GENETIC STRUCTURING OF THE TEMPERATE GORGONIAN CORAL CORALLIUM RUBRUM ACROSS THE WESTERN MEDITERRANEAN SEA REVEALED BY MICROSATELLITES AND NUCLEAR SEQUENCES

Federica Costantini, Cécile Fauvelot, M. Abbiati

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Authors: Costantini F, Fauvelot C, Abbiati M

Address: Centro Interdipartimentale di Ricerca per le Scienze Ambientali and Dipartimento di Biologia Evoluzionistica Sperimentale, University of Bologna, Via S. Alberto 163, I – 48100 Ravenna, Italy

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Corresponding author:
Federica Costantini
Via S. Alberto 163,
I – 48100 Ravenna, Italy
Tel. +39 0544 937401
Fax. +39 0544 937411
E-mail: federica.costantini@unibo.it

Running title: Genetic structuring of *Corallium rubrum*

Abstract

In the past decades, anthropogenic disturbance has increased in marine costal habitats leading to dramatic shifts in population size structure of various marine species. In the temperate region, the gorgonian coral *Corallium rubrum* is one of the major disturbed species, mostly owing to the exploitation of its red skeleton for jewellery purposes. Red coral is a gonocoric species inhabiting subtidal rocky habitats in the Mediterranean and neighbouring Atlantic coasts. In order to investigate the spatial genetic structuring of *C. rubrum*, five microsatellite markers and the ribosomal internal transcribed spacer 1 (ITS-1) sequences were analysed in eleven samples from the North Western Mediterranean Sea. Phylogenetic reconstructions obtained from ITS-1 sequences analysis showed that samples from Minorca and Sardinia islands were the most divergent while the three samples from the Tuscan archipelago (Argentario, Giannutri and Elba) appeared genetically homogeneous. Both markers revealed a strong structuring over large spatial scales (though to a different extent) with no isolation by distance pattern. Microsatellites based F_{ST} estimates appeared much smaller than ITS-based estimates and not significantly correlated, likely due to microsatellite allele size homoplasy typical of these highly polymorphic loci. Our study shows that the absence of clear patterns of genetic structuring over large spatial scales together with strong genetic structuring should be interpreted with caution because such patterns may hide underlying small scale genetic structuring. Our results further confirm that effective larval dispersal in red coral is highly restricted in the North Western Mediterranean Sea, suggesting that an increase of anthropogenic disturbance could aggravate the disappearance of red coral, not only along the Mediterranean coasts but also, and with more intensity, in the main Mediterranean islands.

Introduction

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 coastal habitats (Airoldi & Back 2007). Owing primarily to this disturbance, biogenic reefs in tropical and temperate habitats have suffered long-term degradation affecting both species and genetic diversities (Hellberg *et al.* 2002, Hughes *et al.* 2002, Pandolfi *et al*. 2003, Bellwood *et al.* 2004, Worm *et al.* 2006). Sustainable exploitation and conservation of marine species are among the priorities of modern bio-resource management (Gray 1997). In this context, estimating connectivity among populations is essential because dispersal is a key element warranting population resilience following disturbance (Palumbi 2003, Bellwood *et al.* 2004).

Corallium rubrum is a gorgonian coral (Anthozoa, Gorgonacea) inhabiting subtidal rocky habitats at a depth ranging from about 10 to 600 m (Taviani M, personal communication) in the Mediterranean and neighbouring Atlantic coasts (Zibrowius *et al.* 1984). Due to the high economic value of its red axial carbonate skeleton, red coral populations have been intensively harvested (Santangelo & Abbiati 2001). This major source of disturbance may drive dramatic shifts in population size structure and, eventually, lead to the extinction of local commercial banks (Santangelo & Abbiati 2001, Garrabou *et al.* 2001). Furthermore, red coral populations are vulnerable to water temperature rise (Cerrano *et al.* 2000; Garrabou *et al.* 2001). Therefore, red coral is currently listed among the species of community interest of the European Union Habitat Directive (92/43/EEC, Appendix V).

Within the Mediterranean Sea, the distribution of red coral is mainly restricted to the rocky shores of the western Mediterranean basin, though Chintiroglou *et al.* (1989) reported records along the Greek coasts of the North Aegean Sea. 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). Moreover, red coral has a strong requirement towards its habitat, defined by the morphology and biogenic nature of the substratum, as well as the range of abiotic variables (e.g. light intensity, water temperature and turbidity, sediment loads, current regime) (Weinberg 1979). As a consequence, the distribution of *Corallium rubrum* is highly fragmented at various spatial scales.

Red coral is a gonocoric species, brooding lecithotrophic planulae (Weinberg 1979). In order to investigate the effective larval dispersal of red coral in the western Mediterranean Sea, early population genetics studies have been conducted using allozymes. They have shown the occurrence of significant genetic divergences among samples at a distance of tens of kilometres (Abbiati *et al.* 1993). At shorter distances (about 200 m), no significant genetic structuring was observed using allozymes (Abbiati *et al.* 1997). However, allozyme markers, due to their low mutation rate and polymorphism may have a limited power in revealing a genetic structure at small spatial scales. Indeed, recently, Costantini *et al.* (2007) using four microsatellite loci specifically developed for *C. rubrum* (Costantini & Abbiati 2006) have found a strong genetic structuring at spatial scales of tens of meters among samples within two locations on the Ligurian Sea. These patterns are explained by the reduced swimming ability and geonegative behaviour of the planulae, together with a short larval duration (estimated from 4 to 12 days under laboratory conditions, Vighi 1972, Weinberg 1979), which suggest that, once released, larvae settle in close vicinity of the parental colonies (Vighi 1972). Additionally, differences in genetic variability and in F_{ST} estimates between locations (Costantini *et al.* 2007) suggested that environmental features (e.g. habitat characteristics,

geomorphology, hydrodynamics) strongly influence the genetic structure of red coral populations at larger geographical scales.

