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GENETIC VARIABILITY AND STRUCTURING OF DEEP *CORALLIUM RUBRUM* (Linnaeus, 1758) POPULATIONS

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

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ABSTRACT

Given the decline of shallow-water red coral populations resulting from over-exploitation and mass mortality events, deeper populations below 50 metres depth (mesophotic populations) are currently the most harvested; unfortunately, very little is known about their biology and ecology. The persistence of these populations is tightly linked to their adult density, reproductive success, larval dispersal and recruitment. Moreover, for their conservation, it is paramount understand processes such as connectivity within and among populations. Here, for the first time, genetic variability and structuring of *Corallium rubrum* populations collected in the Tyrrhenian Sea ranging from 58 to 118 metres were analyzed using ten microsatellite loci and two mitochondrial markers (mtMSH and MtC). The aims of the work were 1) to examine patterns of genetic diversity within each geographic area (Elba, Ischia and Praiano) and 2) to define population structuring at different spatial scales (from tens of metres to hundreds of kilometres). Based on microsatellite data set, significant deviations from Hardy-Weinberg equilibrium due to elevated heterozygote deficiencies were detected in all samples, probably related to the presence of null alleles and/or inbreeding, as was previously observed in shallow-water populations. Moreover, significant levels of genetic differentiation were observed at all spatial scale, suggesting a recent isolation of populations. Biological factors which act at small spatial scale and/or abiotic factors at larger scale (e.g. summer gyres or absence of suitable substrata for settlement) could determine this genetic isolation. Using mitochondrial markers, significant differences were found only at wider scale (between Tuscany and Campania regions). These results could be related to the different mutation rate of the molecular makers or to the occurrence of some historical links within regions. A significant isolation by distance pattern was then observed using both data sets, confirming the restricted larval dispersal capability of the species. Therefore, the hypothesis that deeper populations may act as a source of larvae helping recovery of threatened shallow-water populations is not proved. Conservation strategies have to take into account these results, and management plans of deep and currently harvested populations have to be defined at a regional or sub regional level, similarly to shallow-water populations. Nevertheless, further investigations should be needed to understand better the genetic structuring of this species in the mesophotic zone, e.g. extending studies to other Mediterranean deep-water populations.

INTRODUCTION

Mesophotic coral ecosystems (MCEs) have been recently defined as reefs characterized by the presence of light-dependent corals and associated communities that are typically found at depths ranging from about 30 m to over 150 m in tropical and subtropical regions (Lesser *et al*. 2009; Hinderstein *et al*. 2010). The dominant communities, providing structural habitat in the mesophotic zone, can be comprised of coral, sponge, and algal species (Hinderstein *et al*. 2010); moreover, both shallow-water and depth-restricted species can be found (Brokovich *et al*. 2007). In recent years, they have been receiving raising attention from both scientists and managers due to an increasing awareness of their distinctive ecological character and biodiversity (Hinderstein *et al*. 2010; Khang *et al*. 2010; Rooney *et al*. 2010). Species inhabiting mesophotic zone are, in fact, susceptible to threats that display nearly identical signs to those found in shallow reefs (Smith *et al*. 2010; Bongaerts *et al*. 2010). Deeper fishing and harvesting practices, pollution, habitat loss and fragmentation and climate change (Bongaerts *et al*. 2010) can act directly or indirectly on their distribution, population dynamics, growth and genetic structure. Moreover, MCEs are currently under study to evaluate their potential to act as refugia and/or larval supply for shallow-water assemblages (Bongaerts *et al*. 2010; Miller *et al*. 2011; Van Oppen *et al*. 2011; Slattery *et al*. 2011). To act as refugia, MCEs have to be less affected by natural and human disturbances compared to shallow water reefs (Bak *et al*. 2005; Randall 2007; Bongaerts *et al*. 2010). Being more resistant, MCEs could provide a source of propaguls (larvae and recruits) to the shallow water habitats after disturbances, favouring they recovery and resilience against human impacts (Slattery *et al*. 2011). Connectivity between shallow and deep-water populations is a prerequisite for MCE to be considered refugia for shallow-water species. To date, genetic studies provide conflicting results on estimates of larval flow between shallow and deep-water coral reefs. Connectivity is depending primarily on the analyzed coral species and on the geographic areas considered (Van Oppen *et al*. 2011). In some cases shallow and deep populations result isolated (Costantini *et al.* 2010; Costantini *et al.* 2011; Miller *et al*. 2011). Conversely, in other cases, it was demonstrated the occurrence of gene (larval) flow between mesophotic ecosystem and shallow-water assemblages (Armstrong *et al*. 2006).

To understand the capability of deep populations to act as refugia, it is also necessary to investigate what are the horizontal scales of dispersal and the level of connectivity among

populations within the mesophotic zones. Occurrence of small and isolated populations, that are more susceptible to inbreeding depression and genetic drift, could reduce genetic diversity and the evolutionary potential, increasing their risk of extinction (Saccheri *et al*. 1998; Palumbi 2004). For mesophotic coral species, we might predict that open sea would act as barrier to dispersal and that coral populations in different geographic areas would be effectively isolated. However, within regions levels of population connectivity has to be investigated (Miller *et al*. 2011). Up to now, population genetic studies on mesophotic species involved only tropical corals (Bongaerts *et al*. 2010; Slattery *et al*. 2011).

Occurrence in the Mediterranean Sea of temperate biogenic reefs (coralligenous habitats) dwelling in the mesophotic zone was reported since the first deep sea explorations (Marsilli 1725). However, the exploration of these habitats and quantitative and experimental studies on their structure (Balata *et al*. 2005; Linares *et al*. 2005) and dynamics (Airoldi 1998; Virgilio *et al*. 2006) were developed only when SCUBA diving techniques became available for the scientific diving. The development of the technologies for the exploration of the deep sea, such as multibeam sonar and autonomous Remotely Operated Vehicles (ROVs) to be used on small research vessels, allowed the exploration of MCEs in the coastal zone of the Mediterranean Sea (Bo *et al*. 2009; Freiwald *et al*. 2009; Costantini *et al*. 2010; Cerrano *et al*. 2010; Bo *et al*. 2011a, b, c; Gori *et al*. 2011; this study). These studies provided new and valuable data on the reefs occurring in the Mediterranean twilight habitats, which are characterised by high species diversity and a great abundance of cnidarians' species. During some of these surveys, *Corallium rubrum* colonies have been identified and collected for further biological and ecological analyses.

