic populations and for their management and conservation a better understanding of their genetic structuring and connectivity is needed. The present study was carried out to define genetic variability and structuring in *Corallium rubrum* populations located between 60 and 120 metres depth, collected in three different areas of the Tyrrhenian Sea (Liguria, Toscana and Campania). A total of fourteen populations were sampled and analyzed by means of a set of 10 nuclear microsatellite loci and the putative control region of the mitochondrial DNA (MtC). Microsatellite genotyping indicated that all loci were polymorphic and showed significant deviations from Hardy–Weinberg equilibrium, due to an heterozygote deficiency, probably related to the presence of null alleles and/or inbreeding, as was previously observed in shallow-water populations. Both types of molecular markers showed high genetic similarity between Liguria and Toscana populations (Northern Tyrrhenian) compared to Campania populations (Southern Tyrrhenian). The genetic differentiation observed between North and South Tyrrhenian populations follows a weak but significant pattern of isolation by distance. However, low correlation between genetic divergence and geographic distances detected using the mitochondrial marker suggests that observed patterns could also be partially related to the presence of a long term barrier to gene flow, given the hydrodynamic and geological characteristic of the studied area. At smaller spatial scales (within areas) the two molecular markers indicate different structures, probably due to the low polymorphism of MtC or to the occurrence of some historical links within regions. These results show that mesophotic red coral populations are mainly self recruiting, similarly to the shallow water ones, with a limited effective dispersal ability of the larvae, further enhanced by the patchy distribution of suitable habitats. According to our results, management strategies of red coral harvesting in the mesophotic habitats should be defined at a regional level, being highly advisable the creation of deep-sea marine protected areas, in order to maximise the genetic diversity of this highly vulnerable populations of red coral in this unique habitats.

1.INTRODUCTION

1.1 *Population genetics and molecular ecology approaches*

Molecular ecology is the discipline that applies population genetics, phylogenetic, and more recently genomics to traditional ecological questions, e.g., species diagnosis, conservation and assessment of biodiversity, or species-area relationships (Moritz 2002).

Population genetics is a field of biology that studies genetic composition, populations structure, and the changes in genetic variability that result from the interaction of various evolutionary processes. Genetic variability within and between populations, can be assessed by studying the processes influencing the allelic and genotypic frequencies of one or more gene loci. These processes include mutation (the original multi-level source of genetic variation in a population), genetic drift (casual changes in allele frequencies caused by small population size), gene flow (movement between groups that results in genetic exchange), natural selection, and inbreeding (Hedrick 2009). A population or a species that, for any reason, loses part of its gene pool runs into a higher risk of extinction, losing part of its potential adaptability to new environmental conditions (Saccheri et al. 1998; Hughes et al. 2008). In populations of small size, such as in overexploited species, increase of genetic drift and inbreeding leads to an increase of homozygosity which in turn leads to reduction in fitness, defined as an "inbreeding depression" (Newman et al. 1997). In this context dispersive capacity and connectivity between populations, are the key elements to ensure population resilience after a disturbance (Palumbi 2004). Connectivity, in sessile marine species such as corals, is closely associated with dispersal larval capacity, besides the presence of physico-chemical barriers (hydrodynamics, geomorphology, thermal gradients, and lack of suitable habitats for colonization). In coral populations, given the difficulties associated with direct observations of the larval dispersal process, the difficulties in accessibility to populations, an important part of the estimation of their effective larval dispersal capacity may come from molecular data (Moritz & Lavery 1996).

Genetic diversity and connectivity can be measured using various DNA-based methods and other techniques. Nowadays three important components have come together: efficient techniques to examine informative segments of DNA, statistics to analyse DNA data and the availability of easy-to-use computer packages. Genetic

markers such as allozymes, microsatellites and mitochondrial and nuclear DNA sequences can be used to estimate many parameters of interest to ecologists, such as migration rates, population size, bottlenecks, kinship and more (Avise 2004). Based on evolutionary rate of the markers, different genes are chosen to perform different investigations at different taxonomic levels: genes with a low rate of mutation are suitable for interspecific studies (e.g. phylogeny at high taxonomic levels), while genes with high mutation rate are used for intraspecific studies (e.g. population genetics studies). In the phylum Cnidaria mitochondrial DNA, due its low evolutionary rate, is not suitable for the study of population genetics (Costantini et al. 2003, McFadden et al. 2006, Shearer et al. 2002, Hellberg 2006): Nuclear microsatellite loci, tandem repeats of 2-10 base pairs, revealed a high genetic variability. High levels of polymorphism support microsatellites being one of the most useful markers for intraspecific studieson genetic variation. Microsatellites have the potential to provide estimates of migration, to distinguish high rates of migration from panmixia, and to allow estimates of the relatedness among individuals. Several recent reviews detail the variety of molecular techniques now available, and the ecological questions that they can address (Bossart & Prowell 1998; Davies et al. 1999; Luikart & England 1999; Sunnucks 2000; Manel et al. 2003, 2005; Beaumont & Rannala 2004).

Molecular markers allow identifying populations of organisms at risk, or threatened of extinction. These populations are often described as stocks or ESU (Evolutionarily Significant Units). The identification of these units is difficult but important to determine which populations should be subject to legislative interventions and action plans for their conservation, management and sustainable use.

1.2 *Mesophotic coral ecosystem- MCE*

In the past decades, human induced disturbance (e.g. habitat loss and fragmentation, global climate change, overexploitation and other effects due to fishing, pollution and tourism) has increased in marine shallow habitats (Ballesteros 2006; Airoldi & Beck 2007).

With the decline of shallow coastal water resources, due to increasing demands and development of new technologies; human activities, most notably fishing, have expanded offshore and into deeper waters (Morato et al. 2006) Moreover, in the last years, researchers have shown that the deep-sea is more sensitive to human and natural impacts than previously thought (Davies et al. 2007) and that the same threats faced by shallow water species can act directly or indirectly on deep species affecting their distribution, population dynamics, growth and genetic structure (Smith et al. 2010; Bongaerts et al 2010). Therefore, conservation and sustainable management of marine resources in deep-sea ecosystems has become a binding priority for marine scientists.

Mesophotic coral ecosystems (MCEs; Figure 1.1) are characterized by the presence of light dependent corals and associated communities typically found at water depths where light penetration is low (from 30-40 m and extending to around 150 m in tropical and subtropical regions) (Lesser et al. 2009; Hinderstein et al. 2010).The term mesophotic literally means “meso”, for middle, and “photic” for light. The dominant communities providing structural habitat in the mesophotic zone can be made up of corals, sponges, and algal species (Hinderstein et al. 2010). These habitats may be colonized by a number of species that are only found within this depth range and geographical location; moreover, both shallow-water and depth restricted species can be found (Brokovic et al. 2007). Intrinsically valuable as biodiversity hot-spots, they are important from a conservation perspective and provide home for a vast range of marine species (Hinderstein et al. 2010; Khang et al 2010; Rooney et al. 2010) (Fig. 1.2). Moreover, mesophotic coral ecosystem could provide a source of propaguls (larvae and recruits) to the shallow water habitats, favoring their recovery and resilience against human impact (Slattery et al. 2011). In fact, it has been hypothesized that MCEs may serve as potential source to replenish degraded shallow-water coral reef species, and are currently under study to evaluate their potential as refugia and/or larval supplier for shallow-water assemblages (Bongaerts et al. 2010; Miller et al. 2011; Van Oppen et al. 2011; Slattery et al. 2011).

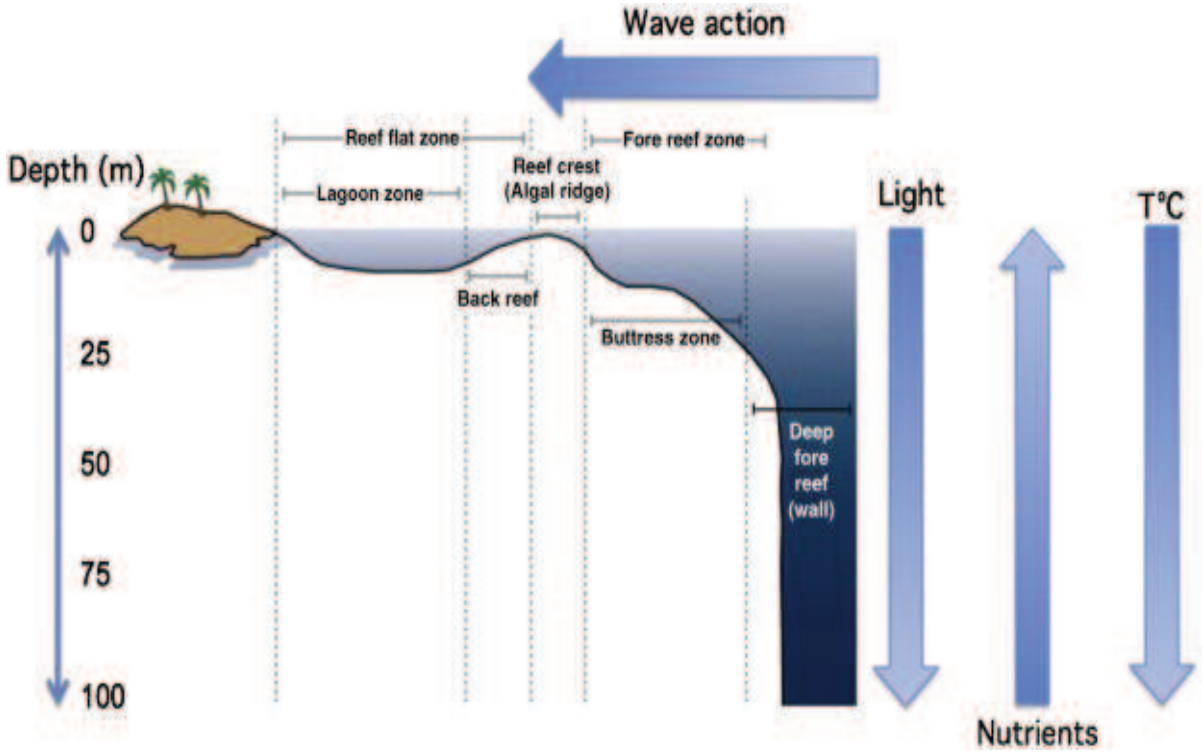

Figure 1.1 Coral reef zonation showing gradients of light, nutrients and temperature with changes in depth from shallow reefs to mesophotic depths (From Lesser 2009).

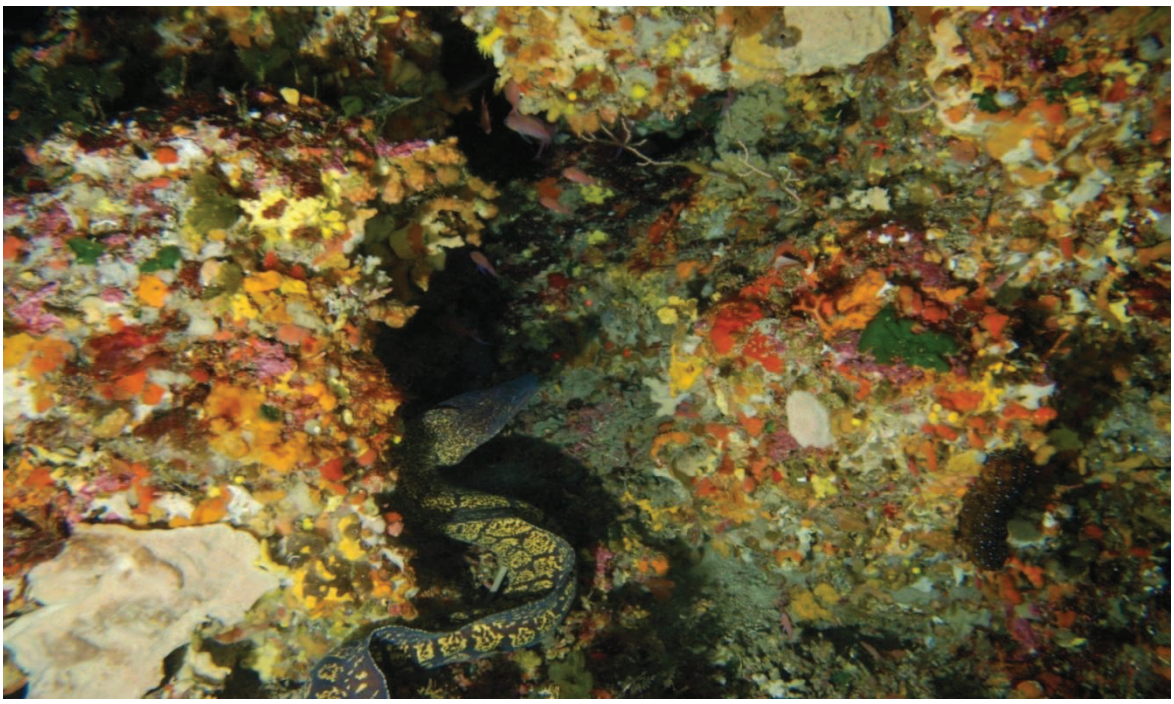

Figure 1.2 Coralligenous habitats dwelling in the mesophotic zone in the Mediterranean Sea (Picture made by Simone Canese, in Capel Rosso site at 60m depth).

Connectivity between shallow-water and deep-water populations is a fundamental prerequisite to consider MCEs as refugia because deep populations can act

as source of propagules for threatened shallow-water populations (Hinderstein et al. 2010). Furthermore, studies addressing these hypotheses provided conflicting results, depending on the analyzed coral species and on the geographic areas considered. Some studies show isolation between shallow and deep-water populations (Costantini et al. 2010; Costantini et al. 2011; Miller et al. 2011), whereas other studies shown the occurrence of gene flow (larval flow) between mesophotic and shallow-water ecosystem (Armstrong et al. 2006).

1.3 *Mediterranean mesophotic habitats*

The occurrence of mesophotic habitat in the Mediterranean Sea was reported since the first deep sea exploration (Marsili 1725). However, the exploration of these habitats and quantitative and experimental studies on their structure (Balata et al. 2005; Lineares et al. 2005) and dynamics (Airoldi 1998; Virgilio et al. 2006) were developed only when SCUBA diving techniques became available for scientific diving.

Remotely operated vehicles (ROVs) (Fig.1.3) allow extensive surveys in a depth range that is inaccessible to scuba diving, increasing the working depth range to more than 100m (Pyle 2000). The use of ROVs on small research vessels, allowed the exploration of MCEs in the coastal zone of the Mediterranean Sea; increasing in the last years the number of studies that have focused on MCE. Some of these studies describe the megabenthic biodiversity of the mesophotic zone in some areas of Mediterranean Sea (Bo et al. 2009; Bo et al. 2011 a, b, c), other investigate their structure, dynamics and genetic characterization (Freiwald et al. 2009; Gori et al. 2011; Cerrano et al. 2010; Costantini et al. 2010; Costantini et al. 2011).

Figure 1.3 The ROVs used in oceanographic cruises (May and June 2012) by the R/V *Astrea*. (Picture made by Simone Canese).

2. *Corallium rubrum*

2.1 *Ecology*

The red coral *Corallium rubrum* (Linneus 1758), belongs to the phylum Cnidaria (Anthozoa, Octocorallia, Scleraxonia), is a long-lived, slow growing gorgonian species. It represents one of the key ecosystem engineering species of coralligenous assemblage (a calcareous formation of biogenic origin), which is one of the richest Mediterranean habitat (Ballesteros 2006; Airoldi & Beck 2007). The origin of the actual distribution of red coral populations may date back to about 5 million years ago, when a coralligenous ecosystem replaced the coral reefs (Mateu 1986). Coralligenous is formed mainly due to the accumulation of encrusting calcareous algae in poorly lit areas and in relatively calm waters (Balata et al. 2005). The complexity of the structure of coralligenous allows the development of extremely heterogeneous communities, exhibiting high species richness and functional diversity (Gili & Coma 1998), dominated by "suspension feeders" (sponges, hydroids, anthozoans, serpulids, molluscs and tunicates) (Fig.2.1).