In order to further investigate the spatial genetic structuring of *C. rubrum*, eleven populations within the distribution range of the species in the North Western Mediterranean Sea have been analysed. Since mitochondrial DNA did not reveal intraspecific variation in red coral (Costantini *et al*. 2003), likely due to its slow evolutionary rate as observed in anthozoans (Romano & Palumbi 1997, Shearer *et al.* 2002, Hellberg 2006), in this study, two nuclear molecular markers with differing levels of polymorphism have been used. Allele frequencies at five microsatellite loci have been analysed in *C. rubrum* samples. Since microsatellites previously revealed genetic structuring at very small scales (Costantini *et al*. 2007), sequences of the internal transcribed spacer 1 region (ITS-1) of the nuclear ribosomal DNA were also analysed in order to test for homoplasy at microsatellite loci over large scales. The results of this study may help understanding how small scale processes may influence patterns over larger spatial scales in *Corallium rubrum* populations, as well as being useful to conservation and sustainable management strategies of the species at the Mediterranean scale.

Materials and methods

Sample collections and molecular analysis

Red coral colonies were collected by SCUBA diving at 11 locations along the Mediterranean coasts, between 25 and 30 m depth (Figure 1). Shoreline distance among samples ranged from 30 km (between Argentario and Giannutri) to about 2650 km (between Medes and Korcula). At each location, branch fragments from 20 to 50 individual colonies were collected and preserved in 80% ethanol at 4°C.

Total genomic DNA was extracted from 2-4 polyps per individual colonies using a cetyltrimethyl ammonium bromide (CTAB) protocol (Winnepennickx *et al.* 1993) with standard phenol-chloroform-isoamyl alcohol (25:24:1) extraction. Five microsatellite loci (COR9, COR15, COR48 and COR58, used in Costantini *et al*. (2007), and COR46) specifically developed for *Corallium rubrum* were amplified (Costantini & Abbiati 2006). Genotyping was carried out on an ABI 310 Genetic Analyser, using forward primers labelled with 6-FAM, HEX or TAMRA (MWG Biotech) and ROX HD400 (Applied Biosystems) as internal size-standards. Allele sizing was performed using GeneScan Analysis Software version 2.02 (Applied Biosystems).

Polymerase chain reaction (PCR) amplifications of the ITS-1 region were carried out using the primers ITS1-new-F 5'–TCGTAACAAGGTTTCCGTA-3' and ITS1-new-R 5'-TAACGGTGGATCTCTTGGCT-3' (Costantini 2001). Each 25 μl PCR reaction contained approximately 20 ng DNA, 1X PCR buffer (Promega), 2 mM $MgCl_2$, 0.5 μM of each primer, 0.4 mM dNTPs and 1 U of *Taq* polymerase (Promega). 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 30s, 57°C for 30s and 72°C for 60s. A final extension at 72°C for 7 min was added. Amplified fragments were purified with an ExoSAP-IT kit (Amersham Pharmacia) and cycle sequenced in both directions using an ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). Sequences were run on an ABI 310 Genetic Analyser (Applied Biosystems). Given that the cloning of PCR products is costly and laborious, and, additionally, artefacts can be produced during the cloning step due to *in vitro* recombination upon transformation of bacterial cells with heteroduplex DNA (Zhang & Hewitt 2003; Vollmer & Palumbi 2004), all ITS-1

fragments were directly sequenced without prior cloning. In order to avoid the term "haplotype", which represents an haploid component of a given sequence, the term "sequence type" was used to refer to every distinct type of ITS-1 sequence detected, as proposed by Worheide *et al.* (2002).

Genetic variability

Microsatellite diversity within samples was estimated using El Mousadik & Petit (1996) allelic richness (A) in FSTAT version 2.9.3.2 (Goudet 2001), and observed (H_{obs}) and Nei 's (1987) unbiased expected heterozygosity (H_{exp}) in GENETIX software package version 4.03 (Belkhir *et al.* 2004). All loci were tested for linkage disequilibrium using GENEPOP version 3.4 (Raymond & Rousset 1995) as implemented for online use (http://genepop.curtin.edu.au/). Single and multilocus F_{IS} were estimated using Weir & Cockerham's (1984) fixation index and, because most of F_{IS} were positive ($H_{obs} < H_{exp}$), deviations from Hardy-Weinberg equilibrium (HWE) were tested using Fisher's exact test, using the null hypothesis H_0 = no heterozygote deficiency, with the level of significance determined by a Markov-chain randomization (1000 dememorizations, 100 batches, and 1000 iterations per batch) in GENEPOP. Significance levels for multiple comparisons of loci across samples were adjusted using a standard Bonferroni correction (Rice 1989). The presence of null alleles was examined by estimating null allele frequencies for each locus and sample following the Expectation Maximization (EM) algorithm of Dempster et al. (1977) using FREENA (Chapuis & Estoup 2007).

ITS-1 sequences were edited and aligned manually using BIOEDIT version 7.0.4 (Hall 1999). Genetic diversity within samples was estimated using sequence type diversity (*h*, Nei 1987) and nucleotidic diversity (π, Nei 1987) using ARLEQUIN ver 3.1 (Excoffier *et al.* 2005).
Since ITS regions have frequent insertions/deletions (indels) that may be phylogenetically informative (Vogler & DeSalle 1994) and may originate from single evolutionary events regardless of their size (Girabet & Wheeler 1999), alignment gaps were treated using a conservative approach. Here, each indel with a different start and/or end position (i.e. not point mutations), which all sequence type either have or lack, was recoded as a new single base and added at the end of the sequence (see Table 3), and gaps were further treated as missing nucleotides.