Corallium rubrum (L.1758) is and endemic Mediterranean and Eastern Atlantic gorgonian coral inhabiting subtidal rocky habitats (Zibrowius *et al*. 1984; Chintiroglou *et al*. 1989; Rossi *et al*. 2008) ranging from shallow waters (10 m), through the mesophotic (80-150 m) zone, down to the deep-water (more than 800 m) (Freiwald *et al*. 2009; Costantini *et al*. 2010). Red coral is a gonochoric slow-growing gorgonian (Octocorallia: Alcyonacea) with internal fertilisation. The planula larvae are brooded in the polyps of the female colonies; after release the larvae have a negative photo and geo taxis and aquaria experiment showed that can have up to 14 days of free live before settling and metamorphose in polyp (Vighi 1972; Weinberg 1979). *Corallium rubrum* is the precious coral par excellence and it was harvested since ancient time (Tescione 1973; Santangelo & Abbiati 2001; Tsounis *et al*.

2010; Bussoletti *et al*. 2010). In fact, data on red coral harvesting in the Mediterranean Sea showed a trend of heavy reduction in total harvest until 2000 with irregular fluctuations suggesting a 'boom and burst' harvesting strategy. By the end of the nineties the harvest remained constant around the 25-35 tons per years (Santangelo & Abbiati 2001, GFCM 2010). More recently mass mortality events linked with thermal anomalies have been affecting shallow-water red coral populations (Cerrano *et al*. 2000; Bramanti *et al*. 2005; Cerrano & Bavestrello 2008; Garrabou *et al*. 2009). To protect these endangered populations GFCM-FAO (2010) imposed a ban of red coral harvesting down to 50 metres depth. Nowadays all around the Mediterranean Sea harvesting is focused on populations dwelling between 60 and 150 metres depth. Their colonies reach larger commercial sizes and are extremely valuable on the market. Our knowledge on the biological and ecological features of these populations are very limited (Rossi *et al*. 2008; Bussoletti *et al*. 2010; Costantini *et al.* 2011), therefore these populations became a priority focus for current research, with the aim to collect data allowing appropriate management of the harvesting. Genetic studies on red coral shallow-water populations revealed strong structuring at both global (Mediterranean) and micro (less than one metre) scales (Costantini *et al*. 2007a, b; Ledoux *et al*. 2010a, b), with some isolation by distance patterns (Ledoux *et al*. 2010a, b). High genetic structuring in shallow-water populations could be related to the limited effective larval dispersal and to the geo-morphologic characteristic of the habitat where the species lives. A more recent study by Costantini *et al.* (2011) showed a reduction of genetic variability along a depth gradient in a range between 20 and 70 metres, suggesting that depth has an important role in determining the patterns of genetic structure of the species. In particular, a barrier to the connectivity was observed among the samples collected across 40–50 metres depth, supporting the hypothesis that discrete shallow-water and intermediate-water red coral populations occur. These results were already observed by a preliminary study on deep-water *C. rubrum* colonies (up to 800 m depth, Costantini *et al*. 2010).

A research project aimed to fill the gap in the knowledge on the biology and ecology of red coral mesophotic populations was supported by the Italian Ministry of Environment. A research cruise was done to sample red coral colonies in a depth range from 58 to 118 metres, to investigate the level of reproductive isolation of currently harvested populations. Three sampling locations have been identified along the Tyrrhenian coast of Italy. The aims of the work were 1) to examine patterns of genetic diversity within each geographic location

and 2) to define population structuring at different spatial scales (from tens of metres to hundreds of kilometres). Moreover, patterns of genetic structuring observed in this study were compared with those found between shallow-water populations. Molecular markers with different levels of polymorphism were used: allele frequencies of microsatellite loci and sequence polymorphism of portions of the mitochondrial DNA (mutS homolog gene, mtMSH, Pont-Kingdon *et al*. 1995; putative control region, MtC, Uda *et al*. 2011). Although mitochondrial DNA is often considered to evolve very slowly in Anthozoa (Shearer *et al*. 2002; Hellberg 2006), these two regions are enough variable to be informative for intra-specific comparisons (Thoma *et al*. 2009; Miller *et al*. 2011; Van Oppen *et al*. 2011).