Corallium rubrum is a suspension feeder with passive filtration (Tsounis et al. 2005). The suspension feeders community evolved towards a complex three-dimensional structure, creating a barrier between the substrate and the water column so it interacts with the water column in littoral ecosystems by depleting food particles and transferring energy from the water column to the benthos (e.g. Cloern 1982; Officer et al. 1982; Fre'chette et al. 1985,1989; Kimmerer et al. 1994; Riisga˚rd et al. 1998). In addition, these organisms create the habitat for vagile fauna of the coralligenous and increase the biomass and biodiversity of communities, structuring and stabilizing the ecosystem with their three-dimensional structure (Gili & Coma 1998; Arntz 1999).

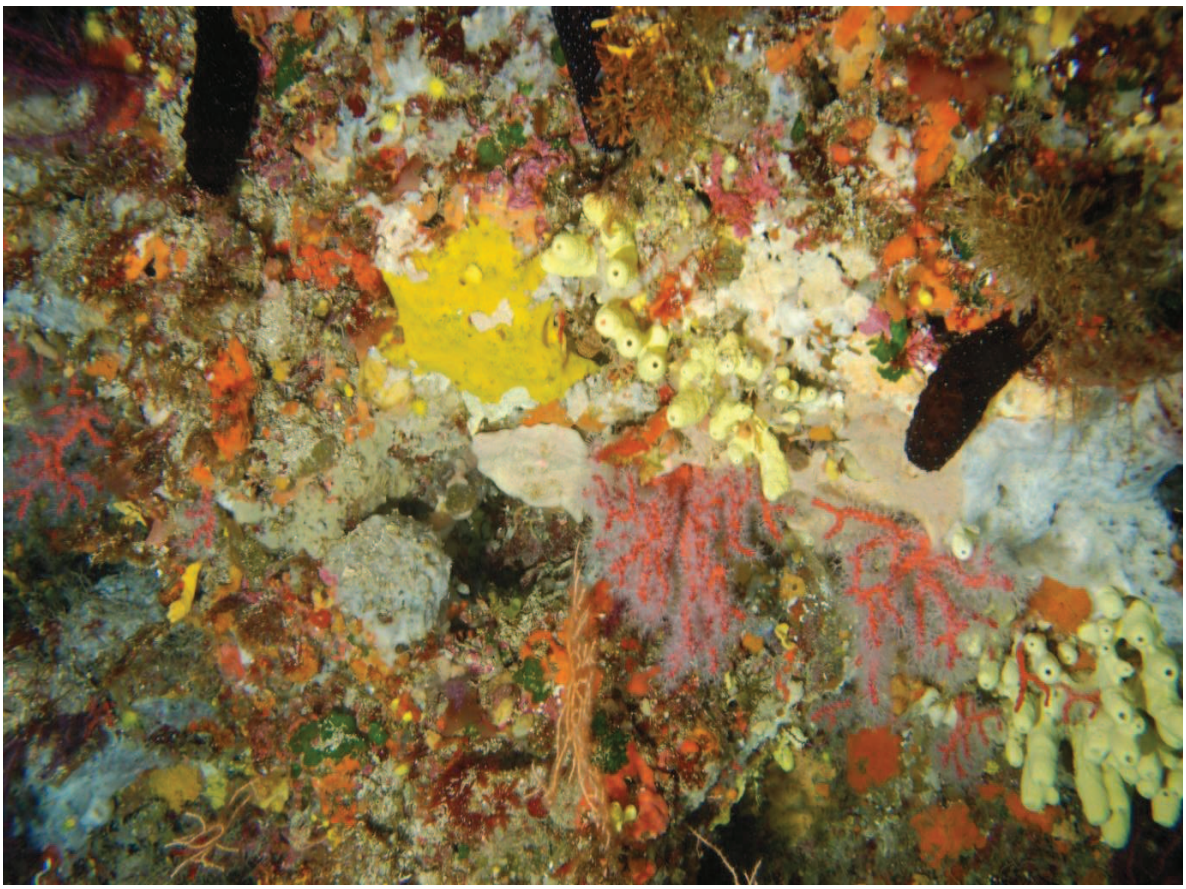

Fig.2.1 Coralligenous assemblages with some colonies of red coral. (Picture made by Simone Canese, in Capel Rosso site at 60m depth).

Corallium rubrum is a endemic Mediterranean species, mainly localized in Western Mediterranean Sea, in some parts of the Eastern Mediterranean Sea (Greek coast of the North Aegean Sea) and in the Atlantic coasts of south Portugal, Canary Islands, Mauritania, Senegal and Cape Verde Islands (Fig.2.2).

Figure 2.2: Geographical distribution of *Corallium rubrum.* The red circles represent the known populations (Marchetti 1965).

The distribution of the species shows major gaps related to the presence of stretches of sandy shores (e.g. Gulf of Valencia, Gulf of Lion, Versilia plain, Gulf of Gaeta) (Chintiroglou et al 1989; Marchetti 1965; Zibrowius et al. 1984). Moreover, red coral has strong habitat requirements , defined by the morphology and biogenic nature of the substratum, as well as by a series of abiotic variables (e.g. light intensity, water temperature and turbidity, sediment loads, current regime) (Weinberg 1979; Laborel & Vacelet 1961; Stiller & Rivoire 1984). It has a wide bathymetric distribution: from a few meters to about 800 m (Costantini et al. 2010). In shallower waters it is frequent in caves and poorly lit overhangs. Deeper down, it is found on underwater cliffs and even on the bottom (Laborel et Vacelet 1961; Zibrowius et al. 1984).

At the moment, three typologies of red coral populations have been described:

1. Shallow-water populations, in a depth range between 15 and 60 m, dwelling in vertical cliffs and inside caves; these populations have been commercially exploited for centuries and, at present, are made by small, short-lived colonies (Santangelo & Abbiati 2001);
2. Intermediate-water or mesophotic populations, at a depth range of 60 - 300 m, may be larger, sparse, long-lived colonies (Santangelo et al. 1999; Tsounis et al. 2006a);
3. Deep-water populations, below 300 m depth. These populations, for obvious practical reasons, are poorly known (Costantini et al. 2010).

Due to its commercial and ecological value, the biological information on the species increased noticeably during the last decades. Several studies have been carried out in different areas of the Mediterranean, mainly on its feeding ecology (Tsounis et al. 2006b), reproductive patterns (Vighi 1972; Santangelo et al. 2003; Torrents et al. 2005), recruitment (Garrabou & Hermelin 2002; Bramanti et al. 2005, 2009), growth (Bramanti et al. 2005), physiology (Rossi & Tsounis 2008), genetic population structure (Abbiati et al. 1993), competition for space (Giannini et al. 2003) and population dynamics (Santangelo et al. 2003). All these studies were carried out on shallow-water populations, and, until now, only few studies have focused on deeper populations (Garrabou et al. 2001; Bramanti et al. 2005; Tsounis et al. 2006b; Rossi et al. 2008; Torrents et al. 2008; Costantini et al. 2010, 2011).

2.2 *Biology and reproduction*

Red coral lives in compact colonies, each coral colony being formed by up it thousands of individual, white transparent polyps, each one bearing eight tentacles that produce a red skeleton of calcareous spicules. All polyps of a same colony are genetically identical (fig. 2.3).

Figure 2.3 Colonies of *Corallium rubrum* with expanded polyps (Picture made by Simone Canese, in Capel Rosso site at 60m depth).

Unlike other Cnidarians, red coral exhibits very limited organ development. It shares two anatomical features with other Cnidarians: a gastrovascular cavity (simple stomach) that opens only on one end, and a ring of tentacles. Red coral has no central nervous system. Unlike many coral species, it doesn't have the symbiotic algae zooxanthellae living within the coral tissue. Lacking zooxanthellae means that the coral must obtain food by another method. It feeds on particles of organic matter, suspended in the water, which are captured by their tentacles (fig. 2.4). They also occasionally capture and consume larger zooplankton.

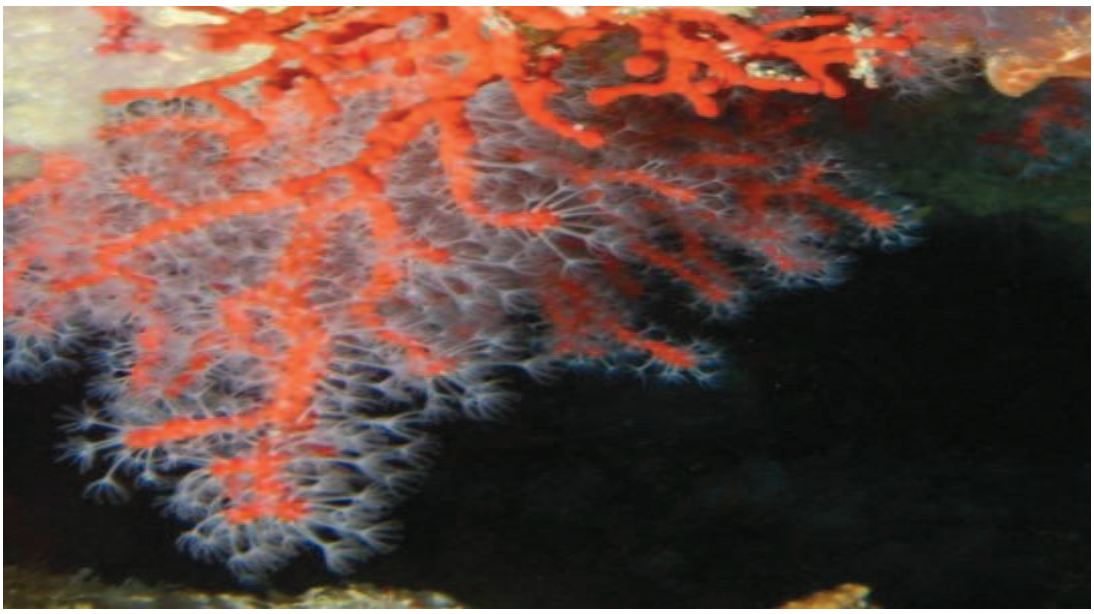

Figure 2.4 White polips (Picture made by Simone Canese).

The reproductive system of the coral is inside the polyps. Although fragmentation is common in modular marine invertebrates (Jackson 1986; Karlson 1986), in red coral this has not been evidenced (Santangelo & Abbiati 2001). Recruitment in this species is therefore considered to depend on larval settlement (Santangelo et al. 2003). Sexes are normally separated in male and female colonies and, fertilization is internal, therefore fecundation happens when free-swimming sperm from male colonies reach the polyps of female colonies. The maturation of the male gametes takes place over one year. It starts at the beginning of summer. The maturation of the female gonads takes place every 2 years. It begins slowly the first year and is completed at the beginning of the second year (Vighi 1972). The fertilized eggs develop into larvae within the female polyp's body cavity. The young larva, known as a planula, develops for 20 or 30 days inside the polyp before going out into the open sea. The emission of larvae takes place from July to the beginning of October, depending on the depth. The larvae swim for 4 to 15 days (Vighi 1972), or until they find a suitable substrate to settle. Like all larvae of sessile marine invertebrates, even the red coral planula requires specific conditions for settlement. Red coral planulae are indifferent to light and show negative geotropism and laboratory experiments suggest that larvae do not spread very far from the parental colonies (Vighi 1972), although, currently, limited data support or refute this hypothesis. Right after the settlement starts the metamorphosis, the mechanisms and control of which are totally unknown (Weinberg 1980). Following metamorphosis, the young larva begins to build-up its structure becoming a new coral colony.

The colony grows by asexual reproduction (fig. 2.5). The sexual maturity of the first polyps is reached after about 2 years (Santangelo et al. 2003).

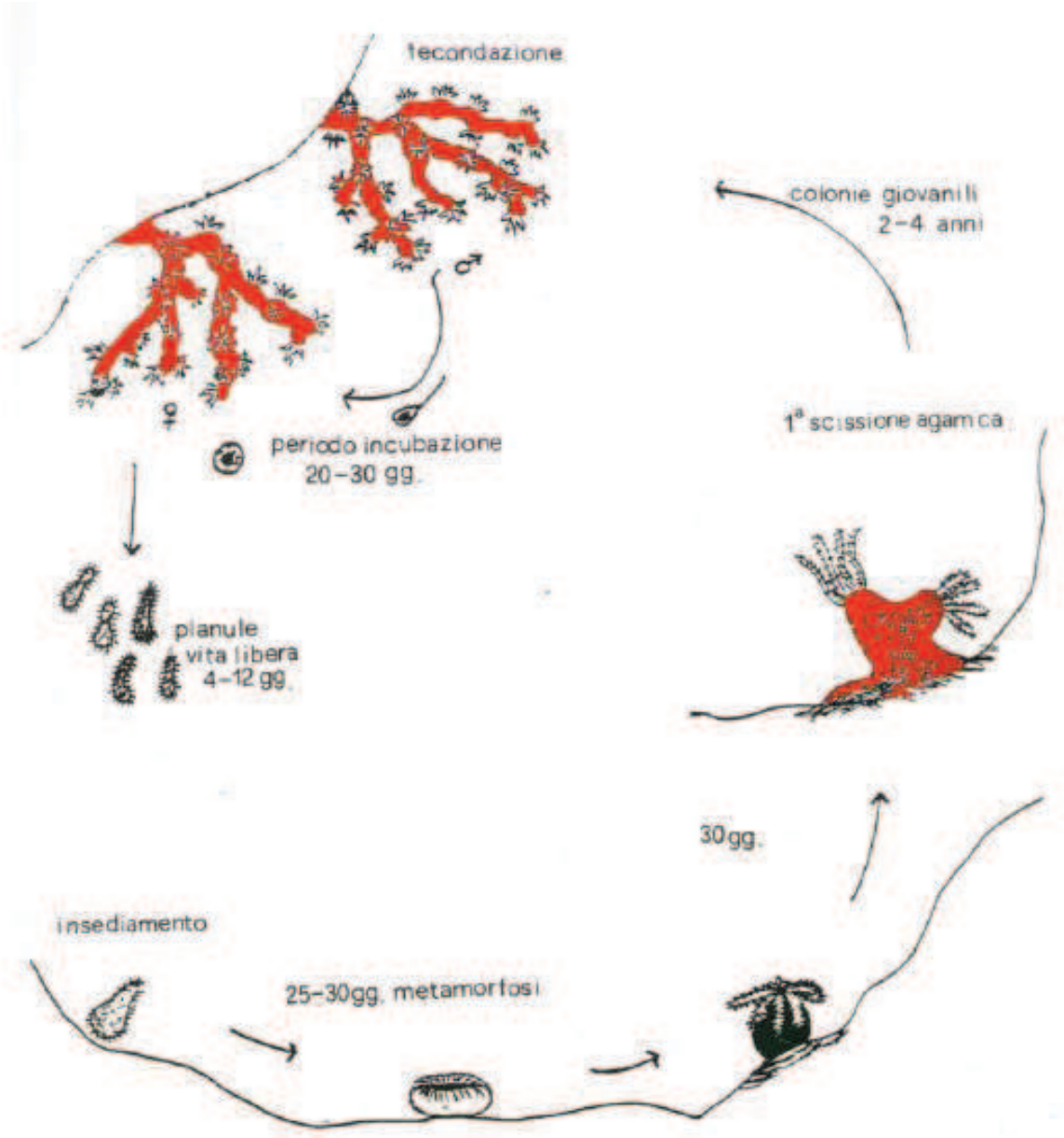

Figure 2.5 Reproductive life cycle of red coral (Vighi 1972).