Hierarchical likelihood ratio tests were used to determine the best-fit model of nucleotide substitution using the program MRMODELTEST version 2.2 (Nylander 2004). The Jukes & Cantor (JC) model (equal nucleotide frequencies and equal rate of nucleotide substitution between nucleotides) was supported by the likelihood ratio test ($P < 0.000001$) and was then used in following analysis. Phylogenetic trees of the relationships among sequence types were constructed using Maximum parsimony (MP) in PAUP 4.0b10 (Swofford 2002) and Bayesian inference (BI) using MRBAYES 3.1 (Ronquist & Huelsenbeck 2003). Maximum parsimony analysis was performed under the heuristic search option employing random stepwise addition, 10 random additions and TBR branch swapping. Nodal support was measured using a non parametric bootstrap method with 100 pseudo replications. BI analysis was performed using one cold chain and three incrementally heated chains with $T = 0.1$. Starting trees for each chain were set as random and all settings were default values of MRBAYES unless stated. Each Metropolis-coupled Markov chain Monte Carlo (MCMCMC) was run for 5 million generations, with trees sampled every 1000 generations, and the first million generations were discarded. Results were summarized as 50% majority rule consensus trees including compatible groupings and branch lengths. Posteriors probabilities were used to asses clade support. Trees were displayed using the software TREEVIEW version 1.6.0 (Page 1996).

Clustering analysis

The number of clusters detected among samples, *K*, was estimated by employing a Bayesian approach implemented in the program STRUCTURE version 2.2 (Pritchard *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 five microsatellite loci. Mean and variance of log likelihoods of the number of clusters for K =1 to K=12 were inferred from multilocus genotypes by running STRUCTURE 5 times with 500 000 repetitions each (burn in = 50 000 iterations) under the admixture ancestry model and the assumption of correlated allele frequencies among samples as suggested in Falush *et al.* (2003). The mean membership of each individual described the likelihood of that individuals belonging to the respective clusters. Following the recommendations of Evanno *et al.* (2005), we calculated the ad hoc statistic ΔK based on the rate of change in the log likelihood of data between consecutive K-values. Due to the presence of null alleles, the clustering analysis was conducted on the original dataset (not in HWE), using the option of null alleles coded as recessive alleles described in Falush *et al*. (2007). Indeed, we could not use a dataset adjusted for the presence of null alleles such as the so-called INA method described in Chapuis & Estoup (2007) since this procedure consider only one null allele common to all populations, which may biased the assignment tests (Chapuis & Estoup 2007, Chapuis personal communication).

Population structure analysis

Genetic divergence among samples at microsatellite loci was estimated in FREENA using the F_{ST} estimates of Weir (1996) and following the so-called ENA method described in (Chapuis & Estoup 2007) which provides unbiased F_{ST} estimates, computed excluding null alleles. Pairwise F_{ST} among samples were also estimated using the original dataset (not corrected for nulls). Genotypic differentiation among samples was tested with an exact test (Markov chain parameters: 1000 dememorizations, followed by 1000 batches of 1000 iterations per batch) with the original dataset, and the P-value of the log-likelihood (G) based on the exact test (Goudet *et al*. 1996) was estimated in GENEPOP. For the ITS-1 sequences dataset, genetic differentiation for each pairwise sample was estimated using Φ statistics (Φ_{ST} based on haplotype frequencies and molecular divergence, using JC model) and its significance determined using a permutation test (10 000 permutations) in ARLEQUIN. Sequential Bonferroni corrections (Rice 1989) for multiple comparisons were applied.

For both microsatellite and ITS-1 sequences datasets, 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 and the logarithm of the geographic distances among samples was tested using 1000 permutations within the original matrices.

An analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) implemented in ARLEQUIN was conducted to examine the partition of the genetic variance among red coral samples. With this purpose, red coral samples were grouped A) according to their sea of origin (six groups: Balearic Sea, Sardinian Sea, Ligurian Sea, North and Southern Tyrrhenian Sea, Adriatic Sea), B) based on the existing gaps in the distribution of the species (seven groups: Minorca; Sardinia; Medes; Plane and San Fruttuoso; Calafuria, Argentario, Giannutri and Elba; Palinuro; Korcula), C) *a priori*, based on the ITS-1 phylogenetic tree (four groups: Minorca; Sardinia; Tuscan archipelago samples and all the other samples, see results), and D) *a priori*, based on the results of the clustering analysis (two groups: Medes, Minorca, Plane, Sardinia, San Fruttuoso, Calafuria and Korcula; Argentario, Giannutri, Elba and Palinuro).

Results

Microsatellite loci variability and null alleles

Over all samples, locus COR15 exhibited the lowest variation (9 alleles in total), while COR9 had the largest number of alleles (33). Overall, observed heterozygosities ranged from 0.26 (in COR9 and COR46) to 0.45 (in COR15 and COR48) and expected heterozygosities ranged from 0.66 (in COR15) to 0.93 (in COR9). No linkage disequilibrium was detected among loci (all $P > 0.05$ after Bonferroni corrections) and all loci were therefore considered genetically independent. *Corallium rubrum* samples showed considerable variation in their allelic richness over all five microsatellite loci, ranging from 5.1 for Giannutri to 10.0 for Minorca (Table 1). Significant multilocus deviations from Hardy-Weinberg expectations (HWE) were observed in all samples (Table 1). Multilocus estimates of F_{IS} ranged from 0.15 to 0.71, showing in all cases heterozygote deficiencies. Significant deviations from HWE were also observed in nearly all samples for each locus, with the exception of COR15, the less polymorphic one. Assuming HWE, estimated null allele frequencies (R) ranged among loci from 0 to 0.78 (Table 1). Within each locus, the number of expected null homozygotes within samples based on HWE ($N*R^2$) was significantly higher than the average number of observed null homozygotes for COR9, COR46 and COR48 (paired t-test, $P < 0.001$, $P = 0.006$ and $P = 0.012$ respectively). Over all samples, the average number of expected null homozygotes per locus was significantly higher than the average number of observed null homozygotes (paired t-test, $P = 0.035$).