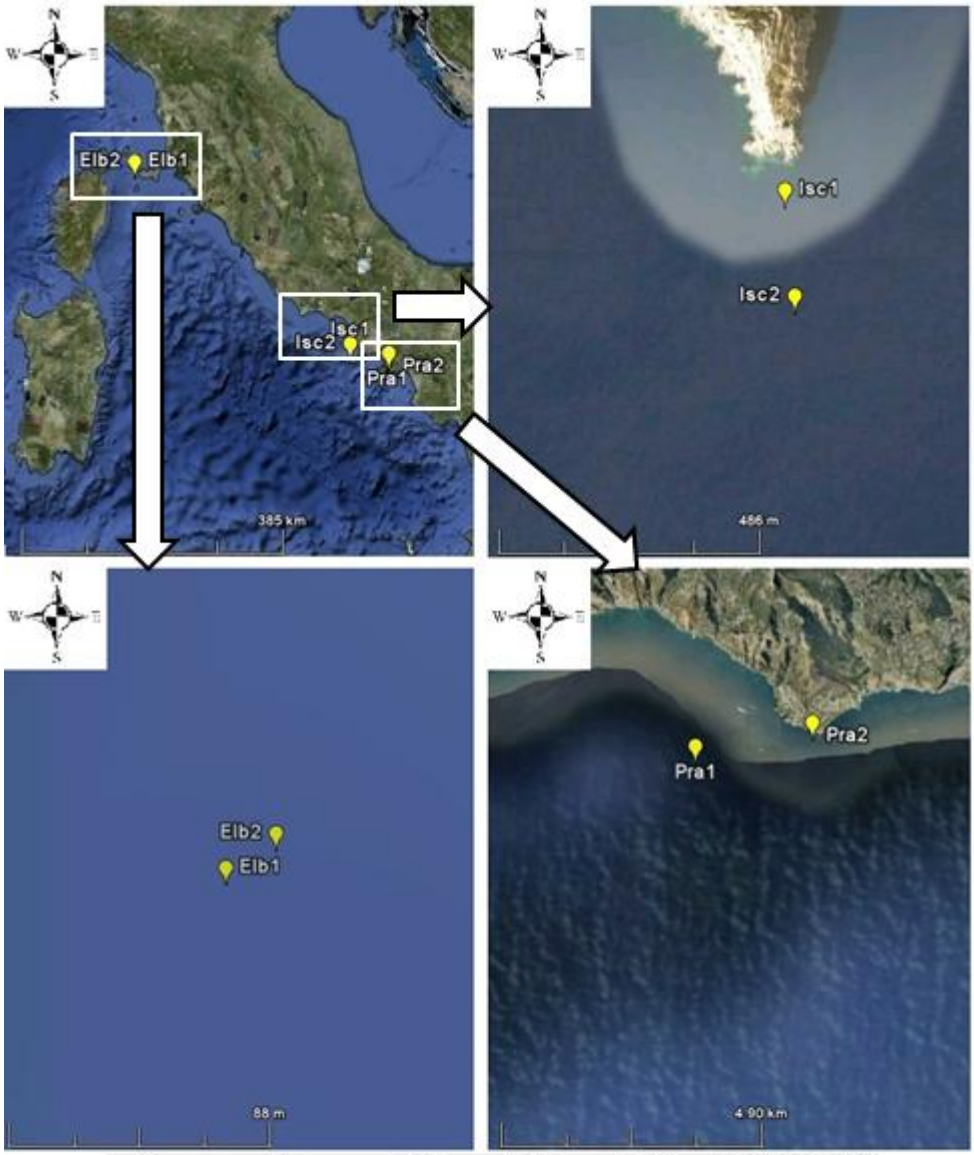
MATERIALS AND METHODS

Samples collections

The oceanographic cruise by the R/ V *Astrea* done in July 2010 was specifically dedicated to the survey and sampling of mesophotic red coral populations. Using both commercial SCUBA divers and a Remotely Operated underwater Vehicle (ROV) 145 red coral colonies were collected in three different areas along the coast of the Tyrrhenian Sea at depth ranging between 58 and 118 metres. The investigated areas were Elba (LI), Ischia (NA), and Praiano (SA). Areas were located following previous records of deep-water red corals colonies made by local fishermen. In each area two dive sites were selected and the occurrence of *Corallium rubrum* was detected by a multibeam echosounder and ROV (Fig. 1).

The Elba area (Tuscany region) is geographically separated from the others by hundreds of kilometres, whereas Ischia and Praiano (Campania region) areas are separated by few tens of kilometres. The two sampling sites defined within each area were separated by tens to hundreds of metres. In Elba area colonies dwelt on scattered rocky boulders spread on a sedimentary bottom, in Ischia and Praiano colonies were found on both vertical rocky cliffs and overhangs, with high sediment/mud depositions. In each site *C. rubrum* was observed in association with colonies of *Eunicella cavolinii* and *Paramuricea clavata*.

For each site, a branch fragment from 15 to 33 live colonies of red coral was collected within an area ranging from 50 to 100 m^2. Samples were preserved in 80% ethanol at 4°C.



Code	Site	Region	Latitude	Longitude	Depth (m)	Samples (n)
Elb1	Rete Bruno (SW Elba)	Tuscany	42°41'20.02'' N	10°1'22.90'' E	84-88	23
Elb2	Rete Bruno (SW Elba)	Tuscany	42°41'20.39'' N	10°1'23.64'' E	83-86	23
Isc1	Punta S. Angelo (Ischia)	Campania	40°41'28.29'' N	13°53'37.59'' E	100-118	22
Isc2	Punta S. Angelo (Ischia)	Campania	40°41'21.91'' N	13°53'38.35'' E	85-95	32
Pra1	Torre di Grado (Praiano)	Campania	40°36'7.36'' N	14°30'10.97'' E	58-62	22
Pra2	Scoglio D'Isca (Praiano)	Campania	40°36'21.02'' N	14°31'41.12'' E	62-65	13

Fig. 1. Geographic positions of the sampling locations: maps, identification codes, sites, regions, geographical coordinates, depths and sample sizes.

Molecular analysis

Total genomic DNA was extracted from two to four polyps per fragment using cetyltrimethyl ammonium bromide (CTAB) following the procedure described in Costantini *et al.* (2007a, b).

Ten microsatellite loci specifically developed for *C. rubrum* (COR9, COR15, COR46, COR48, COR58 by Costantini & Abbiati 2006; MIC20, MIC22, MIC23, MIC24, MIC26 by Ledoux *et al*. 2010a) were analysed. Microsatellite loci were amplified either locus by locus or in multiplex using a QIAGEN® Multiplex PCR Kit (see Table 1 for PCR multiplex set) using polymerase chain reaction (PCR) conditions described in Costantini *et al*. (2011).

Table 1. Summary of genetic diversity at eight microsatellite loci within *Corallium rubrum* samples: n, number of sampled individuals; N, number of genotypes per locus; Ar, allelic richness based on 12 individuals; H_o, observed heterozygosity; H_s, gene diversity (Nei 1987); F_{IS}, Weir and Cockerham's (1984) estimate of Wright's (1951) fixation index. Bold types indicate significant deviations from HWE after FDR correction. [a], loci amplified in multiplex PCR 1; [b], loci amplified in multiplex PCR 2; [c], loci amplified in multiplex PCR 3.

	SAMPLE (n)						
LOCUS	Elb1 (23)	Elb2 (23)	Isc1 (22)	Isc2 (32)	Pra1 (22)	Pra2 (13)	**MEANS**
COR9[a]							
N	21	19	22	31	22	13	21.33
Ar	7.22	7.31	3.00	3.87	5.65	5.84	5.48
H_O	0.10	0.58	0.14	0.16	0.09	0.08	0.19
H_S	0.77	0.81	0.58	0.70	0.68	0.50	0.67
F_{IS}	**0.88**	**0.31**	**0.77**	**0.78**	**0.87**	**0.86**	0.74
COR15[b]							
N	22	23	22	31	21	13	22.00
Ar	2.80	2.00	2.71	2.02	2.57	1.92	2.34
H_O	0.00	0.00	0.09	0.10	0.05	0.08	0.05
H_S	0.42	0.39	0.21	0.09	0.32	0.07	0.25
F_{IS}	**1.00**	**1.00**	**0.58**	-0.02	**0.86**	0.00	0.57
COR46[a]							
N	19	23	20	31	22	13	21.33
Ar	6.81	6.52	4.60	7.45	6.98	9.69	7.01
H_O	0.58	0.43	0.55	0.61	0.95	0.85	0.66
H_S	0.82	0.78	0.74	0.79	0.80	0.87	0.80
F_{IS}	**0.32**	**0.46**	0.28	0.24	-0.16	0.06	0.20
COR48[b]							
N	21	23	22	23	21	12	20.33
Ar	9.38	5.90	5.26	8.58	9.63	9.00	7.96
H_O	0.71	0.43	0.32	0.30	0.57	0.58	0.49
H_S	0.88	0.81	0.74	0.86	0.87	0.85	0.84
F_{IS}	**0.21**	**0.48**	**0.59**	**0.66**	**0.37**	**0.36**	0.44
COR58[a]							
N	21	20	20	32	22	13	21.33
Ar	5.39	2.94	9.73	9.79	7.99	6.84	7.11
H_O	0.52	0.40	0.60	0.50	0.59	0.62	0.54
H_S	0.75	0.45	0.88	0.85	0.82	0.74	0.75
F_{IS}	**0.32**	0.14	**0.34**	**0.42**	**0.30**	**0.21**	0.29