2.3 *Harvesting pressure and natural mortality*

Since prehistoric times, man has been fascinated by red coral which, in spite of its mineral skeleton and vegetal aspect is, in reality, an animal. It was first used in the Upper Palaeolithic (approximately 20.000 B.C.). Later, it was represented on wall paintings and vases, or used to make jewellery and other objects by the Egyptians, the Greeks and the Romans (Morel et al. 2000). In the middle Ages, it was usual to carry a few pieces of coral in a bag to ward off witches. It was also used medicinally for its various supposed virtues. As a powder, for example, it was added to baby food as a protection from epidemics (Liverino 1983; Ascione 1993). Its use as a jewel and talisman has remained constant through the centuries (Roth 2002).

Nowadays, this species is still a resource of richness for many Mediterranean communities. Until quite recently, its professional fishers used a trawling gear called the Saint-André Cross. The Saint-André Cross consists of a wooden or, more recently steel, cross with nets attached. Trawled along the bottom at 50 m by boats, the Cross breaks the coral colonies and the pieces are caught by the nets. Such proceedings bring up 1 to 2 tons of coral a year. But the damage done on the bottom is too strong that in 1994 this kind of harvest method was banned. This important harvesting pressure resulted in profound changes in the species structure, switching from large colonies (base diameters of over 1 cm) into colonies with diameters of a few millimeters (Fig. 2.6.a, b).

Figure 2.6.a Low density of red coral individuals due to overexploitation in Campanian archipelago (picture made by Simone Canese).

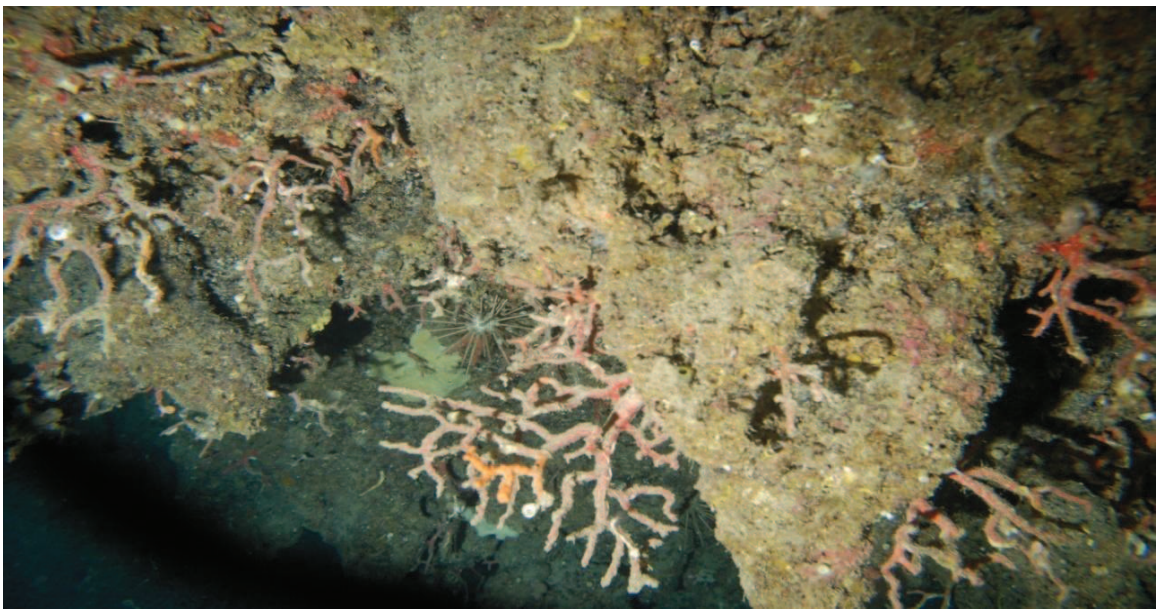

Figure 2.6.b Dead red coral colonies in Campania archipelago (picture made by Simone Canese).

In 2008 was estimated that the annual quantity of red coral fished in the Mediterranean Sea is 400 tons (FAO, 2008, Fig.2.7).

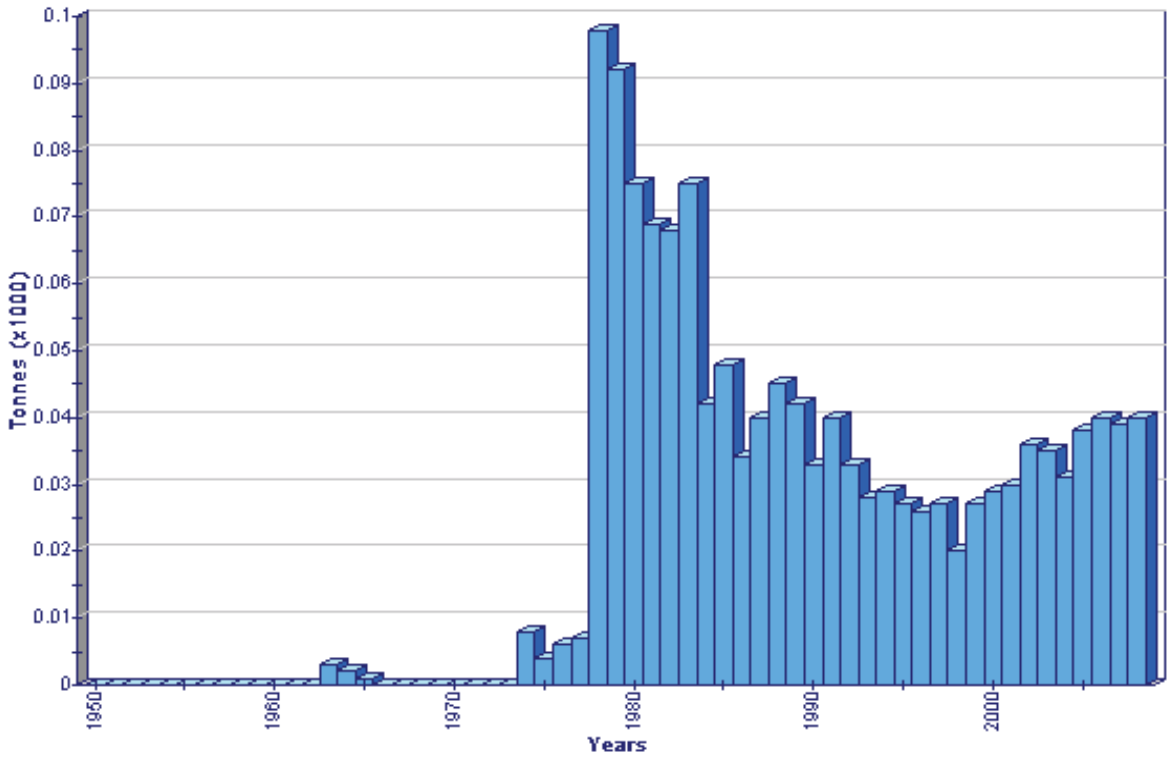

Fig. 2.7 Annual coral exploitation expressed in tonnes between 1950 and 2008 (data FAO, GFCM 2010)

The shallow water populations of the species are not subject to commercial fishing, but its high economic value, or simply its attractiveness to amateur divers has provoked over-exploitation also of these shallower populations, resulting in its complete disappearance from many places along our coasts.

The lack of adequate conservation and management measures could lead to the economical extinction of red coral but not to an ecological extinction (slow growth and density dependent species populations could survive to the overexploitation, but they could not reach the commercially exploitable size) (Santangelo et al. 2007). Meaningful conservation plans can only be built from knowledge of species life history traits (Torrents et al. 2004). Today, the shallow water populations are overexploited all over the Mediterranean Sea (Abbiati et al. 1992; Santangelo & Abbiati 2001; Tsounis et al. 2007; Tsounis et al. 2010; GFCM 2010) and the GFCM on 2010 has imposed a ban on red coral harvesting in populations shallower than 50 metres depth; moving the exploitation towards the mesophotic zone, where the harvesting is done by scuba divers which can immerse over 100m depth, and are capable of collecting up to 200-300kg a year in about 200 dives (http://www.centrescientifique.mc).

More than human overexploitation, there are other causes of natural origin that can lead to mortality of red coral colonies, including: crumbling of the substrate due to dwelling species, parasitism or endosymbiosis by boring sponges, increased sedimentation and mass-mortality events (Harmelin 1984). There are two species closely associated with red coral (Abbiati & Santangelo 1992): *Pseudosimnia carnea* (Poiret 1789), a gastropod that feeds on gorgonacea (Santangelo & Navarra 1984), and *Balssia gasti* (Balss 1921), a decapod crustacean that probably feeds on coenosarc (Santangelo et al. 1993). Moreover, many species of boring sponges (Demospongiae, Clionidae) penetrate in the coral sclerasse, seriously damaging it and reducing its commercial value. These organisms especially affect shallow-water populations, where the overunning by sponges starts from the calcareous substratum and then extends to coral (Corriero et al. 1997).

Furthermore, red coral populations were severely affected by mass mortality events occurring at the end of the summers of 1999 and 2003 along the French and Ligurian coasts. These events might have been related with the increase of temperature recorded in those areas (Cerrano et al. 2000; Perez et al. 2000; Garrabou et al. 2001), greatly impacting the population dynamics of this species (Santangelo et al. 2007).

Red coral shows a good resilience in cases of reduction of the reproductive output and increased mortality, a typical feature in species with low growth rates, high rates of reproduction and overlapping generations. Sporadic mortality events affect its survival, but the increase of fishing pressure and frequent mass mortality events may cause serious damage over time because the recovery potential of the species may not be sufficient to balance them (Santangelo et al. 2007; Tsounis et al. 2007).

2.4 *Population genetic*

Previous genetic studies on red coral populations investigated its effective larval dispersal as well as the spatial genetic structure between and within different geographical areas. Based on the use of allozymes, significant differentiation was observed between samples separated by about 10 km (Abbiati et al. 1993). Successively, using more variable molecular markers, a genetic structuring at scales of less than one meter was observed. Moreover, a high inbreeding rate within populations was also detected (Costantini et al. 2007; Ledoux et al 2010a, b). The inbreeding is the reproduction between closely related individuals or next-to-kin, and probably represents an evolutionary adaptation as it has the effect of bringing to homozygosity genetic loci: this homozygosity makes that both favorable and deleterious genes are enhanced and, if the last are prevalent, could have serious negative effects for the populations survival.

At Mediterranean scale a genetic structuring among shallow-water populations with some isolation by distance patterns (Ledoux et al. 2010a, b) was observed (Costantini et al. 2007b) confirming its patchy distribution. Together, these results suggested that red coral larvae have a short dispersal capability and that the populations tend to be self-recruiting. Recently, studies on deeper populations showed a decline of genetic variability along a depth gradient in a range between 20-70 meters and a genetic isolation between shallow and deep-water populations, suggesting that depth might have an important role in determining the patterns of genetic structure of the species (Costantini et al. 2010).

AIMS

Since shallow water red coral populations are overexploited along all the Mediterranean coasts, today the harvesting pressure has moved towards the deeper populations. Nevertheless, these mesophotic populations are still understudied, when compared to their shallow-water counterparts.

The aim of this thesis is to fill gaps in the knowledge on the biology and ecology of currently harvested red coral populations using a genetic approach. Research cruises were done to sample red coral colonies distributed in three different areas of the Western Mediterranean Sea and in a depth range from 60 to 120 metres. In particular, two molecular markers with different levels of polymorphism, the mitochondrial control region and 12 microsatellite loci were used to evaluate the genetic variability and structuring of the populations.

These data together with those obtained on population structure, biology and ecology will be very useful to allow appropriate management of the harvesting of this precious resource.

4. MATERIALS AND METHODS

3.1 *Sample collection*

Three different geographic areas along the Tyrrhenian Sea were selected for oceanographic cruises done between May and June 2012 by the R/V *Astrea:* Ligurian coast, Tuscan Archipelago, and Campania coast (Figure 3.1. a, b, c). These surveys were specifically dedicated to the sampling of mesophotic red coral populations. *Corallium rubrum* colonies were sampled at 14 sites corresponding to different habitats (overhangs, vertical cliffs and outcrops; Table 3.1). The areas are separated among them by hundreds of kilometers and the sampling sites within each area were separated by tens to hundreds of meters. The occurrence of *Corallium rubrum* was detected by a multibeam echosounder and in total 148 red coral colonies were sampled using a Remotely Operated under water Vehicle (ROV), at depth ranging between 60 and 120 metres (Table 3.1). For each site, a branch fragment from 6 to 23 live colonies of red coral was collected within an area ranging from 50 to 100 m^2. The difference in the number of colonies sampled for each site depends to the abundance of the species. All the fragments were preserved, after collection, in 80% ethanol at 4°C for subsequent molecular analyses.

Figure 3.1 Sampling Areas: Liguria, Toscana and Campania.

Figure 3.1.a Map showing the Liguria sample area. Yellow stars indicate the position of sampled populations.

Figure 3.1.b Map showing the Toscana sample area. Yellow stars indicate the position of sampled populations.

Figure 3.1.c Map showing the Campania sample area. Yellow stars indicate the position of sampled populations.

CODE	SITE	AREA	LATITUDE	LONGITUDE	DEPTH (m)	SAMPLES
LIG1	Portofino P.Faro (GE)	Liguria	44°17.80'N	9°12.140'E	68	6
LIG2	Bordighera (IM)	Liguria	43°44.67'N	7°40.917'E	73	9
TOS1	Isola del Giglio (GR)	Toscana	42°18.64'N	10°55.19'E	105	13
TOS2	Pianosa Nord (LI)	Toscana	42°38.40'N	10°6.49'E	73	6
TOS3	Scogliosante	Toscana	42°25.08'N	10°04.81E	85	11
TOS4	Montecristo (LI)	Toscana	42°30.84'N	10°18.63'E	120	10
TOS5	TunaParadive (LI)	Toscana	42°17'N	10°14'E	94	23
TOS6	Capel Rosso (LI)	Toscana	42°18.07'N	10°19.10'E	60	6
CAM1	Li galli (SA)	Campania	40°34.71'N	14°25.18'E	100	7
CAM2	Li galli (SA)	Campania	40°34.52'N	14°25.03'E	100	6
CAM3	Li galli (SA)	Campania	40°34.62'N	14°24.69'E	90	9
CAM4	Punta Solchiaro (NA)	Campania	40°44.27'N	14°01.09'E	60	15
CAM5	Punta Imperatore (NA)	Campania	40°42.246'N	13°50.94'E	108	6
CAM6	Scoglio D'Ischia (NA)	Campania	40°43.72'N	13°49.52'E	73	6

Table 3.1 Codes, sites, areas, coordinates, depth and number of individuals collected during the oceanographic campaigns.

3.2 *Dna extraction and molecular analysis*

Total genomic DNA was extracted from two to four polyps per individual colonies using cetyltrimethyl ammonium bromide (CTAB) protocol (Winnepenninckx et al. 1993) following the procedure described in Costantini et al. (2007b). Total DNA was visualized in a 0.8% agarose gel, stained with GelRed (BIOTIUM) 1%, after a 30 minutes electrophoresis at 120 V. Extractions were loaded with the loading buffer BLU 6X. Gene Ruler Express DNA Ladder was used for sizing and quantification of DNA fragments. The extraction product was diluted to 1:20 and 1:50 in ultrapure water (SIGMA) for better amplification success.

Eleven microsatellite loci (COR9, COR46, COR58, COR15, MIC23, MIC22, COR48, MIC26, MIC24, MIC13, MIC20) specifically developed for *Corallium rubrum* (Costantini & Abbiati 2006; Ledoux et al. 2010a) and one (CR3Al7) developed for *Corallium lauuense* (Baco et al. 2006) were analyzed. Microsatellite loci were amplified in multiplex with a QIAGEN® Multiplex PCR Kit using polymerase chain reaction (PCR) (conditions described in Costantini et al. 2011). Genotyping of individuals was carried out on an ABI 310 Genetic Analyser (Applied Biosystems), using forward primers labeled with FAM, HEX/VIC, TAMRA/NED, ROX/PET (Sigma) and LIZ HD500 (Applied Biosystems) as internal size standard. Allele sizing was conducted using Peak Scanner Analysis Software v1.0 (Applied Biosystems).