ITS-1 sequence variation and phylogenetic analysis

Intra-individual polymorphism was detected in 17.5% of the 280 amplified individuals (through the presence of 1-3 doublet chromatogram peaks or partially unreadable sequences relatives to indels). This polymorphism was distributed unequally among samples, with a number of failed sequences ranging from 0 in Plane and Argentario samples to 15 in Giannutri sample (Table 2). Individuals showing such polymorphism in at least one nucleotide position were excluded for further analysis, reducing the total number of analyzed individuals to 231.

Across all 231 individuals, the length of the amplified ITS-1 fragment varied among individuals between 228 and 266 bp due to the presence of numerous indels (Table 3). Single base pair indels were mostly associated with the presence of homopolymers but not with microsatellite repeat regions as observed in ITS1 for *Acropora* (van Oppen *et al.* 2000, Vollmer & Palumbi 2004). Including all gaps, the alignment of all individual sequences consisted of 278 positions of which 87 were variable, corresponding to 35 nucleotide substitutions and 52 indels. The sequence alignment (Table 3) showed two highly variable blocks of indels and minor stutters. The first large indel, from 158 to 188 bp, correspond to a 31 bp deletion that was observed only in the Tuscany archipelago samples (Argentario, Giannutri and Elba; sequence types 30, 31, 32, and 45, Table 2). The second highly variable region consist of two smaller indels, situated between 210 to 222 bp and between 244 and 246 bp, that were found only in Minorca sample (sequence types from 10 to 15, Table 2). Out of the 51 different sequence types revealed, nine were found in more than one sample (sequence types 1-5-6-17-29-32-42-49-50, Table 2). Sequence type 5 was the most widespread (seven samples out of 11), though not the most abundant one (12%; Table 2). The most abundant sequence type (sequence type 17, 18.6%) was found only in Plane and Calafuria. The remaining sequence types were unique to a single location and often found in a single individual (28 singletons in total). Genetic variation across locations varied considerably, with a sequence type diversity (h) ranging from 0.273 in Calafuria to 0.890 in Minorca, and a nucleotide diversity (π) ranging from 0.003 in San Fruttuoso to 0.071 in Elba (Table 2).

The two phylogenetic tree reconstruction methods, Maximum Parsimony (MP) and Bayesian

Inference (BI), produced similar trees for the 51 ITS-1 sequence types so that only the BI consensus unrooted tree is presented, with both BI posterior probability values and bootstrap values greater than 50% for MP (Figure 2). Four highly supported clades (with BI posterior probability >0.95) could be identified: one consisting of all but one Minorca sequence types (10, 11, 12, 13, 14, 15), one containing sequence types found exclusively in the Tuscany archipelago samples (Giannutri, Elba and Argentario; 30, 31, 32, 45); one containing sequence types found exclusively in Giannutri (34, 35, 36, 37); and one including two sequences found in Sardinia (23) and Medes (8) (Figure 2).

Population genetic analysis

Testing the significance of the stepwise clustering procedure performed in STRUCTURE resulted in a separation of the samples into 2 clusters (cluster 1: Medes, Minorca, Plane, Sardinia, San Fruttuoso, Calafuria and Korcula; cluster 2: Argentario, Giannutri, Elba and Palinuro, $\Delta K = 104.5$, Figure 3). The lowest proportion of membership to a particular cluster is 0.563 in cluster 2 for Calafuria sample and the highest proportion is 0.973 in cluster 2 for Giannutri sample (Figure 4).

High levels of genetic differentiation were found among Mediterranean Sea samples for both microsatellite loci and ITS-1 sequences datasets (Figure 5). Genetic differentiation estimates between microsatellites loci and ITS-1 sequences varied greatly (Figure 5) and were not significantly correlated ($P = 0.87$). For the microsatellite dataset, pairwise F_{ST} estimates based on the ENA method ranged from 0.053 (Minorca vs. Planes) to 0.305 (Giannutri vs. Sardinia; data not shown) and all pairwise comparisons were highly significant ($P < 0.001$). Though these pairwise F_{ST} estimates were significantly smaller than estimated from the original dataset (paired t-test, $P < 0.001$), they were significantly correlated (Pearson r = 0.974, $P < 0.001$).
For the ITS-1 sequence dataset, Φ_{ST} ranged from 0.004 (Calafuria vs. Plane) to 0.789 (Calafuria vs. San Fruttuoso) and two out of the 55 pairwise Φ_{ST} estimates were not statistically significant after sequential Bonferroni correction (Calafuria vs. Plane, and Giannutri vs. Argentario; data not shown). For both datasets (microsatellite and ITS-1), genetic differentiation estimates were not significantly correlated with the logarithm of geographic distances (Figure 5; $P = 0.273$ for microsatellites, $P = 0.136$ for ITS-1)

The AMOVAs conducted among red coral samples using ITS-1 sequence dataset showed a significant differentiation among samples within all defined groups ($P < 0.001$ for all four analyses, Table 4). A significant variance was observed among groups when grouping samples *a priori* based on the phylogenetic results, representing 33% of the total nuclear variance (i.e. four groups: Minorca vs. Tuscany archipelago vs. Sardinia vs. all others; Va = 0.813, $P = 0.002$, Table 4). Based on the existing gaps in the distribution of the species (i.e. seven groups) or based on the results of the clustering analysis, the nuclear variance attributed among the groups was not significant (Va = 0.483, $P = 0.109$; Va = 0.061, $P = 0.120$, respectively; Table 4). Using the microsatellite dataset, the nuclear variance distributed among groups was not significant except for the grouping based on the clustering analysis (Va = 0.065, $P = 0.020$) and more than 80% of the total variance was observed within samples whatever the grouping (Table 4).