Table 1 continued

LOCUS	SAMPLE (n)						MEANS
	Elb1 (23)	Elb2 (23)	Isc1 (22)	Isc2 (32)	Pra1 (22)	Pra2 (13)	
MIC20[c]							
N	23	23	22	32	22	13	22.50
Ar	6.45	5.76	4.92	7.12	8.74	5.92	6.48
H_O	0.43	0.39	0.55	0.59	0.86	0.77	0.60
H_S	0.64	0.65	0.57	0.77	0.80	0.76	0.70
F_{IS}	**0.34**	**0.42**	0.07	0.25	-0.05	0.03	0.18
MIC24[c]							
N	23	23	22	32	22	13	22.50
Ar	5.03	4.82	7.64	6.35	4.27	3.77	5.31
H_O	0.30	0.48	0.64	0.47	0.27	0.23	0.40
H_S	0.66	0.68	0.75	0.69	0.25	0.21	0.54
F_{IS}	**0.56**	**0.32**	0.17	**0.34**	-0.06	-0.04	0.21
MIC26[c]							
N	22	23	22	32	22	13	22.33
Ar	10.49	8.37	9.02	10.42	10.14	7.84	9.38
H_O	0.95	0.87	0.77	0.72	0.91	0.77	0.83
H_S	0.86	0.84	0.88	0.89	0.88	0.83	0.86
F_{IS}	**-0.08**	-0.02	0.14	**0.21**	-0.01	0.11	0.06
Multilocus							
Ar	6.70	5.45	5.86	6.95	7.00	6.35	6.38
H_O	0.45	0.45	0.46	0.43	0.54	0.50	0.47
H_S	0.73	0.68	0.67	0.71	0.68	0.60	0.68
F_{IS}	**0.40**	**0.36**	**0.34**	**0.40**	**0.23**	**0.22**	0.32

Genotyping of individuals was carried out on an ABI 310 Genetic Analyser (Applied Biosystems), using forward primers labelled with FAM, HEX/VIC, TAMRA/NED, ROX/PET (Sigma) and LIZ HD500 (Applied Biosystems) as internal size standard. Allele sizing was conducted using GENESCAN Analysis Software version 2.02 (Applied Biosystems).

MtMSH sequences were amplified using the primers MUT4759f and MSH5376r and following France & Hoover (2001) and Lepard (2003) protocols. PCR amplifications of MtC were obtained 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 12.5 µL MtC PCR reaction contained approximately 20 ng DNA, 1X PCR buffer (Invitrogen), 2 mM $MgCl_2$, 0.5 µM of each primer, 0.8 mM dNTPs and 1 U *Taq* polymerase (Invitrogen). Amplifications were performed on a GeneAMP PCR System 2700 (Applied Biosystems) as follows: an initial denaturation at 95 °C for 3 min, 30 cycles including 95 °C for 30 s, 59 °C for 30 s and 72 °C for 60 s. A final extension at 72 °C for 5 min was added.

PCR products were sent to Macrogen (South Korea) for purification and sequencing with the same primers and the obtained sequences were edited and aligned manually using MEGA version 5.0 (Tamura et al. 2011).

Genetic variability

Sampling using the ROVs may cause a fragmentation of the colonies; therefore, fragments 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 software MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al*. 2004) was used to test the reliability of the data set and to investigate the presence of null alleles. All loci were tested for linkage disequilibrium using GENEPOP version 4.1 (Rousset 2008) as implemented for online uses (http://genepop.curtin.edu.au/).

Microsatellite diversity within samples for each locus and over all loci was estimated as observed heterozygosity (H_O) and unbiased gene diversity (H_S, Nei 1987) using the GENETIX software package version 4.05 (Belkhir *et al.* 2004). As the number of alleles found in a sample is dependent on sample size, allelic richness (Ar) was estimated using the El Mousadik & Petit (1996) rarefaction index in FSTAT version 2.9.3.2 (Goudet 2001) with a sample size of 12 specimens.

Single and multilocus F_{IS} were estimated using Weir and Cockerham's *f* (Weir & Cockerham 1984) and significant departures from the Hardy–Weinberg equilibrium (HWE) were tested using the exact test implemented in GENEPOP, with the level of significance determinate by a Markov-chain randomization (1000 dememorizations, 100 batches and 1000 iterations per batch).

Moreover, the samples were grouped according to their geographical origin (A: three groups: Elba, Ischia and Praiano; and B: two groups: Tuscany and Campania) and their depth (C:

two groups: 58-65 m and 85-118 m). Significant differences in genetic diversity (H_O, H_S and Ar) among groups of samples were tested using a permutation procedure (1000 iterations) in FSTAT.

Sequence genetic diversity within samples was estimated using haplotype diversity (h, Nei 1987) and nucleotide diversity (π, Nei 1987) implemented in the software ARLEQUIN version 3.5 (Excoffier & Lischer 2010).