The mitochondrial control region (MtC) sequences were amplified using the primers ND618510CkonojF 5'-CCATAAAACTAGCTCCAACTATTCC-3' and COI16CkonojR 5'-GGTTAGTAGAAAATAGCCAACGTG-3' (Sigma). These primers were specifically designed using the online PRIMER3 version 4.0 software (Rozen & Skaletsky 2000) on the *nad6* and *cox1* genes flanking the putative control region, which seems located in the intergenic spacer 12 (IGS12) of the mitochondrial genome of *Paracorallium japonicum* and *Corallium konojoi* (Uda et al. 2011). Each 25.0 µL MtC PCR reaction contain: 2.5 µL of DNA template; 2.5 µL of buffer; 2 µL of $MgCl_2$ 25 mM; 2 µL of dNTPs 10 mM; 1.25 µL of each forward and reverse primers 10 mM; 13.3 µL of ultrapure water (SIGMA) and one unit (0.2 µL) of Taq polymerase enzyme (Invitrogen**).** PCR reaction was performed in a GeneAmp® PCR Sistem 2700 thermocycler (Applied Biosytems) as follows: an initial denaturation at 95 °C for 3 min, 30 cycles including 95 °C for 30 s, other 30s at a specific annealing temperature 59 °C and an extension at 72 °C for 60 s, and a final extension at 72 °C for 7 min. After PCR,

the products were maintained at 4ºC. Products of amplification were visualized in a 1.5% agarose gel as previously described. PCR products were sent to Macrogen (South Korea) for purification and sequencing with the same primers using for the amplification.

3.3 *Microsatellite Markers*

3.3.1 Genetic Variability

Sampling using the ROVs may cause fragmentation of the colonies, and in some cases we can wrongly analyze fragments belonging to the same individual as if they were from different colonies. For this reason first of all individuals sharing the same multilocus genotype (MLG) were checked using GENALEX version 6.1 (Peakall & Smouse 2006). Moreover, the unbiased probability of identity (P_{ID} Kendall & Stewart 1977) that two individuals share the same MLG by chance and not by descent was computed.

The analysis of microsatellite genetic variability within samples were estimated evaluating the number of alleles for locus and populations (Na), observed heterozygosity (Ho) and unbiased gene diversity (Hs, Nei 1987) using the GENETIX software package version 4.05 (Belkhir et al. 2004). Single and multilocus *Fis* were estimated using Weir and Cockerham's model (Weir & Cockerham 1984) implemented in GENETIX. *Fis* give us information about populations equilibrium, with values ranging from -1 to 1 (where values near to 0 means that Ho is close to Hs). Since several *Fis* values obtained are positive (Ho < Hs), the departures from Hardy-Weinberg equilibrium (HWE) were tested using "Fisher's exact test" in GENEPOP version 3.4 (Raymond & Rousset 1995) as implemented for online use (http://genepop.curtin.edu.au/), with the level of significance determinate by a Markov-chain randomization. For this analysis 1000 steps of dememorization, 100 batches and 1000 iterations per batch, were set, with the aim to obtain a significance level of 1%. Moreover, significant differences in genetic diversity (Ho, Hs, *Fis*) among populations grouped according to their geographical origin (two populations for Liguria; six populations for Toscana and Campania respectively) were tested using a permutation procedure (1000 iterations) in FSTAT version 2.9.3.2 (Goudet 2001).

3.3.2 Population genetic differentiation

The genetic divergence among populations was determined from the original dataset using Weir & Cockeram's (1984) F_{ST} estimator in the ARLEQUIN Version 2.0 software (Schneider et al. 1999). F_{ST} values ranges from 0 to 1. A zero value implies complete panmixia; while a value of 1 would implies that the two populations are completely separate. The significance of the estimator was determined using bootstrap resampling with 10000 permutations.

To evaluate the isolation by distance pattern, and so the presence of correlation among genetic differentiation estimates (F_{ST}) and geographical distance (Log transformed), a Mantel test (Mantel 1967) computed using the Isolde program implemented in GENEPOP (using 1000 permutations to test the significance level of correlation) was used.

The programme STRUCTURE v. 2.3 was used to detect the number (K) of genetically homogeneous populations in the microsatellite dataset (Pritcherd et al. 2000, Falush et al. 2003, 2007). Each individual was assigned to probable common clusters based on the similarity of their multilocus genotypes at 10 microsatellite loci. Mean and variance of log likelihoods of the number of clusters for K=1 to K=16 were inferred from multilocus genotypes by running STRUCTURE five times with 200000 repetitions each (burn-in=50000 iterations) under the admixture ancestry model and the assumption of correlated allele frequencies among samples (as suggest in Falush et al. 2003).

In order to have a visual assessment of between-population differentiation, we performed a discriminant analysis of principal components (DAPC, Jombart et al. 2010) as implemented in the ADEGENET package for R (Jombart 2008). This technique extracts information from genetic datasets (multivariate in nature) by first performing a principal component analysis (PCA) on pre-defined groups or populations, and then using the PCA factors as variables for a discriminant analysis (DA), which seeks to maximize the intergroup component of variation. The optimal number of clusters (populations) was predicted using the *k*-means clustering algorithm, find.clusters', retaining all principal components. In all analyses 50 principal components of PCA were retained as input to DA.

The partition of genetic variance among samples was conducted through an analysis of molecular variance (AMOVA) implemented in ARLEQUIN. For this purpose three different AMOVA were computed: the first, grouping populations based on their geographical origin (three different areas: Liguria-LIG, Toscana-TOS and Campania-CAM); the second, grouping populations according to the STRUCTURE results (two groups: LIG-TOS populations and CAM populations) and the third grouping populations according to the DAPC results (three group: LIG1-2 and TOS1-2 populations; TOS3-4-5-6; and CAM populations). The AMOVA assigns percentages of variability explained and a significance to the variability among groups, within populations inside the groups and within populations without grouping, giving information on the degree of homogeneity of the groups set and how differentiated are from each other.

3.4 *Mitochondrial Markers*

3.4.1 Genetic variability

The obtained sequences were edited and aligned manually using BioEdit Sequence Alignment Editor v. 7 (Hall 1999). The alignment was performed with ClustalX (Thompson et al. 1997) and corrected by hand.

Sequence genetic diversity within samples was estimated using number and of haplotype (*h*), haplotype diversity (*Hd*, Nei 1987) and nucleotide diversity (π, Nei 1987) using DnaSP v5.10 software (Librado & Rozas 2009). The value of *Hd* ranges from 0 to 1. A value of 0 indicates that all haplotypes are identical (no diversity), while a value of 1 (very high diversity) indicates no shared haplotypes among individuals (Grant & Bowen 1998). The nucleotide diversity (*Pi* or π) is the average sequence divergence among haplotypes (Grant & Bowen 1998).

3.4.2 Population genetic differentiation

Population differentiation was analyzed using pairwise genetic distances (F_{ST}) between populations, in ARLEQUIN 3.5 (Excoffier et al. 2005). The significance of the genetic distances was tested by permuting 10000 times the haplotypes between the populations, assuming as null hypothesis (H_0) the absence of population differentiation. The P-value of the test was the proportion of permutations leading to F_{ST} values larger or equal to the observed ones. If P-values <0.05 (significant) or 0.01 (highly significant), the H_0 is rejected hence the pair of populations compared are significantly different.

In order to have a visual assessment of between-population differentiation a Multidimensional Scaling (MDS) were performed using the software PRIMER v6 (Clarke 1993).

With the intent to detect the effect of isolation by geographical distance, we compared the correlation of genetic distances (Reynold's distance: F_{ST} /1- F_{ST}) with $\log_{10}$-transformed geographical distances using the Mantel test procedure implemented in GENEPOP version 4.1 (Rousset 2008).

The partition of the genetic variance among areas was conducted through an analysis of molecular variance (AMOVA) implemented in the ARLEQUIN software v3.5.1.2 (Excoffier & Lischer 2010) grouping population for their geographical origin (three groups) and grouping populations according MDS results.

4. RESULTS

4.1 *Microsatellite Markers*

4.1.1 Genetic Variability

A total of 148 *Corallium rubrum* colonies were analyzed. The twelve microsatellite loci analyzed were polymorphic in all fragments. Loci MIC22 and MIC23 were excluded from subsequent analysis, because they correctly amplified in Toscana and Liguria populations, but did not give any amplification in Campania populations. Over all samples, the number of alleles per locus ranged from 1 (COR15, COR58 and MIC13) to 17 (MIC26). Within populations, over all loci, the mean number of alleles ranged between 3.7 (in CAM5) and 8.5 (in TOS5). Mean observed heterozygosity ranged between 0.22 (in TOS3) to 0.46 (in TOS2, CAM3, CAM4), and gene diversity from 0.53 (in TOS6, CAM5) to 0.70 (in TOS5) (Tab. 4.1).

	SAMPLE (n)													
LOCUS	LIG1 (6)	LIG2 (9)	TOS1 (13)	TOS2 (6)	TOS3 (11)	TOS4 (10)	TOS5 (23)	TOS6 (6)	CAM1 (7)	CAM2 (6)	CAM3 (9)	CAM4 (15)	CAM5 (6)	CAM6 (6)
COR9 bis														
N	6	7	10	6	5	8	16	5	7	6	8	14	6	5
Na	5	3	4	5	5	5	9	3	7	5	4	7	6	5
Ho	0.16	0.14	0.20	0.33	0.40	0.12	0.31	0	0.28	0.50	0.75	0.64	0.66	0.40
Hs	0.79	0.35	0.68	0.72	0.68	0.57	0.81	0.56	0.75	0.72	0.67	0.67	0.73	0.60
F_{IS}	**0.82**	0.64	**0.73**	**0.60**	0.50	**0.80**	**0.63**	1	**0.66**	0.38	-0,05	0.07	0.18	0.42
COR15														
N	5	7	13	6	10	10	19	4	5	5	4	15	6	4
Na	2	3	2	2	1	1	2	4	2	4	3	3	2	3
Ho	0	0.42	0.15	0.33	0	0	0	0.25	0.20	0.20	0.25	0.06	0	0.25
Hs	0.48	0.50	0.26	0.27	0	0	0	0.21	0.66	0.18	0.65	0.18	0.27	0.59
F_{IS}	1.00	0.21	0.44	-0.11	-	-	-	-	**0.75**	-	0.70	0.65	1.00	**0.66**
COR46bis														
N	3	7	8	2	11	10	20	6	7	6	9	11	6	3

Na	3	8	6	2	4	9	12	8	10	6	9	15	5	6
Ho	0.66	0.71	0.25	0.50	0	0.60	0.45	0.50	0.71	0.50	0.66	0.81	0.33	1
Hs	0.61	0.84	0.71	0.37	0.62	0.81	0.87	0.84	0.88	0.77	0.86	0.92	0.77	0.83
F_{IS}	0.11	**0.23**	**0.68**	-	**1.00**	**0.31**	**0.50**	**0.48**	0.26	**0.43**	0.28	0.15	**0.62**	0
COR48														
N	6	9	9	6	10	7	10	4	7	6	5	8	5	5
Na	7	9	9	8	8	6	10	7	4	6	7	8	8	6
Ho	0.33	0.55	0.33	0.66	0.40	0.71	0.60	0.75	0.14	0.50	0.60	0.37	0.80	0.40
Hs	0.83	0.86	0.75	0.86	0.78	0.74	0.84	0.84	0.72	0.75	0.84	0.82	0.86	0.80
F_{IS}	**0.52**	**0.40**	**0.60**	**0.31**	**0.52**	0.11	**0.33**	0.25	**0.82**	0.41	0.38	**0.58**	0.17	**0.57**
COR58														
N	4	6	10	4	4	7	8	3	4	3	6	13	0	4
Na	3	4	4	5	4	6	8	1	4	3	3	5	0	3
HO	0	0.33	0.30	0.50	0	0.28	0.25	0	0	0	0.16	0.07	0	0.25
Hs	0.62	0.41	0.74	0.78	0.75	0.69	0.82	0	0.75	0.66	0.40	0.61	0	0.59
F_{IS}	**1.00**	0.28	**0.62**	0.47	**1.00**	**0.63**	**0.73**	-	**1.00**	**1.00**	0.64	**0.88**	-	**0.66**
MIC13														
N	5	7	10	3	7	6	22	6	2	3	4	7	5	3
Na	1	2	4	1	2	2	4	3	2	2	3	4	3	3
Ho	0	0	0.20	0	0	0	0.27	0.33	0.50	0	0.50	0.28	0	0.33
Hs	0	0.24	0.66	0	0.40	0.27	0.57	0.29	0.37	0.44	0.40	0.66	0.64	0.50
F_{IS}	-	1.00	**0.72**	-	**1.00**	1.00	**0.54**	-0,05	-	**1.00**	-0,09	**0.61**	**1.00**	0.50
MIC20														
N	6	7	11	6	10	10	20	6	7	6	9	13	3	4
Na	4	5	3	8	9	7	8	4	4	8	8	7	4	4
Ho	0.83	0.42	0.09	0.66	0.50	0.40	0.50	0.66	0.28	0.83	0.22	0.69	0.66	0.75
Hs	0.58	0.76	0.16	0.86	0.83	0.80	0.76	0.68	0.64	0.84	0.82	0.81	0.66	0.65
F_{IS}	-0.35	0.50	0.50	**0.31**	**0.44**	**0.53**	0.37	0.11	**0.60**	0.10	**0.75**	0.18	0.20	0
MIC24														
N	6	8	6	6	10	6	23	6	7	6	9	14	6	5
Na	3	4	3	5	7	8	7	3	5	4	2	6	2	4
Ho	1	0.75	0.66	0.50	0.50	1	0.43	0.66	0.42	0.33	0.11	0.28	0.16	0.40
Hs	0.56	0.67	0.56	0.61	0.65	0.84	0.71	0.48	0.61	0.51	0.10	0.61	0.15	0.58
F_{IS}	-0.71	-0.05	-0.08	0.26	**0.28**	-0.09	**0.41**	-0.29	0.37	0.43	-	**0.56**	-	0.40
MIC26														
N	6	6	12	6	10	7	17	6	7	6	9	12	5	6
Na	6	4	6	6	10	9	17	10	8	8	8	11	3	3
Ho	0.33	0.33	0.58	0.66	0.40	0.71	0.76	0.83	0.71	0.66	0.33	0.58	0.40	0
Hs	0.77	0.68	0.78	0.81	0.90	0.85	0.90	0.87	0.79	0.83	0.78	0.85	0.54	0.50
F_{IS}	**0.62**	**0.57**	0.29	0.27	**0.59**	0.24	0.18	0.13	0.17	0.28	**0.61**	**0.35**	0.36	**1.00**
CR3AL7														
N	6	6	11	2	9	7	20	3	7	6	9	15	5	5
Na	6	5	9	3	3	2	8	3	4	3	6	7	4	4
Ho	0.33	0.66	0.63	0.50	0	0	0.15	0.33	0.42	0.66	1	0.86	0.40	0.20
Hs	0.75	0.66	0.79	0.62	0.56	0.40	0.65	0.50	0.53	0.48	0.67	0.65	0.66	0.70
Fis	**0.61**	0.09	0.24	0.50	**1.00**	1.00	**0.78**	0.50	0.26	-0.29	-0.42	-0.3	0.48	**0.76**

Multilocus														
Na	4	4.7	5	4.5	5.3	5.5	8.5	4.6	5	4.9	5.3	7.3	3.7	4.1
Ho	0.36	0.43	0.34	0.46	0.22	0.38	0.37	0.43	0.36	0.42	0.46	0.46	0.34	0.40
Hs	0.60	0.60	0.61	0.60	0.62	0.60	0.70	0.53	0.67	0.62	0.62	0.68	0.53	0.63
F_{IS}	**0.30**	**0.39**	**0.47**	**0.26**	**0.62**	**0.45**	**0.45**	**0.21**	**0.49**	**0.40**	**0.28**	**0.37**	**0.40**	**0.50**

Table 4.1 Summary of genetic diversity at ten microsatellite loci within *Corallium rubrum* populations: n, number of sampled individuals; N, number of genotypes per locus; Na, number of alleles per locus; Ho, observed heterozygosity; Hs, gene diversity (Nei 1987); F_{IS}, Weir and Cockerham's (1984) estimate of Wright's (1951) fixation index. Bold types indicate significant deviations from HW equilibrium.