Discussion

Despite new molecular genetic tools and the plethora of information that is now available for population-level processes describing marine species, our knowledge of evolutionary processes governing Anthozoan coral populations is surprisingly poor, mainly due to the problem in accessing appropriate genetic markers in these species (see van Oppen & Gates 2006 for a review). Indeed,

whereas in most animal groups the mitochondrial genome provides an appropriate tool for such investigations, the use of mitochondrial DNA in Anthozoa is limited due to its extremely low evolution rates (Romano & Palumbi 1997, Shearer *et al.* 2002, Hellberg 2006, Calderon *et al.* 2006). In particular, in *Corallium rubrum*, no sequence variability was observed in the mitochondrial COI and 16S genes (Costantini *et al.* 2003, Calderon *et al.* 2006). Consequently, for anthozoans, nuclear markers have been mostly used for intraspecific studies (van Oppen & Gates 2006), with microsatellites and ITS-1 sequences being nowadays predominant (Rodriguez-Lanetty & Hoegh-Guldberg 2002, Maier *et al.* 2005, Magalon *et al.* 2005, Costantini *et al.* 2007).

The main result of our study is that *Corallium rubrum* samples in the Mediterranean Sea are highly genetically heterogeneous. However, the two assessed nuclear markers provide different genetic divergence estimates among samples. Polymorphism levels found in *C. rubrum* samples using microsatellites (H_{exp} = 0.59 – 0.79) are comparable with those observed in the gorgonian *Pseudopterogorgia elisabethae* (H_{exp} = 0.09 – 0.78, Gutierrez-Rodriguez & Lasker 2004) and in the range of those observed for scleractinian corals (Maier *et al.* 2005, H_{exp} = 0.73 ± 0.04; Magalon *et al.* 2005, H_{exp} = 0.75 ± 0.01). However, they are much higher than those obtained with allozyme for the same species (H_{exp} = 0.073 – 0.104, Abbiati *et al.* 1993), as expected from the higher mutation rates and polymorphism of microsatellite loci compared to allozyme loci (Estoup & Angers 1998). Heterozygote deficits (strong positive deviations from HWE) were detected for all samples and at almost all microsatellite loci. Heterozygosity deficits have frequently been observed in marine invertebrates (Duran *et al.* 2004a; Addison & Hart 2004), including in many shallow-water Anthozoa (Ayre & Hughes 2000; Ridgway *et al.* 2001; Le Goff-Vitry *et al.* 2004). In *Corallium rubrum*, the heterozygote deficit observed may result from technical factors, such as the presence of null alleles, or biological factors, such as inbreeding, Wahlund effect or selection. In our case, as in Costantini *et al.* (2007), non amplifying individuals (null homozygotes) were found in all samples suggesting the occurrence of null alleles. However, observed frequency of null homozygotes were significantly smaller than expected if the heterozygote deficiencies were generated only by null alleles under HWE. In a previous study, Costantini *et al.* (2007) showed that heterozygosity deficiencies found in four proximate (less than 100 meters) samples of red coral collected both within San Fruttuoso and Calafuria were explained by the combined occurrence of null alleles (detected by the presence of non amplifying individuals), inbreeding (more inbred individuals were found than under the hypothesis of random mating) and Wahlund effect (revealed using a maximum-likelihood partitioning method). The heterozygote deficiencies observed in the present analysis further suggest that the latter two biological processes co-occur within all Mediterranean populations of red coral.

ITS-1 region sequences investigation may be successful in resolving phylogeographic relationships at large spatial scales, such as in other coral studies (Le Goff-Vitry *et al.* 2004, on *Lophelia pertusa*; Rodriguez-Lanetty & Hoegh-Guldberg 2002, on *Plesiastrea versipora*). However, in Anthozoan, ITS-1 sequence markers may suffer from intra-individual rDNA variability overlapping with within-species rDNA variation (Wei *et al*. 2006, Vollmer & Palumbi 2004, Marquez *et al*. 2003). Indeed, although concerted evolution is expected to homogenize all copies of the rDNA families within individuals (Takabayashi *et al*. 1998), intra-individual variability has been detected in various scleractinian species through cloning and sequencing of ITS PCR products (Vollmer & Palumbi 2004, Ridgway & Gates 2006). For the gorgonian *Corallium rubrum*, we observed 17.5% of unreadable sequences (due to the presence of 1-3 doublet chromatogram peaks or a frame shift in the sequences relative to indels), which may be interpreted as cases of intra-individual polymorphism. In order to avoid including cloning errors and intragenomic variants as sources of

additional sequence types (van Oppen & Gates 2006, but see Vollmer & Palumbi 2004 concerning scleractinians corals), we have decided to exclude the individuals showing intra-individual variability from the analyses rather than clone and sequence ITS PCR products. Though excluding individuals showing intra-individual variability (i.e heterozygotes) may reduce the overall frequency of rare alleles, making samples more heterogeneous than they truly are, the fact that the percentage of excluded individuals vary greatly among samples (from 0 to 59%), with the highest values observed in samples where the indels were observed among individuals (Minorca, Sardinia, San Fruttuoso and Giannutri), suggests that the sequences carrying indels were found at very low frequency elsewhere than currently observed, minimizing the bias of our method.

Despite the intragenomic variation of the ITS-1 and the overestimated genetic differentiation among samples resulting from our method to handle it, some clear patterns are drawn from the phylogenetic analyses. Minorca and Sardinia samples both showed a high genetic variability (0.8 – 0.9 sequence type diversity, 1.3 - 3.3% nucleotide diversity) with seven and six private sequence types respectively. Phylogenetic reconstructions showed that Minorca sample appear the most divergent, as expected from its particular insular situation and the complex atmospheric and hydrological systems observed in the Balearic Islands (La Violette *et al.* 1990). Sardinia appears less divergent from the coastal samples compared to Minorca, as expected from its closer location to Corsica and Ligurian coastlines. However, for both islands, we could not propose hypothesis explaining patterns of genetic divergence, neither date the origin of divergence. Indeed, ITS-1 region is a nuclear marker experiencing recombination and, to our knowledge, no proper evolutionary rate has been defined in Anthozoa for this marker.