Population structure analysis

Owing to deviation from the Hardy–Weinberg equilibrium, genotypic differentiation between samples was tested with an exact test (Markov chain parameters: 1000 dememorizations, followed by 100 batches of 1000 iterations per batch) using GENEPOP. As null alleles can induce overestimation of genetic distance (Chapuis & Estoup 2007), pairwise F_{ST} estimates were computed following the excluding null alleles (ENA) method in FREENA (Chapuis & Estoup 2007). The significance of pairwise genotyping differentiation between samples was tested with an exact test as implemented in GENEPOP. Recent analyses suggest that F_{ST} may be poorly suited as an estimator of population divergence for data sets in which allelic diversity is high (Hedrick 2005; Jost 2008). Given the high variability of the markers used here, differentiation among samples was estimates using Jost's actual measure of differentiation D_{est} calculated with the package DEMEtics version 0.8.3 (Gerlach *et al*. 2010) within the statistical package R v2.13.1 (R Development Core Team 2009). Overall estimates of D_{est} were calculated from individual loci using a harmonic mean approximation. P-values (indicating the strength of evidence against the null hypothesis of no genetic differentiation) are obtained from bootstrap methods with 1000 pseudoreplications.

For the mtMSH and MtC sequences data sets, genetic differentiation between samples was estimated using pairwise F_{ST} estimator and its significance determined using a permutation test (10000 permutations) in ARLEQUIN.

For both microsatellite and sequences data sets, isolation by distance model between samples was tested through a Mantel test (Mantel 1967) computed using the Isolde program implemented in GENEPOP. A significant correlation between genetic differentiation estimates (F_{ST} and D_{est}) and the geographical distances (Log transformed) among samples was tested using 1000 permutations.

The partition of the genetic variance among samples based on the three data set was conducted through an analysis of molecular variance (AMOVA) implemented in the software ARLEQUIN. For this purpose, the same groups of samples used for the analysis of genetic diversity were adopted.

Significance levels were corrected using a false discovery rate (FDR) correction for multiple tests (Benjamini and Hochberg 1995) when necessary.

RESULTS

Microsatellite loci variability

Six multilocus genotypes (MLGs) were found twice and one was found triple. One MLG was encountered twice in sample Elb2, two MLGs in Pra2 and one MLG in Isc2. Within Pra1 two MLGs were encountered twice and one triple. The probability that each of these genotypes was produced through sexual reproduction resulted small (1.16×10^{-11}), indicating that shared genotypes derive from a single individual, and, therefore, were included only once, obtaining a final data set of 135 different multilocus genotypes. This choice was supported also by the fact that the eight excluded samples showed identical mtMSH and MtC sequences.

Loci MIC22 and MIC23 were excluded from subsequent analysis, because they correctly amplified in Elba samples, but did not give any amplification in the Ischia and Praiano samples, even though repeated attempts were made.

According to MICRO-CHECKER, null alleles may be present in some loci, but no evidence of scoring errors due to stuttering or large allele dropout was found in the whole data set. The eight microsatellite loci analysed were polymorphic in all samples. One locus (COR46) was in linkage disequilibrium with all the other ($P = 0.00036$) after false discovery rate correction. Eliminating this locus from the following analysis did not change the results.

Over all samples, the number alleles per locus ranged from 6 (in COR15) to 22 (in COR48), and allelic richness from 2.34 (in COR15) to 9.38 (in MIC26). Within samples, the allelic richness based on a minimum sample size of 12 diploid individuals, ranged between 5.45 (in Elb2) and 7 (in Pra1). Mean observed heterozygosity ranged between 0.43 ± 0.08 (in Isc2) to 0.54 ± 0.13 (in Pra1), and gene diversity from 0.60 ± 0.11 (in Pra2) to 0.73 ± 0.05 (in Elb1). Highly significant deviations from HWE ($P < 0{,}001$) were observed in all samples.

Multilocus estimates of F_{IS} ranged from 0.22 (in Pra2) to 0.4 (in Elb1 and Isc2), showing heterozygote deficiencies in all analyzed samples (Table 1).

Values of all the considered indexes of genetic variability were comparable among groups of samples; indeed, observed heterozygosity, gene diversity, allelic richness and F_{IS} were not significantly different among groups ($P > 0.05$, see materials and methods for group definition).

Mitochondrial sequence variation

Across all 135 individuals the mtMSH fragment was 567 bp in length. The sequence alignment showed the presence of both the two haplotypes (GenBank accession number GQ304902, GQ304903) previously recorded by Costantini *et al*. (2010). Haplotype GQ304903 was present in all samples and was the most abundant (94%), whereas haplotype GQ304902 was detected only in Praiano area, with six individuals in Pra1 and two individuals in Pra2. These two samples showed a haplotype diversity of 0.42 and 0.28, respectively (Table 2).

Table 2. Sequence differences, distribution and genetic diversity of the two mtMSH and three MtC haplotypes found in *Corallium rubrum* samples: dots indicate identical bases; H, total number of haplotypes; *h*, haplotype diversity (*h*, Nei 1987); π, nucleotide diversity (π, Nei 1987).

	Nucleotide position	mtMSH					
	156	Elb1	Elb2	Isc1	Isc2	Pra1	Pra2
GQ304903	G	23	23	22	32	16	11
GQ304902	A					6	2
H		1	1	1	1	2	2
h		0	0	0	0	0.42	0.28
π		0	0	0	0	0.0007	0.0005

	Nucleotide position				MtC					
	28	82	274	275	Elb1	Elb2	Isc1	Isc2	Pra1	Pra2
Hap_1	C	A	A	G	19	14				
Hap_2	T	G	.	.	4	9	12	16	6	4
Hap_3	.	.	C	T			10	16	16	9
H					2	2	2	2	2	2
h					0.3	0.5	0.52	0.52	0.42	0.46
π					0.0021	0.0035	0.0036	0.0036	0.0029	0.0032

The putative control region (MtC) in *Corallium rubrum* was 290 bp in length, corresponding 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 four nucleotide substitution (1.4% variation) which defined three different haplotypes (Table 2). Hap_2 was present in all samples and was the most abundant, together with Hap_3 (37.8% each). However, Hap_3 was detected only in Ischia and Praiano areas, whereas Hap_1 was found only in Elba area with a high abundance (72%) compare to Hap_2 (28%). Low and comparable values of haplotype and nucleotide diversity of MtC were found among samples, with mean values of 0.45 (± 0.03) and 0.0031 (± 0.0002), respectively.