Highly significant deviations from HWE ($P < 0.001$) were observed in all populations with Hs significantly higher than Ho. All multilocus estimates of F_{IS} were significant different from 0 and ranged from 0.21 (in TOS6) to 0.62 (in TOS3), showing heterozygote deficiencies in all analyzed samples (Table 4.1).

No significant difference in genetic diversity (Ho, Hs, F_{IS}) were observed among populations belonging the different areas ($P > 0.05$, data not shown).

4.1.2 Population genetic differentiation

Analysis of genetic differentiation between pairs of populations showed value of F_{ST} between 0 (TOS2 with TOS6 and CAM5, CAM4 with CAM2 and CAM5) to 0.24 (among CAM6 and TOS1) (Table 4.2) with major pairwise comparison significantly different ($P<0.01$).

Mantel test showed a statistically significant correlation between geographic distance and genetic differentiation among populations ($p=0$; Fig.4.1).

	LIG1	LIG2	TOS1	TOS2	TOS3	TOS4	TOS5	TOS6	CAM1	CAM2	CAM3	CAM4	CAM5	CAM6
LIG1														
LIG2	**0.08**													
TOS1	**0.17**	**0.12**												
TOS2	**0.08**	0.01	0.05											
TOS3	**0.09**	**0.12**	**0.08**	0.3										
TOS4	**0.17**	**0.21**	**0.12**	**0.10**	0.05									
TOS5	**0.08**	**0.07**	**0.06**	0.5	0.01	0.01								
TOS6	0.05	**0.08**	**0.13**	0	0.05	**0.15**	**0.07**							
CAM1	**0.14**	**0.13**	**0.15**	**0.10**	**0.13**	**0.17**	**0.06**	**0.11**						
CAM2	**0.12**	**0.12**	**0.13**	0.03	**0.09**	**0.16**	0.05	**0.07**	0.07					
CAM3	**0.11**	**0.14**	**0.18**	0.05	**0.12**	**0.20**	**0.12**	**0.09**	**0.10**	0.02				
CAM4	**0.15**	**0.14**	**0.14**	**0.06**	**0.06**	**0.11**	**0.05**	0.05	**0.06**	0	**0.08**			
CAM5	0.09	**0.14**	**0.07**	0	0.09	**0.12**	**0.09**	**0.10**	0.08	0.01	-0.02	0		
CAM6	**0.16**	**0.18**	**0.24**	**0.14**	**0.16**	**0.22**	**0.10**	**0.11**	0.02	0.04	0.05	**0.08**	0.04	0

Table 4.2 Pairwise multilocus estimates of F_{ST} (Weir & Cockerham 1984) between all samples. Bold values are statistically significant ($P < 0.01$).

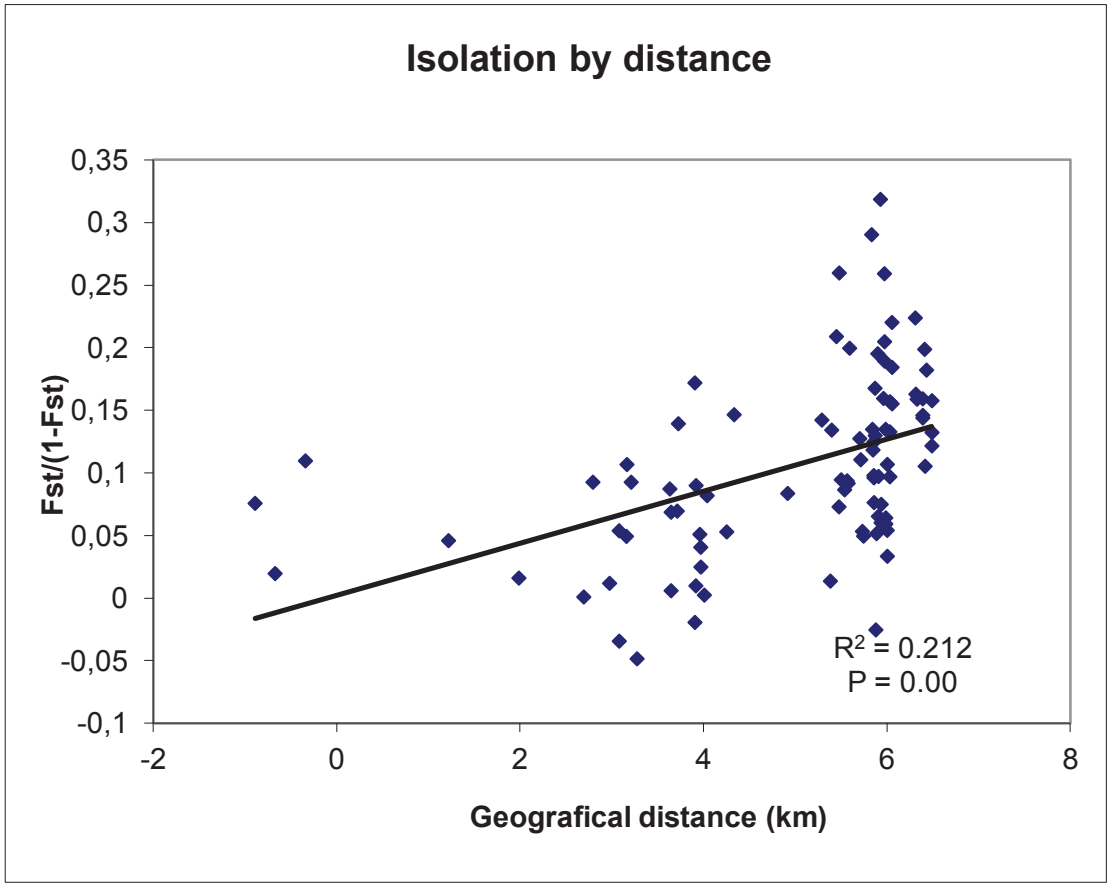

Figure 4.1 Relationship between genetic differentiation and the logarithm of geographical distance among *Corallium rubrum* populations for microsatellites markers.

Testing the significance of the stepwise clustering procedure performed in STRUCTURE resulted in a separation of the populations into two clusters (K=2, Δk=128.26) (Fig.4.2). First cluster contained Liguria and Toscana populations, while the second contained the remaining Campania populations (in according with geographical distance).

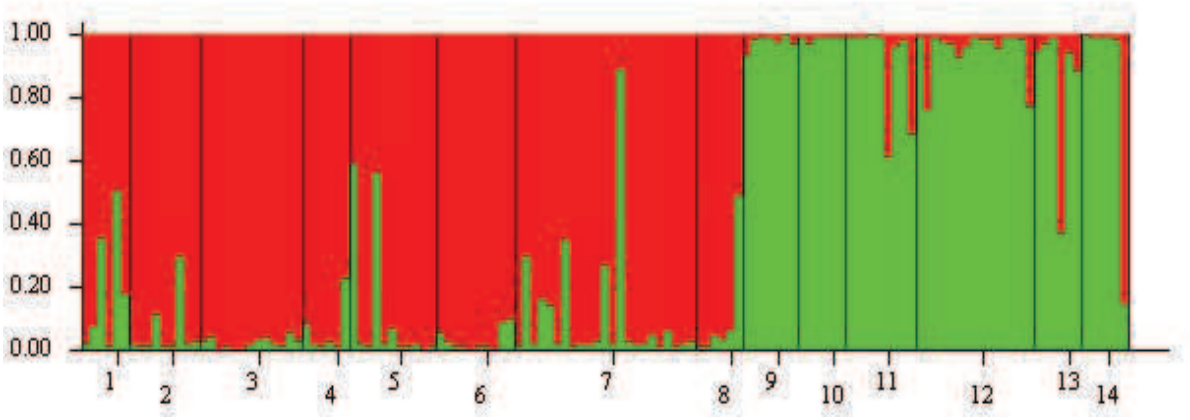

Figure 4.2 Results of the clustering analysis conducted in STRUCTURE. Each individual is represented by a vertical line partitioned into k-colored segments that represent the individual's membership fraction in k clusters. Each population is delineated by black vertical lines and number representing populations in ascending order from Liguria to Campania.

The DAPC showed that the 50 principal components of the retained PCA explained 95 % of the total variance. The scatterplot of the first two components of the DA showed the same results of STRUCTURE. Nevertheless, from the graphic representation (Fig.4.3) can be seen as inside the first cluster there is a slight differentiation between one small cluster including all the samples from Liguria and two populations from Toscana (TOS1 and TOS2) and other small of only Toscana populations (TOS3, TOS4, TOS5, TOS6).

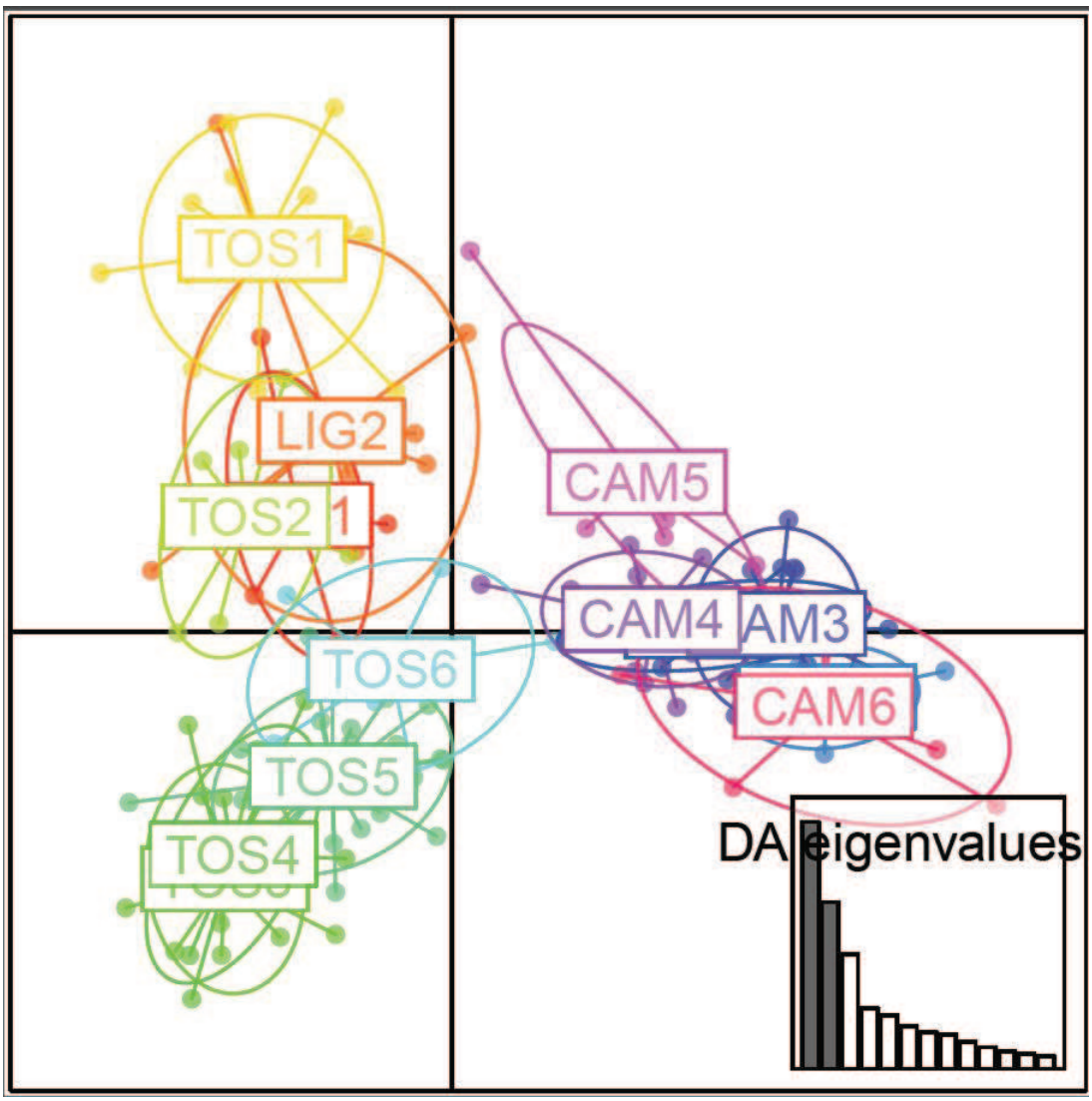

Figure 4.3 Plots of the first two axes obtained in the Discriminant Analysis of Principal Components using microsatellite dataset. Dots represent individuals.

The AMOVA test (Table 4.4) conducted among populations showed similar results independently or the grouping considered (see Materials for grouping). Major variability is explained within populations (between 89.06% and 89.93% depending of the grouping). Moreover, the variance among groups was always significant even if the percentage of variation is very low (between 4.43 and 6.09% depending of the grouping).

Source of variation	*d.f.*	*Sum of squares*	*Variance component*	*Percentage of variation*
Among groups	2	32.985	0.15	6.09***
Among sample within groups	11	49.320	0.12	4.86***
Within samples	252	563.496	2.24	89.06***
Total	265	645.8	2.51	

*** P<=0.001

Table 4.3 Analysis of molecular variance (AMOVA) among *Corallium rubrum* populations using microsatellite data set. Red coral populations were grouped according to their geographical distribution (three groups: Liguria, Toscana and Campania populations).

4.2 *Mitochondrial Markers*

4.2.1 Sequence variation

Across 101 individuals amplified for the putative control region of the mitochondrial DNA (Table 4.5) the fragments was 290 bp in length. MtC in *Corallium rubrum* correspond to positions 18541-18913 of the mitochondrial genome sequence of *Paracorallium japonicum* (GenBank accession number AB595189, Uda et al. 2011) and to positions 18538-18702 and 18815-18969 of the mitochondrial genome sequence of *Corallium konojoi* (GenBank accession number AB595190, Uda et al. 2011). The sequence of the MtC in *C. rubrum* had a percentage of identity of 99% and 92% with those of *P. japonicum* and *C. konojoi*, respectively. The alignment of all individual sequences showed the presence of five nucleotide substitution which defines four different haplotypes (Table 4.5). The sequence alignment showed the presence of the three haplotypes (Hap1, Hap2, Hap3) previously recorded by Costantini et al. (2011). Haplotype 1 and 2 were present in almost all populations and were the most abundant (36% and 37% respectively). Haplotype 4 is only present in a population of Tuscany (TOS1), where it was the most abundant. Nucleotide diversity is slow, but this depends of the low number of variable position (only five). So, low and comparable value of haplotype and nucleotide diversity of MtC were found among populations, with mean values of 0.38 (± 0.14) and 0.0033 (± 0.0001) respectively.