Corallium rubrum analysis revealed a high degree of genetic differentiation between samples throughout the North Western Mediterranean Sea, with apparent genetic homogeneity within the Tuscan archipelago (revealed using ITS-1 sequences, though microsatellite revealed significant differentiation). Microsatellites and ITS-1 sequences showed no correlation between genetic differentiation and geographical distance among the analysed samples. In some cases, for ITS-1 sequence data, small geographical distances were associated with relatively small F_{ST} estimates, such as was found when comparing the Argentario and Giannutri samples using ITS-1. In other cases, F_{ST} estimates were large despite geographical proximity, such as was found when comparing the Calafuria and Elba samples for both markers. Such lack of correlation between pairwise F_{ST} estimates and geographical distances may support an island model of dispersal, where larvae move from one area to another with equal probability, rather then an isolation by distance model, where the probability of dispersal between sites declines with increasing geographical distance.

While microsatellite data showed no correlation between F_{ST} estimates and geographic distances ranging from 30 to 2650 km, Costantini *et al.* (2007) have shown the occurrence of significant genetic structuring at spatial scales of ten meters with a significant isolation by distance at the spatial scale of less than one kilometer, suggesting that larval dispersal in red coral is highly restricted (likely few meters). Therefore, due to their high variability and polymorphism, microsatellites likely appear useless in revealing a clear genetic structuring pattern at the Mediterranean scale. Indeed, microsatellites and ITS-1 are non-coding nuclear markers that should reveal the same patterns under neutrality. However, microsatellites based F_{ST} estimates appeared much smaller as compared to ITS-based estimates (Figure 5).This discrepancy is likely due to allele size homoplasy typical of microsatellites (identity by state but not by descent) resulting from their high mutation rates and allele size range constraints (Goldstein *et al.* 1995, Slatkin 1995). Similar results have been obtained when using loci differing in heterozygosities (O'Reilly *et al.* 2004) or

when using highly polymorphic loci over spatial scales similar to the geographical range of the species (Peijnenburg *et al.* 2006).

Overall, the AMOVAs conducted on the ITS-1 sequences indicated a partitioning of the total genetic variability in four groups: one main group gathering samples belonging to the western/Ligurian Sea (Medes, Plane, San Fruttuoso, Calafuria samples), a second main group of samples from the Tyrrhenian/eastern Seas (Tuscany archipelago, Palinuro and Korcula samples), and two other groups representing an island sample each: Minorca and Sardinia respectively. Using microsatellites data, a subdivision in two clusters identified with a clustering analysis method was confirmed by the AMOVA. However, samples within clusters were not homogeneous (high genetic differentiation for all pairwise comparisons was found), and these results are likely biased by microsatellite size homoplasy (see previously). Strong patterns of genetic divergence over the North Western Mediterranean Sea have already been reported for other marine invertebrates, and mainly result from large or medium-scale hydrodynamic process or by geomorphologic characteristics of Mediterranean Sea. Duran et al. (2004a, 2004b), using both microsatellite loci and ITS sequences, showed that Mediterranean populations of the marine sponge *Crambe crambe* are highly divergent due to the restricted gene flow and follow the isolation by distance model. In this sessile invertebrates, currents and behaviour of the lecitotrophic larvae have a strong influence on the effective larval dispersal, as it may be the case for red coral. Similarly, Lejeusne & Chevaldonné (2006) have found high genetic structuring at the Mediterranean scale in two brooding cave-dwelling mysids. While restricted gene flow with isolation by distance explains the patterns observed at the finest scale, habitat disjunction (natural fragmentation) and coastal geomorphology strongly influence population structuring since, in these mysids, dispersal is only possible at the adult stage.

The results obtained in this study on population genetics of *Corallium rubrum* in the Mediterranean Sea showed the occurrence of a strong large scale structuring and the saturation of the microsatellite loci as compared to the ITS-1 sequences. Lack of clear patterns at the Mediterranean scale together with the results on small scale structuring from Costantini *et al*. (2007) have major implications for the conservation and the sustainable management of this species. Indeed, populations of *C. rubrum* are genetically differentiated at the small scale so that management plans have to be developed at the local scale. Our findings also suggest that, for species with restricted dispersal, absence of clear patterns of genetic structuring over large spatial scales derived form highly polymorphic markers such as microsatellite loci should be interpreted with caution. In fact, chaotic genetic structure at large scales may reveal underlying small scale genetic structuring. Use of different markers (e.g. nuclear or mitochondrial sequences) together with analyses at small spatial scales may help understanding the effective larval dispersal and prevent erroneous interpretation of the genetic patterns observed.

These results, together with studies on demography (e.g. Santangelo & Abbiati 2001; Garrabou & Harmelin 2002; Torrents *et al.* 2005) and reproductive structure (Santangelo *et al.* 2003) have important consequences for red coral populations resilience. Indeed, an increase of natural and anthropogenic source of mortality such as over harvesting (Santangelo & Abbiati 2001) or water temperature anomalies (Cerrano *et al.* 2000; Garrabou *et al.* 2001) could lead to the disappearance of red coral in shallow water habitats, not only along the Mediterranean continental coasts (Torrents *et al.* 2005) but also along the Mediterranean islands. Further studies that include additional Mediterranean and Atlantic regions, as well as deep sea and peripheral populations of red coral are

needed to identify bio-geographical discontinuities and refugia populations of this highly valuable species.

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Figure Legends:

Figure 1: Location of the eleven *Corallium rubrum* samples (represented by black dots). Sample names are given based on the name of the proximate island or based on the proximate city. Minorca (MIN) and Medes (MED) are located in the Balearic Sea; Sardinia (SAR) is located in the Sardinian Sea; Plane (PLA), San Fruttuoso (SAN) and Calafuria (CAL) are located in the Ligurian Sea; Elba (ELB), Giannutri (GIA) and Argentario (ARG) are located in the Tuscan archipelago, north Tyrrhenian Sea; Palinuro (PAL) is located in the southern Tyrrhenian Sea; Korcula (KOR) is located in the Adriatic Sea. Dominating surface currents are indicated as grey arrows (based on Pinardi & Masetti 2000).