Genetic differentiation between samples

The three molecular markers used in this study provided slight different level of genetic differentiation among samples. Variability between markers is due to variability in the level of polymorphism. For the microsatellite data set, F_{ST} estimates and F_{ST} estimates based on the ENA method to correct for the presence of null alleles (Chapuis & Estoup 2007) gave similar results. The pairwise F_{ST} estimated ranged from 0.05 (Elb1 vs. Elb2) to 0.24 (Pra2 vs. Elb2 and Isc1) and all pairwise comparisons were significant ($P < 0.01$) after FDR correction. D_{est} values were substantially higher, ranging from 0.05 (Isc1 vs. Isc2) to 0.59 (Elb2 vs. Pra2) and all pairwise comparisons were significant ($P < 0.01$) after FDR correction (Table 3). Overall, higher values of genetic differentiation were observed using D_{est} rather than F_{ST} ($D_{est} = 0.37$; $F_{ST} = 0.13$). The sample Pra2 appeared to be the most differentiated from all of the other samples, both based on pairwise F_{ST} estimates and on actual measure of differentiation D_{est}. Moreover, the lowest values of F_{ST} and D_{est} were observed between samples belonging to the same area (Table 3). No correlation between F_{ST} and the natural logarithm of the geographical distances ($P = 0.08$) was observed, whereas a significant isolation by distance pattern was detected using D_{est} ($P < 0.01$) (Fig. 2).

Table 3. Pairwise multilocus estimates of F_{ST} (Weir & Cockerham 1984) below the diagonal, and D_{est} (Jost 2008) above the diagonal between all *Corallium rubrum* samples; all are statistically significant ($P < 0.01$) after FDR correction.

	Elb1	Elb2	Isc1	Isc2	Pra1	Pra2
Elb1		**0.10**	**0.21**	**0.30**	**0.34**	**0.54**
Elb2	**0.05**		**0.30**	**0.30**	**0.36**	**0.59**
Isc1	**0.10**	**0.14**		**0.05**	**0.18**	**0.49**
Isc2	**0.15**	**0.16**	**0.14**		**0.20**	**0.28**
Pra1	**0.12**	**0.15**	**0.08**	**0.15**		**0.17**
Pra2	**0.22**	**0.24**	**0.24**	**0.10**	**0.14**	

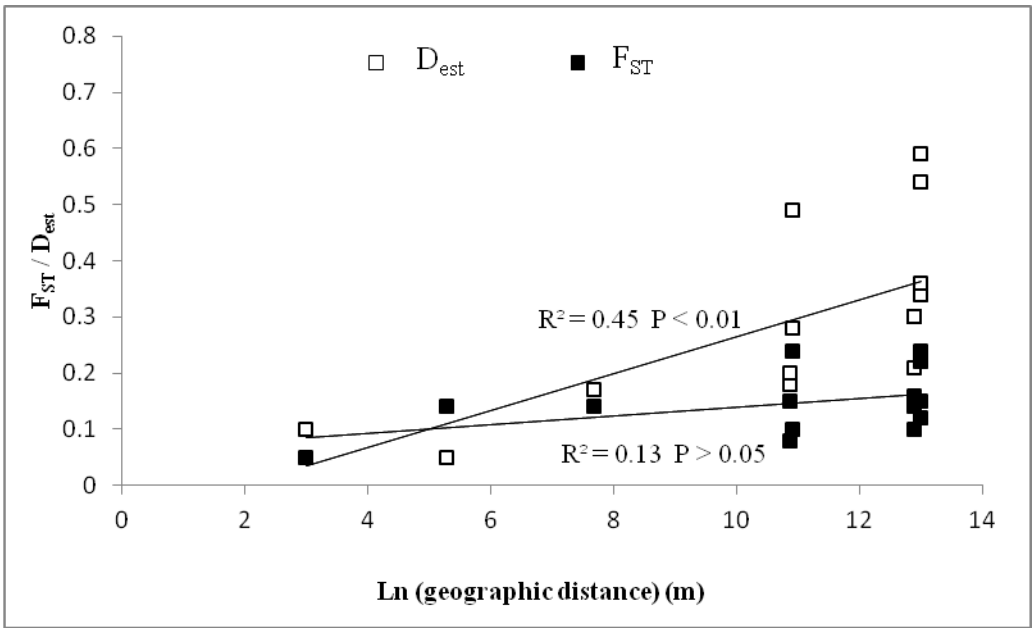


Fig. 2. Relationship between genetic differentiation estimates (F_{ST} and D_{est}) and the logarithm of geographical distance among *Corallium rubrum* samples for microsatellites markers.

The mtMSH marker provided the lowest values of genetic differentiation between samples. Pairwise F_{ST} estimated ranged from -0.02 (Pra1 vs. Pra2) to 0.29 (Pra1 vs. Isc2) and all the comparisons were not significant after FDR correction (Table 4). The MtC marker revealed intermediate values of genetic differentiation, compared to microsatellites and mtMSH. Pairwise F_{ST} estimated ranged from -0.06 (Pra1 vs. Pra2) to 0.77 (Pra1 vs. Elb1). Global estimates of F_{ST} were not significant both within Tuscany ($F_{ST} = 0.07$, $P > 0.01$) and within Campania ($F_{ST} = 0.02$, $P > 0.01$). Pairwise multilocus estimates of F_{ST} between Tuscany and Campania samples were generally high (range: 0.52 to 0.77) and significantly different from zero after FDR correction (Table 4). Significant isolation by distance pattern was observed using F_{ST} based on MtC data set ($P = 0.01$) (Fig. 3), whereas correlation between F_{ST} based

on mtMSH data set and the natural logarithm of the geographical distances (P = 0.12) was not significant.

Table 4. Pairwise multilocus estimates of both MtC F_{ST} (below the diagonal) and mtMSH F_{ST} (above the diagonal) between all *Corallium rubrum* samples; bold types indicate statistically significant values (P < 0.01) after FDR corrections.