Nucleotide position						LIG1	LIG2	TOS1	TOS2	TOS3	TOS4	TOS5	TOS6	CAM1	CAM2	CAM3	CAM4	CAM5	CAM6
	28	82	129	274	275	(6)	(9)	(13)	(6)	(11)	(10)	(23)	(6)	(7)	(6)	(9)	(15)	(6)	(6)
N.amp						6	6	13	6	2	5	15	4	7	5	9	13	6	4
Hap 1	C	A	C	A	G	4	4	0	5	2	4	14	0	2	0	0	1	0	0
Hap 2	T	G	.	.	.	2	2	0	0	0	1	1	1	2	4	7	8	5	4
Hap 3	T	G	.	C	T	0	0	5	1	0	0	0	3	3	1	2	4	1	0
Hap 4	T	G	T	G	T	0	0	8	0	0	0	0	0	0	0	0	0	0	0
h						2	2	2	2	1	2	2	2	3	2	2	3	2	1
H						0.53±0.17	0.53±0.17	0.51±0.08	0.33±0.21	0	0.41±0.20	0.13±0.11	0.50±0.26	0.76±0.11	0.40±0.21	0.3±0.1	0.56±0.11	0.33±0.21	0
π						0.007±0.002	0.007± 0	0.005± 0	0.002±0.001	0	0.005±0.003	0.002±0.001	0.003±0.001	0.006±0.001	0.002±0.001	0.002±0.002	0.004±0.001	0.002±0.001	0

Table 4.4 Sequence differences, distribution and genetic diversity of the four MtC haplotypes found in *Corallium rubrum* populations: dots indicate identical bases; H, total number of haplotypes; *h*, haplotype diversity (*h*, Nei 1987); π, nucleotide diversity (π, Nei 1987).

4.2.2 Genetic differentiation between populations

The MtC marker revealed low values of genetic differentiation, compared to microsatellites. Pairwise F_{ST} estimated ranged from -0.01 (CAM1 vs LIG1 and LIG2) to 1 (CAM6 vs TOS3) (Table 4.5).

.

	LIG1	LIG2	TOS1	TOS2	TOS3	TOS4	TOS5	TOS6	CAM1	CAM2	CAM3	CAM4	CAM5	CAM6
LIG1														
LIG2	-0.2													
TOS1	**0.45**	**0.45**												
TOS2	0.03	0.03	**0.65**											
TOS3	-0.09	-0.09	**0.66**	-0.3										
TOS4	-0.17	-0.17	**0.55**	-0.12	-0.29									
TOS5	0.15	0.15	**0.73**	-0.07	-0.33	-0.04								
TOS6	0.24	0.24	0.28	0.63	0.71	0.42	**0.73**							
CAM1	-0.01	-0.01	0.31	0.29	0.29	0.12	**0.47**	-0.1						
CAM2	0.4	0.4	0.36	**0.78**	0.83	0.59	**0.82**	0.31	0.23					
CAM3	0.45	0.46	**0.39**	**0.77**	0.81	**0.62**	**0.81**	0.33	0.28	-0.18				
CAM4	0.3	0.3	**0.31**	**0.61**	0.63	**0.46**	**0.69**	0.05	0.08	-0.08	-0.04			
CAM5	0.45	0.45	**0.39**	**0.8**	0.85	0.63	**0.83**	0.39	0.29	-0.22	-0.15	-0.03		
CAM6	0.53	0.53	**0.47**	**0.89**	1	0.72	**0.89**	0.67	0.42	-0.05	-0.01	0.11	-0.08	0

Table 4.5 Pairwise multilocus estimates of MtC between all *Corallium rubrum* populations. Bold values are significantly different from zero (P <=0.01).

The Multidimensional Scaling (MDS) representation (Fig.4.4), utilized with the intent to have a visual assessment of between-population differentiation, showed three groups of samples. First group includes Liguria (LIG1, LIG2) and several Toscana populations (TOS2, TOS3, TOS4, TOS5), second group with TOS1 and TOS6 (probably in according with the presence of Hap 4 only in TOS1, and the low number of haplotypes in TOS6) and third group with all Campania populations.

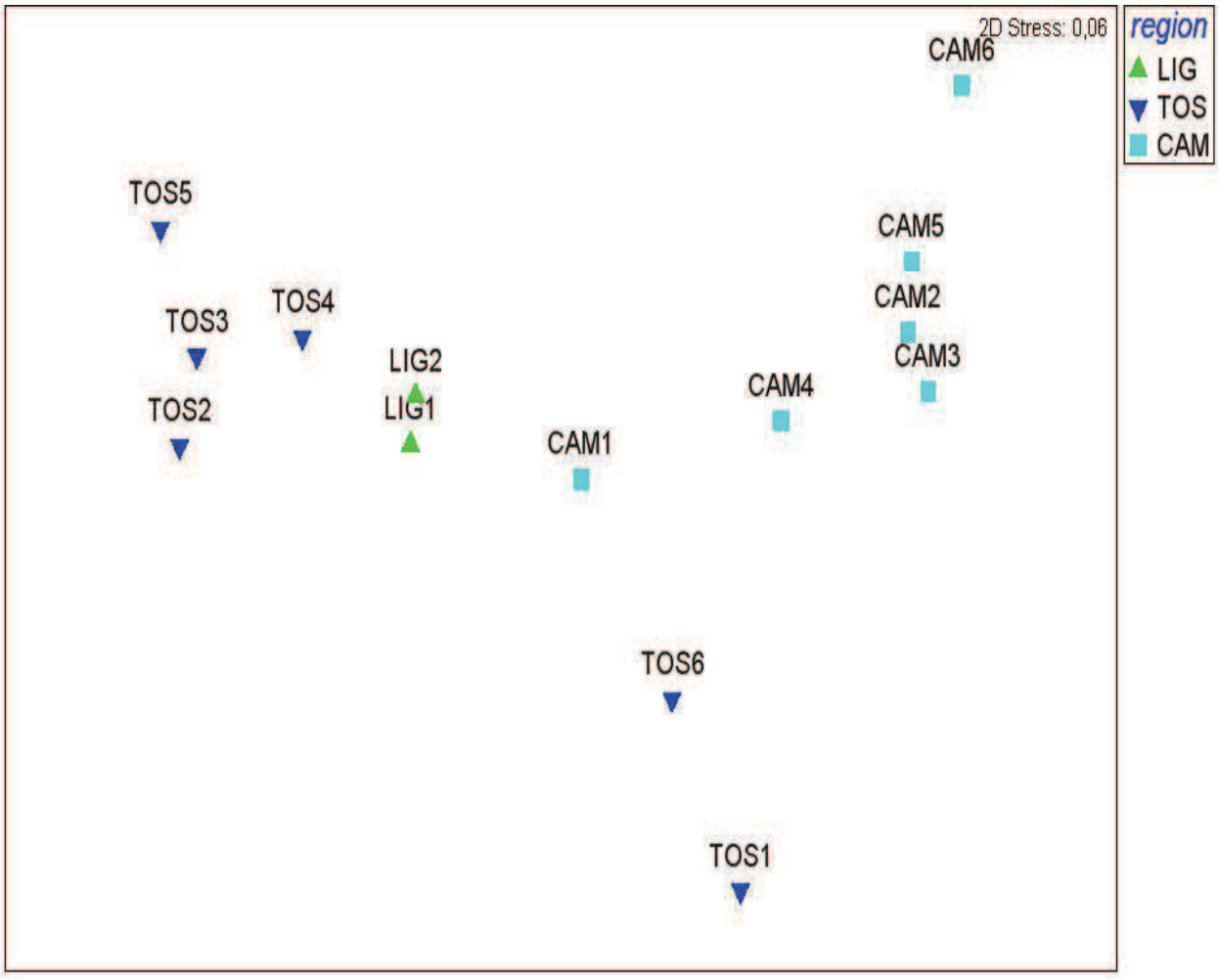

Figure 4.4 Multidimensional scaling of the pairwise F_{ST} among populations. Green triangle represents Liguria populations, blue triangle Toscana populations and blue square Campania populations.

Even in this case, as in microsatellite dataset, the Mantel test was significant, so there is correlation between genetic and geographical distances (Fig.4.5).

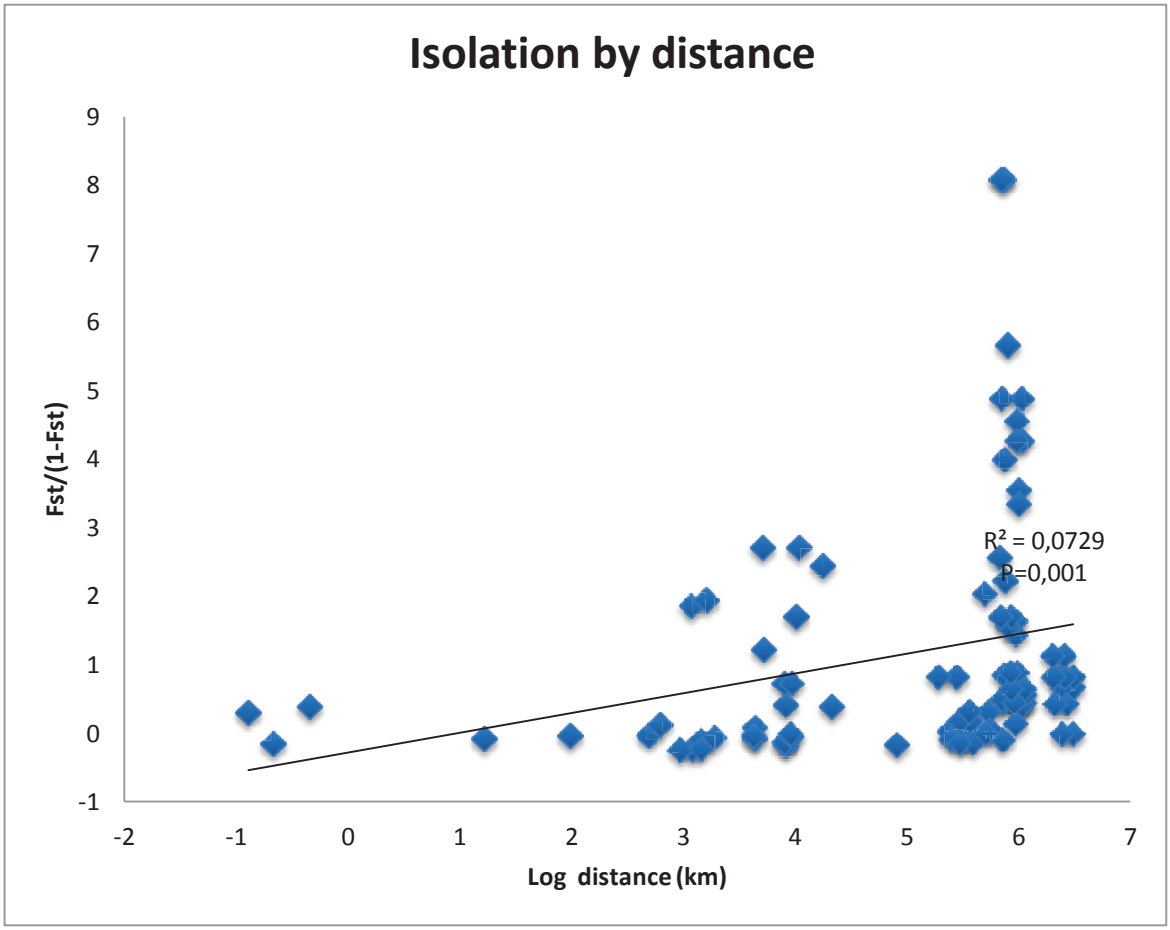

Figure 4.5 Relationship between genetic differentiation estimates and the logarithm of geographical distance among *Corallium rubrum* populations using MtC dataset.

The AMOVA test (Table 4.7) conducted among populations even in this case showed major genetic variation within population (47.35%; P<0.001), while about 25% of variations is observed among groups (three geographical areas: Liguria, Toscana and Campania) and among populations within groups. The percentage of variation among group increase significantly grouping populations according the F_{ST} values and MDS results (53.45% , P = 0, data not shown).

Source of variation	*d.f.*	*Sum of squares*	*Variance component*	*Percentage of variation*
Among group	2	25.231	0.312	25.68*
Among populations within group	11	31.215	0.328	26.96**
Within populations	87	50.089	0.57	47.35**
Total	100	106.5	1.21	

*= P<0.05

**=P<0.001

Table 4.6 Analysis of molecular variance (AMOVA) among *Corallium rubrum* populations using MtC marker. Red coral populations were grouped according to their geographical area.

5. DISCUSSION

This research provides new data on genetic variability and structuring of harvested red coral populations dwelling in the depth range from 60 to 120 meters. For this purpose two markers with different evolutionary rates were used. Indeed, both markers gave comparable results. The results showed evidence of:

1) high inbreeding rate within *Corallium rubrum* populations;

2) a genetic structure fitting isolation by distance model among all populations;

3) a higher genetic similarity between Liguria and Toscana populations compared to Campania populations.

1.1 *Genetic variability*

The multilocus polymorphism found in *Corallium rubrum* microsatellite genotypes resulted comparable with that observed in samples collected from 50 meters depth by Costantini et al. (2011), and lower compared to shallow-water populations, confirming a reduction of genetic variability below 50 meter depth (Costantini et al. 2011). Conversely, the microsatellite Hs values were much higher than those obtained for the allozymes (mean Hs=0.088; Abbiati et al. 1993). This is an expected result given the higher rates of polymorphism of microsatellites relative to allozymes. Moreover, the obtained values of Hs still fall within the range of those previously reported for other cnidarians species (e.g. *Acropora palmata*, Hs from 0.58 to 0.85) (Baums et al. 2005).

Strong deviations from Hardy-Weinberg equilibrium (emphasized by high positive F_{IS} estimates) were detected for all populations and at all microsatellite loci. Heterozygote deficit was already found in previous studies on *C. rubrum* (Abbiati et al. 1993; Costantini *et al*. 2007a, b; Ledoux *et al*. 2010a, b; Costantini *et al*. 2011), and confirms the occurrence in this species of processes affecting intra-population gene flow, either due to the mixing of differentiated gene pools (Wahlund effect), or to high levels of consanguineous mating (inbreeding). We cannot reject as well the possible occurrence of technical problems such as failures of amplification or null alleles. As already observed in Costantini *et al*. (2007a), in shallow-water red coral populations, deficiencies of heterozygotes have been described in other shallow-

water Anthozoa populations, and it has been hypothesized that it might be a consequence of localized recruitment (restricted dispersal of gametes or larvae) and inbreeding (Magalon et al. 2005). These results are in agreement with the distribution of mesophotic red coral populations, which at that depth are found in scattered rocky boulders separated by sedimentary bottom or patchily distributed on vertical rocky cliffs, suggesting a low capacity of the larvae to reach distant suitable settlement areas. Nevertheless, despite the different habitat features found between Liguria (red coral mainly found in overhangs), Toscana (mainly found on vertical rocky cliffs) and Campania (red coral found in scattered rocky boulders on a sedimentary bottom), no differences in genetic variability were observed between the red coral populations of these three areas (with similar and comparable values of the genetic parameters of variability). All together, these results suggest that habitat features do not greatly influence the levels of genetic diversity, which seems to be much more influenced by intrinsic characteristic of the species, namely larval behavior (Costantini et al. 2007b).