Figure 2: Unrooted phylogenetic tree of 51 ITS-1 sequence types of *Corallium rubrum* using Bayesian inference (BI). The MP tree showed the same pattern. Posterior probability (BI) and bootstrap values are indicated (BI/MP). Numbers in brackets denote the number of individuals per sample for each corresponding clade.

Figure 3: Values of Δ*K*, calculated as in Evanno *et al*. (2005) and based on the log likelihood of the data given by STRUCTURE version 2.2 for each number of cluster assumed (*K*).

Figure 4: Results of the clustering analysis conducted in STRUCTURE 2.2. In the bar plot, each of the 370 individuals is represented by a vertical bar indicating its estimated proportion of membership to each cluster (represented by different colours). Under the bar plot is indicated the mean proportion of membership of each sample to each cluster.

Figure 5: Relationship between genetic differentiation estimates and the logarithm of geographical distance among *Corallium rubrum* samples for both microsatellite and ITS-1 markers.

Authors Information Box

This paper was part of the PhD work of Dr Federica Costantini supervised by Prof. Marco Abbiati, which aimed to focus on population genetics and molecular systematic of marine invertebrates in coastal habitats, mainly for biodiversity conservation purposes. Dr Cécile Fauvelot is a molecular ecologist who has worked on both coral reef fish and rainforest butterfly population genetics and contributed to this work during her post doctoral position in Ravenna.

Table 1: Summary of genetic diversity at five microsatellite loci from *Corallium rubrum* samples; n: number of sampled individuals; N: number of genotypes per loci; A: allelic richness based on 16 ind.; H_{obs}: observed heterozygosity; H_{exp}: expected heterozygosity; F_{IS}: Weir and Cockerham's (1984) estimate of Wright's (1951) fixation index (bold type indicate significant deviations from HWE after standard Bonferroni correction); *R*: null allele frequency based on Dempster *et al*. (1977) estimator.

	MED	**MIN**	**PLA**	**SAR**	**SAN**	**CAL**	**ARG**	**GIA**	**ELB**	**PAL**	**KOR**
n	24	24	20	28	50	50	48	36	32	24	34
COR9											
N	23	21	18	26	46	47	44	35	30	23	32
A	12.3	9.1	11.8	9.3	7.5	12.2	7.6	7.8	7.1	6.7	5
*H*obs	0.39	0.24	0.22	0.31	0.26	0.21	0.2	0.34	0.37	0.22	0.12
*H*exp	0.86	0.8	0.87	0.84	0.62	0.89	0.79	0.85	0.74	0.73	0.72
F_{IS}	**0.56**	**0.71**	**0.76**	**0.64**	**0.58**	**0.77**	**0.75**	**0.60**	**0.52**	**0.71**	**0.83**
R	0.29	0.42	0.42	0.35	0.32	0.40	0.40	0.30	0.29	0.34	0.40
COR15											
N	24	24	18	28	47	50	48	35	32	24	34
A	3.6	4.8	1	3	3	3.6	3	2.4	3.2	3.7	3.8
*H*obs	0.5	0.54	0	0.46	0.36	0.68	0.42	0.37	0.25	0.83	0.41
*H*exp	0.53	0.5	0	0.44	0.53	0.67	0.56	0.32	0.34	0.67	0.62
F_{IS}	0.08	-0.06	-	-0.04	0.33	-0.001	0.27	-0.13	**0.28**	-0.22	0.35
R	0.01	0.00	0.32	0.02	0.22	0.00	0.08	0.09	0.09	0.00	0.11
COR46											
N	22	22	18	16	20	42	46	35	29	24	29
A	9.6	12.4	6	9	7.9	7.7	8.5	3.9	4.6	9.5	8
*H*obs	0.27	0.5	0.17	0	0.1	0.48	0.37	0.08	0.24	0.79	0.31
*H*exp	0.87	0.87	0.75	0.86	0.82	0.74	0.81	0.48	0.55	0.83	0.83
F_{IS}	**0.70**	**0.44**	**0.79**	**1**	**0.88**	**0.94**	**0.55**	**0.83**	**0.57**	0.07	**0.64**
R	0.39	0.28	0.42	0.71	0.78	0.52	0.29	0.32	0.33	0.03	0.41
COR48											
N	21	23	18	24	47	48	48	36	29	24	34
A	11.1	15.3	8.8	8.8	5.8	6.6	9.8	5.4	8.2	10.4	8.2
*H*obs	0.43	0.96	0.33	0.17	0.25	0.27	0.42	0.64	0.27	0.92	0.56
*H*exp	0.85	0.91	0.79	0.82	0.66	0.73	0.82	0.65	0.8	0.81	0.78
F_{IS}	**0.51**	-0.03	**0.60**	**0.80**	**0.62**	**0.64**	**0.50**	0.04	**0.67**	-0.11	**0.30**
R	0.34	0.05	0.35	0.47	0.32	0.31	0.23	0.03	0.38	0.00	0.12
COR58											
N	23	22	18	27	45	46	46	34	29	21	32
A	9	8.4	5	2.7	6.6	13.3	7.5	6.4	7.8	10.4	7.1
*H*obs	0.39	0.41	0.28	0	0.2	0.5	0.54	0.29	0.65	0.67	0.34
*H*exp	0.82	0.8	0.76	0.14	0.76	0.89	0.59	0.63	0.81	0.88	0.67
F_{IS}	**0.54**	**0.51**	**0.65**	**1**	**0.74**	**0.45**	0.09	**0.54**	0.20	**0.26**	**0.50**
R	0.28	0.30	0.37	0.26	0.41	0.28	0.11	0.29	0.21	0.25	0.27
Multilocus											
A	9.1	10	6.5	6.5	6.2	8.7	7.3	5.2	6.2	8.1	6.4
*H*obs	0.4	0.53	0.2	0.19	0.23	0.34	0.39	0.35	0.36	0.68	0.35
*H*exp	0.79	0.78	0.63	0.62	0.68	0.78	0.71	0.59	0.65	0.78	0.72
F_{IS}	**0.51**	**0.34**	**0.70**	**0.71**	**0.66**	**0.57**	**0.46**	**0.42**	**0.46**	**0.15**	**0.53**

Table 2: Distribution of sequence types and genetic diversity in *Corallium rubrum* samples; number of individuals amplified per sample (N), number of individuals per sample with analysed sequence (n), total number of sequence type (H), sequence type diversity (*h*, Nei 1987), nucleotide diversity (π, Nei 1987).