	Elb1	Elb2	Isc1	Isc2	Pra1	Pra2
Elb1		0.00	0.00	0.00	0.24	0.15
Elb2	0.07		0.00	0.00	0.24	0.15
Isc1	**0.68**	**0.52**		0.00	0.24	0.14
Isc2	**0.68**	**0.54**	-0.04		0.29	0.20
Pra1	**0.77**	**0.66**	0.10	0.07		-0.02
Pra2	**0.76**	**0.63**	0.05	0.02	-0.06	

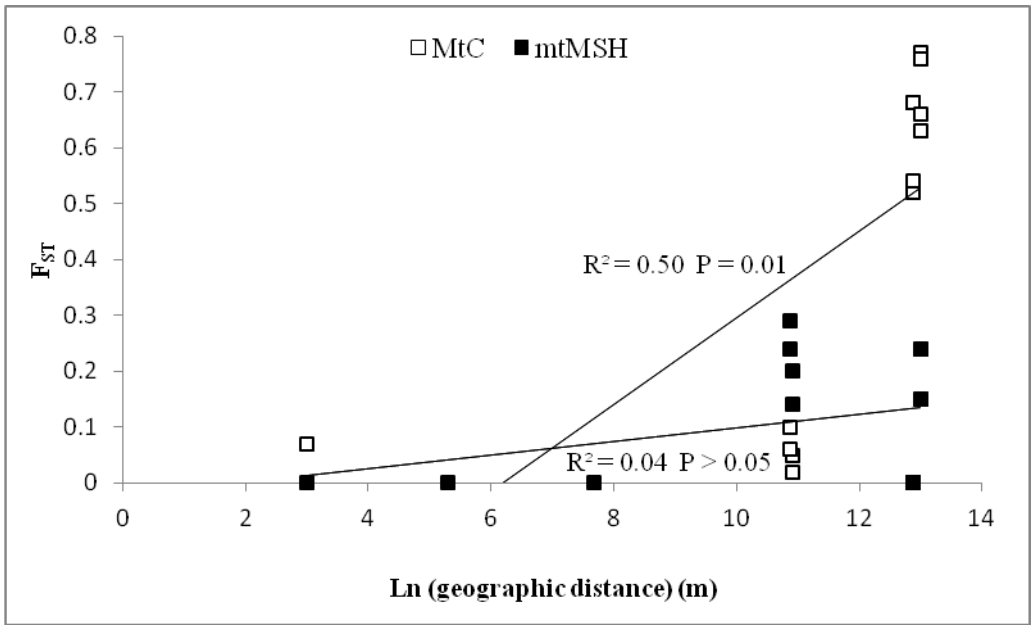


Fig. 3. Relationship between genetic differentiation estimates and the logarithm of geographical distance among *Corallium rubrum* samples for both mtMSH and MtC markers.

The AMOVA test conducted among samples showed different results, depending on which data set was adopted and which grouping were considered. Microsatellites showed major genetic variation within samples (from 83.99 to 85.17%; P < 0.001), and among samples within all considered groups (from 10.49 to 12.68%; P < 0.001). Using mtMSH data set, variation within samples was also high (from 65.49 to 81.69%; P < 0.001) together with the genetic variation among groups within samples, where grouped based on their depth,

(35.62%; $P > 0.05$). By contrast, using MtC data set no significant genetic variation was observed among groups (from 56.78 to 63.6%; $P > 0.05$). When samples were grouped according to their depth the genetic variation within samples and among samples within groups (45.31 and 39.97% respectively; $P < 0.001$) assumed a primary role (Table 5).

Table 5. Analysis of molecular variance (AMOVA) among *Corallium rubrum* samples using microsatellites, mtMSH and MtC data sets. Red coral samples were grouped according to (A) their area (three groups: Elba, Ischia and Praiano), (B) their region (two groups: Tuscany and Campania), and (C) their depth (two groups: 58-65 m and 85-118 m). $^{*}P < 0.05$, $^{**}P < 0.001$.

		Microsatellites			mtMSH			MtC		
	Source of variation	d.f.	Variance components	%	d.f.	Variance components	%	d.f.	Variance components	%
(A)	*Three groups*									
	Among groups	2	0.132	4.34	2	0.014	23.65	2	0.594	56.78
	Among samples within groups	3	0.320	10.49**	3	0.000	-0.64	3	-0.003	-0.30
	Within samples	264	2.597	85.17**	129	0.047	76.99**	129	0.455	43.52**
(B)	*Two groups*									
	Among groups	1	0.153	4.96	1	-0.001	-2.27	1	0.828	63.60
	Among samples within groups	4	0.342	11.05**	4	0.012	20.58*	4	0.019	1.44
	Within samples	264	2.597	83.99**	129	0.050	81.69**	129	0.455	34.96**
(C)	*Two groups*									
	Among groups	1	0.079	2.57	1	0.026	35.62	1	0.148	14.72
	Among samples within groups	4	0.389	12.68**	4	-0.001	-1.11	4	0.402	39.97**
	Within samples	264	2.597	84.75**	129	0.047	65.49**	129	0.455	45.31**

DISCUSSION

This study provides the first data on the genetic variability and structuring of harvested red coral populations dwelling in the depth range of *c.* 60-120 m (the mesophotic zone). For this purpose markers with different evolutionary rates were used. The results show evidence that 1) mesophotic red coral populations are isolated at scales of tens of metres following isolation by distance pattern; 2) migration events within both Tuscany and Campania regions occurred in past time.

Genetic variability

The multilocus polymorphism found in *Corallium rubrum* using microsatellites (H_S ranging from 0.60 ± 0.11 to 0.73 ± 0.05, with a mean value of 0.68 ± 0.02) resulted comparable with that observed in samples collected from 50 metres depth down by Costantini *et al*. (2011) (Student's *t*-test: $P > 0.05$) and lower compared to shallow-water populations, confirming a reduction of genetic variability respect to these latter (Costantini *et al*. 2011).

Strong deviations from Hardy-Weinberg equilibrium (showed by high positive F_{IS} estimates) were detected for all samples and at all microsatellite loci. Deficit of heterozygotes was already found in previous studies on *C. rubrum* (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 (e.g. inbreeding and Wahlund effect), as well as the possible occurrence of technical problems, such as the presence of non-amplifying or null alleles (aspects discussed in Costantini *et al*. 2007a). It is important to notice that the microsatellite loci MIC22 and MIC23 amplified exclusively in the samples Elb1 and Elb2. This suggested that some mutations could have affected the flanking regions of these loci in the samples Isc1, Isc2, Pra1 and Pra2, leading to different sequences which could not be matched by the primers. Similar results were found by Aurelle *et al*. (2011): 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). Despite differences in the habitat features between Tuscany (characterized by scattered rocky boulders on a sedimentary bottom) and Campania (vertical rocky cliffs and overhangs; Canese pers. comm.), no differences in genetic variability were observed between the two regions.