Mitochondrial markers showed low genetic variability among populations, compared to microsatellite markers, probably due to the fact that in Anthozoa mitochondrial DNA has extremely low mutation rate and it is very conserved (Shearer et al. 2002; Costantini et al. 2003; Calderon et al. 2006; Hellberg 2006). Nevertheless, the putative control region of the mitochondrial DNA, the MtC, showed a higher variability compared to other mitochondrial genes (e.g. COI, 16S and MSH Calderon *et al*. 2006; Costantini *et al*. 2003; Costantini et al. 2010, 2011). MtC has been successfully used to investigate levels of genetic structuring among populations of two scleractinian corals: *Desmophyllum dianthus* and *Seriatopora hystri*x (Miller *et al*. 2011; Van Oppen *et al*. 2011). Here, four haplotypes were observed: three of them were previously described on red coral mesophotic populations (Costantini et al. 2012) and a fourth haplotype never observed before. The first three were widespread along all samples, while the fourth (Hap4) was observed only in a single Tuscany population (TOS1), were it was the most common haplotype, found in eight out of 13 individuals sequenced. TOS1 population was collected around Giglio Island, which is the more distant sample site with respect to the rest of Toscana sites. Furthermore TOS1 site is the sampling site closest to the coastline and is also one of the deepest. All these differences, together with the

presence of the private haplotype, suggest that in this Island further research is needed to understand the particular genetic pattern of the red coral population. Not only genetic data, but also information on abundance, distribution and age of the colonies in this site could be useful.

The mitochondrial marker MtC shows a low number of haplotypes (four haplotypes on 101 individual sequenced), compared to those shown by scleractinian corals (26 haplotypes on 162 individual sequenced; Miller et al. 2011). However also in those cases, haplotypes showed little differences between them. The low nucleotide diversity observed was comparable to that found by Miller *et al*. (2011) in *Desmophyllum dianthus*. However, this level of variation is much higher than that found in other mitochondrial markers investigated in red coral populations, and it allowed detecting differences between the populations studied.

.

1.2 *Genetic differentiation*

While having different mutation rates, both molecular markers, showed comparable results, and both allowed detecting the genetic structuring among mesophotic red coral populations. In fact both types of markers evidenced a higher similarity between Liguria and Toscana populations (hereafter North Tyrrhenian populations) compared to Campania populations (hereafter Southern Tyrrhenian populations). This genetic structure could be explained by isolation by distance pattern among populations, although the two markers showed different levels of correlation. This differentiation pattern could be explained by the failure of amplification of loci MIC22 and MIC23 in Campania populations, opposed to amplification success obtained with Liguria and Toscana populations. This suggests that some mutations could have affected the flanking regions of these loci only in Campania populations making impossible the union of the specific primers and hence the amplification. Similar results were found by Costantini et al. (2012) on other mesophotic red coral populations, and by Aurelle et al. (2011) analyzing shallow-water populations: these authors did not get any amplification of the loci MIC22 and MIC23 in the Adriatic and North African samples, whereas they normally amplified in all samples from the north-western Mediterranean Sea. These

results suggest the occurrence of major divergence between the northern and southern areas of the Tyrrhenian Sea (see also below).

The genetic differentiation observed between North and South Tyrrhenian populations could be related to the geographic distance, as suggested by the significant isolation by distance pattern observed. However we cannot reject the occurrence of a barrier to gene flow probably related to the geomorphologic and hydrodynamic characteristics of the studied areas (Costantini et al. 2012, Ledoux et al. 2010a, b). Tuscany and Campania populations are located in different biogeographical areas according to species distribution, one including the Northern Tyrrhenian Sea between Corsica, Sardinia and Tuscany, and the other including the southern Tyrrhenian Sea (Bianchi & Morri 2000; Bianchi 2007) The currents in this area flow northward in winter, connecting southern and northern Tyrrhenian, whereas in summer, when red coral larvae are released (Santangelo et al. 2003), this Tyrrhenian circulation has a more fragmented pattern with some local gyres (Astraldi & Gasparini 1994) that could act as a barrier to gene flow.

The lower correlation value between genetic and geographic distance using MtC compared to that obtained with microsatellites suggests that the isolation by distance pattern is weaker for the marker with the lowest mutation rate, and that some historical events might have played a role in the differentiation found between populations located in northern and southern Thyrrenian.

Events occurred during the the so-called 'Messinian salinity crisis' in the late Miocen (5.6 mya), when large parts of the Mediterranean Sea was desiccated, probably transforming it into a series of large evaporitic lakes isolated from each other also in our study area (Bianchi & Morri 2000). These past history events need to be taken in account in order to explain the observed pattern. In fact, the present day deep-sea fauna was established in the Mediterranean at least as far back as the glacial Pleistocene (as evidenced by the fossil record of molluscs and scleractinian corals) (Bouchet & Taviani 1992).

At smaller spatial scales (within areas) the two molecular markers gave different results, probably due to the low polymorphism of MtC found within the populations. This gives further evidence that this marker is not informative for corals at these geographical scales of less than tens of kilometers. In fact, Miller et al. (2011) did not evidence a genetic structuring in *Desmophyllum dianthus* populations

at similar spatial scales. The low level of genetic differentiation observed with shared haplotypes among all samples and low levels of nucleotide diversity, could suggest historical links within regions. As suggested by Aurelle et al. (2010), a scenario of long-term divergence does not seem probable for red coral at this spatial scale and so, although populations within regions have to be considered as separated management units (see microsatellites results below) they should be viewed as unique significant evolutionary units (sensu Moritz 2002).

Biological, ecological and/or abiotic factors which act at small spatial scale could determine the genetic structuring observed within areas using microsatellite markers. Red coral larvae have a negative photo and geo-taxis (Weinberg 1979) and a low dispersal capability (Vighi 1972) that could influence the larval retention at local scale. Larval retention processes could be caused by small-scale habitat heterogeneity. In Campania scattered rocky boulders were separated by flat sedimentary bottom, where red coral was not able to settle. In Toscana and Liguria overhangs and fissures could enhance a larval retention and protect colonies from smothering due to the abundant sedimentation rates in these areas.

6. CONCLUSION

While shallow water (less then 50m depth) red coral populations have been over harvested in the past, mesophotic populations are nowadays actively and intensively commercially harvested.

The results of the present study provide information that could significantly enhance the management of mesophotic populations of *C. rubrum*. The high inbreeding rate within populations, together with the genetic structuring between nearby samples implies that mesophotic red coral populations are mainly self recruiting. This could be important for the resilience of these populations at long term scale since their survival has to rely on local recruitment. Moreover, due to its restricted dispersal abilities and the isolation by distance pattern observed, recolonization from different geographical areas probably occurred following a gradual process. Therefore, management of red coral harvesting in the mesophotic habitats should be defined at a regional (or sub regional) level as already observed for shallow water populations. The first step should be the development of a marine reserve network based on the restricted dispersal abilities of the species (Palumbi 2004) followed by the creation of deep-sea marine protected areas. Marine reserves are expected to reduce harvesting and other human pressures, supporting the adaptive capacity of long-lived species to survive disturbances. In particular, establishment of marine reserves can increase the size of populations of commercially exploited, long-lived, sessile invertebrates (Gorrabou & Harmelin 2002; Davies et al. 2007). Previous studies showed an increase in the maximum size of shallow red coral colonies and a shift toward more mature populations in marine reserves (Linares et al. 2010; Linares et al. 2012).

The knowledge of the principal ecological aspects of Mediterranean mesophotic red coral populations, are just beginning, so in the future could be necessary to map other Mediterranean deep red coral populations and using a multidisciplinary approach to study population genetic , structure and dynamics of these precious populations.

REFERENCES

Abbiati M, Buffoni G, Caforio G, Di Cola G, Santangelo G (1992) Harvesting, predation and competition effects on a red coral population. Neth J Sea Res 30:219-228.

Abbiati M, Santangelo G, Novelli S (1993) Genetic variation within and between two Tyrrenian population of the alcyonarian *Corallium rubrum*. Mar Ecol Prog Ser 95:245-250.

AiroldiL(1998)Roles of disturbance, sediment stress,and substratum retention on spatial dominance in algal turf. Ecol. 8:2759-2770.

Airoldi L, Beck M (2007) Loss, status and trends for coastal marine habitats of europe. Ocean Mar Biol, 44:345-405.

Armstrong RA, Singh H, Torres J, Nemeth RS, Can A, Roman C, Eustice R, Riggs L, Garcia-Moliner G (2006) Characterizing the deep insular shelf coral reef habitat of the Hind Bank Marine Conservation District (US Virgin Islands) using the Seabed autonomous underwater vehicle. Cont Shelf Res 26:194–205.

Arntz WE, Gili JM, Reise K (1999) Unjustifiably ignored: reflections on the role of benthos in marine ecosystems. In: Gray JS et al (eds) Biochemical cycling and sediment ecology. Kluwer, Netherlands, pp 105–124.

Ascione C (1993) The art of corals: myth, history and manufacture from ancient times to the present. In: Cicogna F, Cattaneo-Vietti R (eds) Red coral in the Mediterranean Sea, art, history and science. Min Ris Agric, Alim e Forest , Rome, p 25–36.

Astraldi M Gasparini GP (1994) The seasonal characteristics of the circulation in the Tyrrhenian Sea. In: La Violette, P.E. (Ed.). Seasonal and interannual variability of the Western Mediterranean Sea. Coast and Estuar Stud 46:115–134.

Aurelle D, Ledoux JB, Rocher C, Borsa P, Chenuil A, Feral JP (2011) Phylogeography of the red coral (*Corallium rubrum*): interferences on the evolutionary history of a temperate gorgonian. Genetica 38:227-232.

Avise JC (2004) Molecular Markers, Natural History, and evolution (second edition).

Baco AR, Clark AM, Shank TM (2006) Six microsatellite loci from the deep-sea coral *Corallium lauuense* (Octocorallia: Coralliidae) from the island and seamounts of the Hawaiian archipelago. Mol Ecol Notes 6:147-149.

Balata D, Piazzi L, Cecchi E, Cinelli F (2005) "Variability of Mediterranean coralligenous assemblages subject to local variation in sediment deposition. Mar Environ Res 60:403-21.

Ballasteros E (2006) Mediterranean coralligenous assemblages: a synthesis of present knowledge. Ocean Mar Biol 44:123-195.

Baums IB, Miller MW, Hellberg ME (2005) Regionally isolated populations of an imperiled Caribbean coral, Acropora palmata. Molecular Ecology, 14: 1377–1390.

Beaumont MA, Rannala B (2004) The Bayesian revolution in genetics. Nat Rev Genet, 5:251–261.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (2004*)* GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier, France.

Bianchi CN, Morri C (2000) Marine biodiversity of the Mediterranean Sea: situation, problems and prospects for future research. Marine Pollution Bulletin, 40:367-376.

Bianchi CN (2007) Biodiversity issues for the forthcoming tropical Mediterranean Sea. Hydrobiologia, 580:7-21.

Bo M, Bavestrello G, Canese S, et al. (2009) Characteristics of a black coral meadow in the twilight zone of the central Mediterranean Sea. Mar Ecol Prog Ser, 397:53-61.

Bo M, Bertolino M, Borghini M et al. (2011a) Characteristics of the Mesophotic Megabenthic Assemblages of the Vercelli Seamount (North Tyrrhenian Sea). PLoS ONE, 6: e16357. doi:10.1371/journal.pone.0016357.

Bo M, Bavestrello G, Canese S, Giusti M, Angiolillo M, Cerrano C, Salvati E, Greco S (2011b) Coral assemblage off the Calabrian Coast (South Italy) with new observations on living colonies of Antipathes dichotoma. Ita Jour of Zool, 78:231-242.

Bo M, Baker AC, Gaino E, Wirshing HH, Scoccia F, Bavestrello G, (2011c) First description of algal mutualistic endosymbiosis in a black coral (Anthozoa: Antipatharia). Mar Ecol Prog Ser, 435:1-11.

Bongaerts P, Ridgeway T, Sampayo EM, Hoegh-Guldberg O (2010) Assessing the "deep reef refugia" hypothesis: focus on Caribbean reefs. Coral Reefs 29:309-327.

Bossart JL, Prowell DP (1998) Genetic estimates of population structure and gene flow: limitations, lessons and new directions. Trends Ecol Evol 13:202–206.

Bouchet P, Taviani M (1992) The Mediterranean deep-sea fauna: pseudopopulations of Atlantic species? Deep Sea Research Part A. Oceanographic Research Papers 39:169-184.

Bramanti L, Magagnini G, De Maio L, Santangelo G (2005) Recruitment, early survival and growth of the Mediterranean red coral *Corallium rubrum* (L 1758), a 4-year study. J Exp Mar Biol Ecol, 314:69–78.

Bramanti L, Iannelli M, Santangelo G (2009) Mathematical modelling for conservation and management of gorgonian corals: Young and olds, could they coexist? Ecol Mod, doi:10.1016/j.ecolmodel.2009/01.031.

Brokovich E, Einbinder S, Kark S, Shashar N, Kiflawi M (2007) A deep nursery for juveniles of the zebra angelfish *Genicanthus caudovittatus*. Env Biol Fishes 80: 1–6.

Calderon I, Garrabou J, Aurelle D (2006) Evaluation of the utility of COI and ITS markers as tools for population genetic studies of temperate gorgonians. Journal of Exp Mar Biol and Ecol, 336:184–197.

Cerrano C, Bavestrello G, Bianchi CN, et al. (2000) A catastrophic mass-mortality episode of gorgonians and other organisms in the Ligurian Sea (NW Mediterranean). Ecol Let 3:284–293.

Cerrano C, Danovaro R, Gambi C, Pusceddu A, Riva A, Schiaparelli S. (2010) Gold coral (Savalia savaglia) and gorgonian forests enhance benthic biodiversity and ecosystem functioning in the mesophotic zone. Biod and Cons, 19:153–167.

Chintiroglou H, Dounas C, Koukouras A (1989) The presence of *Corallium rubrum* (Linnaeus, 1758) in the eastern Mediterranean Sea. Mitt Zool Mus Berlin 65:145–149.

Clarke K.R., 1993. Non parametric multivariate analyses of changes in community structure. Aus Jour of Ecol 18:117- 143.

Cloern JE (1982) Does the benthos control phytoplankton biomass in south San Francisco Bay? Mar Ecol Prog Ser 9:191–202.

Corriero G, Abbiati M, Santangelo G (1997) The sponge complex inhabiting a Mediterranean red coral population. P.S.Z.N. Mar Eco, 18(2):147-155.

Costantini F, Tinti F, Abbiati M (2003) Sistematica molecolare e filogenesi di *Corallim rubrum*. Bio Mar Med, 10:73-75.

Costantini F., Abbiati M. (2006) Development of microsatellite markers for the Mediterranean gorgonian coral *Corallium rubrum*. Mol Ecol Notes 6:521-523.

Costantini F, Fauvelot C, Abbiati M (2007a) Genetic structuring of the temperate gorgonian coral (*Corallium rubrum*) across the western Mediterranean Sea revealed by microsatellites and nuclear sequences. Mol Ecol 16:5168–5182.

Costantini F, Fauvelot C, Abbiati M (2007b) Fine-scale genetic structuring in *Corallium rubrum:* evidence of inbreeding and limited effects of larval dispersal. Mar Ecol Prog Ser 340:100–119.

Costantini F, Taviani M, Remia A, Pintus E, Schembri PJ, Abbiati M (2010) Deep-water *Corallium rubrum* (L., 1758) from the Mediterranean Sea: preliminary genetic characterization. Mar Ecol 31:261–269.

Costantini F, Rossi S, Pintus E, Cerrano C, Gili JM, Abbiati M (2011) Low connectivity and declining genetic variability along a depth gradient in *Corallium rubrum* populations. Coral Reef 30:991-1003.

Costantini F, Carlesi L, Abbiati M (2012) Conservation genetics of the precious coral *Corallium rubrum*: spatial structuring in mesophotic populations, PLOS One, submitted

Davies N, Villablanca FX, Roderick GK (1999) Determining the source of individuals: multilocus genotyping in non equilibrium population genetics. Trends Ecol Evol 14:17–21.

Davies AJ, Roberts JM, Hall-Spencer J (2007) Preserving deep-sea natural heritage: emerging issues in offshore conservation and management. Biol Cons 138:299-312.

Excoffier, L. G. Laval, and S. Schneider (2005) Arlequin ver. 3.5: An integrated software package for population genetics data analysis. Evol Bioinf Online 1:47- 50.

Excoffier L, Lischer HEL (2010) ARLEQUIN suite version 3.5: a new series of programs to perform population genetics analysis under Linux and Windows. Mol Ecol Res 10:564–567.

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. Genetics 164:1567–1587.

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes 7:574–578.

Fre´chette M, Bourget E (1985) Energy flow between the pelagic and benthic zones: factors controlling particulate organic matter available to an intertidal mussel bed. Can J Fish Aquat Sci 42:1158–1165.

Fre´chette M, Butman CA, Geyer WR (1989) The importance of boundary-layer flow in supplying phytoplankton to the benthic suspension feeder, Mytilus edulis L. Limnol Ocean 34:19–36.

Freiwald A, Beuck L, Ruggeberg A, Taviani M, Hebbeln D and R / V Meteor M70-1 Participants (2009) The white coral community in the Central Mediterranean Sea revealed by ROV surveys. Ocean, 22:58–74.

Garrabou J, Perez T, Sartoretto S, Harmelin JG (2001) Mass mortality event in red coral (*Corallium rubrum*, Cnidaria, Anthozoa, Octocorallia) population in the Provence region (France, NW Mediterranean). Mar Ecol Prog Ser 217:263–272.

Garrabou J, Harmelin G (2002) A 20 year study on life-history traits of a harvested long lived temperate coral in the NW Mediterranean: insights into conservation and management needs. J An Ecol 71:967-978.

GFCM - General Fisheries Commission for the Mediterranean (2011) Second transversal workshop on Red Coral. Ajaccio, Corsica (France), 5-7 October 2011.

Giannini F, Gili JM, Santangelo G (2003) Relationship between the spatial distribution of red coral *Corallium rubrum* and coexisting suspension feeders at Medas Islands Marine Protected Area (Spain). Ital J Zol 70:233-239.

Gili JM, Coma R (1998) Benthic suspension feeders in marine food webs. Trends Ecol Evol 13:316–321

Gori A, Rossi S, Berganzo E, Pretus JL, Dale MRT, Gili JM (2011) Spatial distribution patterns of the gorgonians Eunicella singularis, Paramuricea clavata and Leptogorgia sarmentosa (Cape of Creus, Northwestern Mediterranean Sea). Marine Biology, 158:143–158.

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices version 2.9.3. Available from. http://www.unil.ch/izea/softwares/fstat.html (updated from Goudet 1995).

Grant WS, Bowen BW (1998) Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. Journal of Heredity 1998;89:415-426.

Hall TA (1999) bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/97/ NT. Nucleic Acids Symp Series, 41:95–98.

Harmelin JG (1984) Biologie du corail rouge. Paramètres de populations, croissance et mortalité naturelle. Etat des connaissances en France. FAO Fish Rep 306:99–103.

Hedrick P.W. (2009) *Genetics of populations* fourth edition Jones and Barlett Publishers.

Hellberg ME (2006) No variation and low synonymous substitution rates in coral mtDNA despite high nuclear variation. BMC Evol Bio, 6-24.

Hinderstein LM, Marr JCA, Martinez FA, et al. (2010) Theme section on "Mesophotic Coral Ecosystems: Characterization, Ecology, and Management". Coral Reefs 29:247–251.

Hughes AR, Inouye BD, Johnson MTJ, Underwood N, Vellend M (2008) Ecological consequences of genetic diversity. Ecol Lett 11:609–623

Jackson J (1986) Models of dispersal of clonal benthic invertebrates: consequences for species distributions and genetic structure of local population. Bull Mar Sci 39:588-606.

Jombart T (2008) Adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405.

Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genetics 11:94.

Karlson RH (1986) Disturbance, colonial fragmentation, and size –dependent life history variation in two coral reef cnidarians. Mar. Ecol. Prog. Ser.

Kendall M, Stewart A (1977) *The Advanced Theory of Statistics*, vol. 1. Mac NY.

Khang SE, Garcia-Saís JR, Spalding HL, Brokovich E, Wagner D, Weil E, Hinderstein L, Toonen RJ (2010) Community ecology of mesophotic coral reef ecosystems. *Coral Reefs* 29:255-275.

Kimmerer WJ, Gartside E, Orsi JJ (1994) Predation by an introduced clam as the likely cause of substantial declines in zooplankton of San Francisco Bay. Mar Ecol Prog Ser 113:81–93.

Laborel J, Vachelet J (1961) Etude de Peuplement d'une grotte sous-marine du golfe de Marseille. Bull Ist Ocean Monaco 1120:1-20.

Ledoux JB, Mokhtar-Jamaï K, Roby C, Féral JP, Garrabou J, Aurelle D (2010a) Genetic survey of shallow populations of the Mediterranean red coral *Corallium rubrum* (Linnaeus, 1758): new insights into evolutionary processes shaping nuclear diversity and implications for conservation. Mol Ecol 19:675–690.

Ledoux JB, Garrabou J, Bianchimani O, Drap P, Féral JP, Aurelle D (2010b) Fine-scale genetic structure and inferences on population biology in the threatened Mediterranean red coral, *Corallium rubrum*. Mol Ecol 19:4204–4216.

Lesser MP, Slattery M, Leichter JJ (2009) Ecology of mesophotic coral reefs. J Exp Mar Biol Ecol 375:1–8.

Librado P., Rozas J. (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data Bioinf 25:1451-1452.

Linares C, Coma R, Diaz D, Zabala M, Hereu B, Dantart L (2005) Immediate and delayed effects of a mass mortality event on gorgonian population dynamics and benthic community structure in the NW Mediterranean Sea. Mar Ecol Prog Ser, 305:127-137.

Linares C, Bianchimani O, Torrents O et al. (2010) Marine Protected Areas and the conservation of long-lived marine invertebrates: the Mediterranean red coral. Marine Ecol Prog Ser 402:69–79.

Linares C, Garrabou J, Hereu B, et al (2012) Assessing the Effectiveness of Marine Reserves on Unsustainably Harvested Long-Lived Sessile Invertebrates. Cons Biol 26:88–96.

Liverino B (1983) Il corallo. Li Causi (Ed), Torre del Greco.

Luikart G, England PR (1999) Statistical analysis of microsatellite DNA data. Trends Ecol Evol 14:253–256.

Magalon H, Adjeroud M, Veuille M (2005) Patterns of genetic variation do not correlate with geographical distance in the reef-building coral *Pocillopora meandrina* in the South Pacific. Mol Ecol 14:1861–1868.

Manel S, Schwartz MK, Luikart G, Taberlet P (2003) Landscape genetics: combining landscape ecology and population genetics. Trends Ecol Evol 18:189–197.

Manel S, Gaggiotti OE., Waples RS (2005) Assignment methods: matching biological questions with appropriate techniques. Trends Ecol Evol 20:136–142

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research* 27: 209–220.

Marchetti R (1965) Ricerche sul corallo rosso della costa ligure e toscana. Distribuzione geographica. Rendiconti dell'Istituto Lombardo, Acc Scien Lett 99:255–278.

Marsili LF (1725) Histoire physique de la mer. De'Pens, Amsterdam, Netherlands pp.570.

Mateu G, Traveria A, Fontarnau P, Masso C (1986) Biodiagenesis mineralogica del *Corallium rubrum* (L.,1758). Boll Inst Esp Oceanogr 3:1-12.

McFadden CS, France SC, Sanchez JA, Alderslade P (2006) A molecular phylogenetic analysis of the octocorallia (Cnidaria:Anthozoa) based on mitochondrial protein-coding sequences. Mol Phyl and Evol, 41:513-527.

Miller KJ, Rowden AA, Williams A, Häussermann V (2011) Out of their depth? Isolated deep populations of the cosmopolitan coral *Desmophyllum dianthus* may be highly vulnerable to environmental change. *PLoS ONE*, e19004. doi:10.1371/journal.pone.0019004.

Morato T, Watson R, Pitcher TJ, Pauly D (2006) Fishing down the deep. Fish Fisher 7:24–34.

Moritz C, Lavery S (1996) Molecular ecology: Contributions from molecular genetics to population ecology. Pp. 433-450 in Frontiers of Population Ecology (eds. by R.B. Floyd, A.W. Sheppard and P.J. De Barro). CSIRO, Melbourne.

Moritz C (2002) Strategies to protect biological diversity and the evolutionary processes that sustain it. Syst Biol 51:238–254.

Nei M (1987) Molecular Evolutionary Genetics. Columbia University Press, New York.

Newman D, Pilson D(1997) Increased probability of extinction due to decreased genetic effective population size: experimental populations of *Clarkia pulchella*. Evol. 51:354-362.

Officer CB, Smayda TJ, Mann R (1982) Benthic filter feeding: a natural eutrophication control. Mar Ecol Prog Ser 9:203–210.

Palumbi SR (2004) Marine reserves and ocean neighborhoods: the spatial scale of marine populations and their management. An Rev Envir Res 29:31–68.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Not, 6, 288–295.

Perez T, Garrabou J, Sartoretto S, Harmelin JG, Francour P, Vacelet J (2000) Mortalité massive d'invertébrés marins: un événement sans précédent en Méditerranée nord-occidentale. Comptes Rendus Académie des Sciences Série III, Life Sciences, 323:853-865.

Pyle RL (2000) assessing undiscovered fish biodiversity of deep coral reefs using advanced self-contained diving technology. Mar Techno J 34:82-91.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics, 155:945–959.

Raymond M, Rousset F (1995) An exact test for population differentiation. Evolution 49:1280–1283.

Riisga˚ rd HU, Jensen AS, Ju¨ rgensen C (1998) Hydrography, nearbottom currents, and grazing impact of the filter-feeding ascidian Ciona intestinalis in a Danish fjord. Ophelia 49:1–16.

Rooney J, Donham E, Montgomery A, Spalding H, Parrish F, Boland R, Fenner D, Gove J, Vetter O (2010) Mesophotic coral ecosystems in the Hawaiian Archipelago. Coral Reefs 29:361–367.

Rossi S, Tsounis G, Orejas C, Padron T, Gili JM, Bramanti L, Teixido N, Gutt J (2008) Survey of deep dwelling red coral (*Corallium rubrum*) populations at Cap de Creus (NW Mediterranean). Mar Biol 154:533-545.

Roth J (2002) The Leviathan. In: The collected stories of Joseph Roth. Norton & Company, London.

Rousset F (2008) GENEPOP 007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Res, 1:103-106.

Rozen S, Skaletsky HJ (2000) PRIMER3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Meth in Mol Biol. Humana Press, Totowa, NJ, 365-386.

Saccheri I, Kuussaari M, Kankare M, Vikman P, Fortelius W, Hanski I (1998) Inbreeding and extinction in a butterfly metapopulation. Nature 392:491–494.

Santangelo G, Abbiati M, Giannini F, Cicogna F (1993) Red coral fishing trends in the western Mediterranean sea during the period 1981-1991, Sci Mar 57(2-3):139-143.

Santangelo G, Bongiorni L, Giannini F, Abbiati M, Buffoni G (1999) Structure analysis of two red coral populations dwelling in different habitats. In: Cicogna F., Bevestrello G., Cattaneo-Vietti R. (Eds), Red coral and other mediterranean octocorals: Biology and Protection, Ministero delle Poliche Agricole Alimentari e Forestali, Rome 23-43.

Santangelo G, Abbiati M (2001) Red coral: conservation and management of an overexploited Mediterranean species. Aq Cons: Mar Fresh Ecos 11:253–259.

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

Santangelo G, Bramanti L, Iannelli M (2007) Population dynamics and conservation biologyof the over-exploited Mediterranean red coral. J. of Theor Biol. 244:416-423.

Schneider S., Excoffier L (1999) Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates very among sites: Application to human mitochondrial DNA GENETICS Vol 152(3):1079-1089.

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

Slattery M, Lesser MP, Brazeau D, Stokes MD , Leichter JJ (2011) Connectivity and stability of mesophotic coral reefs. J Exp Mar Biol Ecol 408:32-41.

Smith TB, Blondeau J, Nemeth RS, Pittman SJ, Calnan JM, Kadison E, Gass J (2010) Benthic structure and cryptic mortality in a Caribbean mesophotic coral reef bank system, the Hind Bank Marine Conservation District, U.S. Virgin Islands. Coral Reefs 29:289-308.

Stiller M.A., Rivoire G. 1984 Biologie et ecologie du corail rouge en Méditerranée française. FAO Fish Rep 306:89-93.

Sunnucks P (2000) Efficient genetic markers for population biology. Trends Ecol Evol 15:199–203.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. (1997): The ClustalX windows interface:flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 56:604-614.

Torrents O, Garrabou J, Marschal C, Harmelin JG (2004) Age and size at first reproduction in the commercially exploited red coral *Corallium rubrum* (L.) in the Marseilles area (France, NW Mediterranean). Bio Cons 121:391–397.

Torrents O, Garrabou J, Marschal C, Harmelin JG, (2005) Age and size at first reproduction in the commercially exploited red coral *Corallium rubrum* (L.) in the Marseilles area (France, NW Mediterranean). Biol Cons 121:391-397.

Torrents O, Tambutté E, Caminiti N, Garrabou J (2008) Upper thermal thresholds of shallow vs. deep populations of the precious Mediterranean red coral Corallium rubrum (L.): Assessing the potential effects of warming in the NW Mediterranean, Exp Mar Bio 357:7-19.

Tsounis G, Rossi S, Aranguren M, Gili JM, Arntz W (2005) Effects of spatial variability and colony size on the reproductive output and gonadal development cycle of the Mediterranean red coral (*Corallium rubrum* L.), Mar Biol 148:513–527.

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

Tsounis G, Rossi S, Laudien J, Bramanti L, Fernandez N, Gili JM, Artnz W (2006b) Diet in a seasonal prey capture rate in the Mediterranean red coral. Mar Biol 149:1059-1070.

Tsounis G, Rossi S, Gili JM, Arntz W, (2007) Red coral fishery at the Costa Brava (NW Mediterranean): case study for an over harvested precious coral. Ecosyst 10:975-986.

Tsounis G, Rossi S, Grigg R, Santangelo G, Bramanti L, Gili JM (2010) The exploitation and conservation of precious corals. Ocean and Mar Bio Annual Review 48:161–211.

Uda K, Komeda Y, Koyama H, Koga K, Fujita T, Iwasaki N, Suzuki T (2011) Complete mitochondrial genomes of two Japanese precious corals, *Paracorallium japonicum* and *Corallium konojoi* (Cnidaria, Octocorallia, Coralliidae): notable differences in gene arrangement. Gene 476: 27–37.

Van Oppen MJH, Bongaerts P, Underwood JN, Peplow LM, Cooper TF (2011) The role of deep reefs in shallow reef recovery: an assessment of vertical connectivity in a brooding coral from west and east Australia. Mol Ecol, 20:1647–1660.

Vighi M (1972) Etude sur la reproduction du *Corallium rubrum* (L). Vie et Milieu 23:21–32.

Virgilio M, Airoldi L, Abbiati M (2006) Spatial and temporal variations of assemblages in a Mediterranean coralligenous reef and relationships with surface orientation. Coral Reefs 25:265–272.

Weinberg S (1979) The light dependent behaviour of planulae larvae of *Eunicella singularis* and *Corallium rubrum* and its implication for octocorallian ecology. Bijdr Dierkd 49:145-151.

Weinberg S (1980) Autoecology of shallow-water octocorallia from Mediterranean rocky substrata. II. Marseille, Cote d'Azur and Corsica. *Bijdr Dierk* 50: 73-86.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evol, 38: 1358–1370.

Winnepenninckx B, Backeljau T, De Wacher R (1993) Extraction of high molecular weight DNA from mollusks. Trends Genet 9:407.

Zibrowius H., Montero M, Grashoff M (1984) La ripartition du *Corallium rubrum* dans l'Atlantique. Thetis 11:163-170.