Mitochondrial markers showed low genetic variability among samples, compare to microsatellite markers, probably due to the fact that in Anthozoa mitochondrial DNA has extremely low mutation rate and it is much conserved (Shearer *et al*. 2002; Costantini *et al*. 2003; Calderon *et al*. 2006; Hellberg 2006). The newly developed mitochondrial marker, the MtC, showed a higher variability compared to other mitochondrial genes (Calderon *et al*. 2006; Costantini *et al*. 2003). MtC have been successfully used to investigate levels of genetic structuring between populations of two scleractinian corals: *Desmophyllum dianthus*

and *Seriatopora hystri*x (Miller *et al*. 2011; Van Oppen *et al*. 2011). MtC red coral shows a lower number of haplotypes, compared to those of scleractinian corals, with low levels of differences between them; in fact, only four substitutions were found. Moreover, the very low nucleotide diversity observed was comparable to those found by Miller *et al*. (2011) in *Desmophyllum dianthus* (Student's *t*-test: $P > 0.1$). However, this level of variation is much higher compared to mtMHS and it allows quantifying differences between samples. In fact, whereas one haplotype was widespread along all samples, the other two were observed one in Tuscany samples and one in Campania samples.

Previous study on genetic structure of red coral populations sampled at a depth ranging between 600 and 800 metres (Costantini *et al*. 2010) showed the occurrence of a private mtMSH "deep-water" haplotype GQ304902, which was never found in shallow-water populations. Indeed, they found the "deep-water" mtMHS haplotype only in deep-water colonies from Linosa and Malta. In our study, the mtMSH "deep-water" haplotype was found only in samples Pra1 and Pra2, suggesting a lack of connectivity along a geographical and/or a depth gradient. This founding, together with the fact that MIC22 and MIC23 did not amplify in the southern part of the Mediterranean Sea, support evidences of the occurrence of a geographical barrier to gene flow between Tuscany and Campania.

Moreover, Praiano samples were the shallowest among those investigated in the present study, so a more detailed research of red coral populations around 50 metres deep is needed to confirm the lack of connectivity along a depth gradient and the particularity of this bathymetry close to the thermocline, as already observed by Costantini *et al*. (2011). The high genetic differentiation (using both data sets) detected among Praiano samples respect the others suggest that this area has some peculiarities which have to be taken in account in the future.

Genetic structuring

Using microsatellite loci, a significant genetic differentiation among *C. rubrum* samples at all the spatial scales analyzed was detected, suggesting that mesophotic red coral populations are genetically structured at scale of less than ten of metres with a highly restricted larval dispersal, as was already observed in shallow-water populations (Costantini *et al*. 2007a, b; Ledoux *et al*. 2010a, b; Costantini *et al*. 2011). Both the two estimators of population divergence used in this study (F_{ST} and D_{est}) and AMOVA give similar results on

genetic structuring. Nevertheless, in this species, characterized by high levels of within population variation (as evidenced by high values of heterozygosity and allelic richness), D_{est} is expected to illustrate more accurately the actual magnitude of differentiation between populations. Indeed, microsatellites based F_{ST} estimates appeared much smaller compare to D_{est} estimates, probably due to the fact that F-statistics tend to underestimate differentiation between populations as variation increases (Jost 2008).

Biological and ecological factors which act at small spatial scale and/or abiotic factors at larger scale could determine the isolation observed among populations. 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 Tuscany scattered rocky boulders were separated by flat sedimentary bottom, where red coral was not able to settle. In Campania overhangs and fissures could enhance a larval retention and protect colonies from smothering due to the abundant sedimentation rates in these areas.

The great genetic differentiation observed between Campania and Tuscany compared to those within regions could be related to the occurrence of barrier to gene flow, such as geomorphologic and hydrodynamic characteristic of the studied areas or the geographic distance, as suggested by the isolation by distance pattern observed (see also Ledoux *et al*. 2010a, b). Tuscany and Campania are at both side of the putative biogeographical barrier which divides the Ligurian Sea and the Tyrrhenian Sea (Bianchi & Morri 2000; Bianchi 2007) and between the two regions red coral is patchily distributed with colonies living on sparse rocks arising from the detritical bottom (Marchetti 1965; see also Bramanti 2011). In addition, it should be considered that in winter a northward current along the Italian coast connects southern and northern Tyrrhenian, whereas in summer, when the larvae are released (Santangelo *et al*. 2003), the Tyrrhenian circulation has a more fragmented pattern with some local gyres (Astraldi & Gasparini 1994) that could act as a barrier to gene flow.

Analyzing mitochondrial sequences a genetic structuring within regions was not observed. It was probably due to the low polymorphism of mtMSH and MtC found in our samples, demonstrating that they could be not informative markers for corals at these geographical spatial scales (e.g. Tuscany samples were very close). In fact, Miller *et al*. (2011) did not evidence a genetic structuring in *Desmophyllum dianthus* populations at similar spatial scales, whereas they evidence differences in mitochondrial sequences distribution along a

depth gradient, as observed also in Costantini *et al*. (2010). Further studies have to be carried out to understand the ability of these mitochondrial markers to detect genetic structuring at these scales.

Based on mtMSH data set, high F_{ST} values were observed only among the two Praiano sites and all the others, but all values were not significant after FDR correction ($P > 0.01$). By contrast, using MtC data set, a high genetic structuring between Tuscany and Campania was obtained, whereas low and not significant F_{ST} values were observed within the two regions. The low level of genetic differentiation observed with shared haplotypes among samples and low level of differences between haplotypes could suggest historical links within regions and no recent dispersal events. 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) they should be viewed as unique evolutionary units (sensu Moritz 2002).

CONCLUSION

The results of this study contribute to the knowledge on the actively harvested red coral populations, as well as being useful to develop future effective management strategies, which may allow the exploitation of this resource and, at the same time, its preservation.

The high genetic structuring observed in mesophotic *C. rubrum* populations, together with previous results which indicate lack of connectivity among these and the shallow ones (Costantini *et al*. 2011), suggest that the hypothesis that deeper populations may act as refugia and/or larval supply for some shallow-water populations is unlikely to be true. Raising harvesting pressure and anthropogenic and natural disturbances could increase the fragmentation of these populations in smaller and isolated patches, reducing their genetic diversity and, consequently, their evolutionary potential and increasing the risk of extinction. Therefore, in the mesophotic habitat the management units should be defined at a regional or sub regional level, similarly to shallow-water populations (Costantini *et al*. 2007a; Ledoux *et al*. 2010a). Nevertheless, further investigations should be carried out to understand better the genetic structuring of this species in the mesophotic zone, e.g. extending studies to other deep-water populations.

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