	MED	**MIN**	**PLA**	**SAR**	**SAN**	**CAL**	**ARG**	**GIA**	**ELB**	**PAL**	**KOR**	***Total***
N	**24**	**15**	**20**	**17**	**22**	**34**	**34**	**36**	**20**	**24**	**34**	***280***
1	**11**		**1**									***12***
2	**1**											***1***
3	**1**											***1***
4	**1**											***1***
5	**4**					**4**	**8**	**1**	**4**	**5**	**2**	***28***
6	**3**		**1**							**4**		***8***
7	**1**											***1***
8	**1**											***1***
9		**1**										***1***
10		**1**										***1***
11		**3**										***3***
12		**1**										***1***
13		**1**										***1***
14		**3**										***3***
15		**1**										***1***
16			**2**									***2***
17			**15**			**28**						***43***
18			**1**									***1***
19				**1**								***1***
20				**1**								***1***
21				**5**								***5***
22				**2**								***2***
23				**1**								***1***
24				**1**								***1***
25					**7**							***7***
26					**2**							***2***
27						**1**						***1***
28							**2**					***2***
29							**15**	**7**				***22***
30							**2**					***2***
31							**1**					***1***
32							**4**	**4**	**7**			***15***
33							**2**					***2***
34								**1**				***1***
35								**2**				***2***
36								**1**				***1***
37								**1**				***1***
38								**1**				***1***
39								**1**				***1***
40								**2**				***2***
41									**1**			***1***
42									**1**		**26**	***27***
43									**2**			***2***
44									**1**			***1***
45									**1**			***1***
46										**1**		***1***
47										**4**		***4***
48										**1**		***1***
49										**4**	**2**	***6***
50										**1**	**1**	***2***
51											**1**	***1***
n	**23**	**11**	**20**	**11**	**9**	**33**	**34**	**21**	**17**	**20**	**32**	***231***
H	**8**	**7**	**5**	**6**	**2**	**3**	**7**	**10**	**7**	**7**	**5**	***51***
h	**0.747**	**0.89**	**0.442**	**0.8**	**0.39**	**0.273**	**0.747**	**0.862**	**0.794**	**0.853**	**0.341**	***0.92***
π	**0.01**	**0.033**	**0.005**	**0.013**	**0.003**	**0.005**	**0.046**	**0.044**	**0.071**	**0.012**	**0.006**	***0.046***

Table 3: Sequence differences amongst the 51 sequence types found in *Corallium rubrum* samples. Dots indicate identical bases and hyphens (-) indicate indels. The entire sequence of sequence type 1 was deposited in Genbank, accession number XXXXX. From 275 bp to 296 bp are showed the 22 added nucleotide positions corresponding to recoded indels (see Material and Methods section).

	1111111111111111111111111111111111111111111111111111222222222222222222222222222	22222222222222222222
	1122333444456990234444455556666666666777777777788888888889999011111122223444555566677	77777888888888999999
	4707268124832379943567847890123456789012345678901234567893478701467890126645601670570	56789012345678901234
1	TTTGA-C-GAT---ACGTGG---AGAATGGGG-TAACGGGTCACA--TATAAATCGCTGCC-GTTT-----ATT---TA--ATTTTT	TCAGCAGACACACTTACAGTGT
2	.....-.-...---.....---.........-.............--.............C....-----..---..--.....	.............C........
3	.....-.-...---.....---.........-.............--.............G-....-----..---..--.....	....
4	.....-.-...---...A..---.........-.............--.............G-....-----..---..--.....	....
5	.....-.-...---...A..---.........-.............--.............-....-----..---..--.....	....
6	.....-.-...---...A..---.........-.............--.............C....-----..---..--.....	.............C........
7	.....-.-...---...A..---.........-.............--.........G....C....-----..---..--.....	.............C........
8	.....-.-...---...A..---.........-.............--.........G....-A...-----T..---C.-C.....	C
9	.....-.-...-A-...A..---.........-.............--.............-....-----..---..--.....G	....A..............
10	.....-T-...---...A..G--.........-.............--.............-AA..TTT--T..ACG..--..C..G	.......G.......TT..A..
11	.C..G-.-..C---...A.----.........-.............--.............-.AA.TTTTTC..ACG.G--.....G	......T........TTTTA..
12	.....-T-...---...A..G--.........-.............--...........C..-AA..TTTT-...ACG..--.....G	.......G.......TTT.A..
13	.C..G-.-C.C---...A.----.........-.............--.............-.AA.TTTTTC..ACG.G--.....G	......T........TTTTA..
14	.....-T-...---...A..G--.........-.............--...........C..-AA..TTT--T..ACG..--..C..G	.......G.......TT..A..
15	.....-T-...---...A..G--.........-.............--...........C..-AAA.TTTT-T..ACG..--..C..G	.......G.......TTT.A..
16	.....-.-...---...A.-----.T.....-.............--.............C....------..C---..--.....	......T......C........
17	.....-.-...---...A.----.........-.............--.............C....-----..---..--.....	......T......C........
18	.....-.-...---...A..G--.........-.............--.............C....-----..---..--.....	.......G.....C........
19	.....-.-...T--...A.----.........G.............--.............C....-----..---..--.....	...T..T...G..C........
20	C....-.-...---...A.----.........G.............--.............C....-----..---..--.....	......T...G..C........
21	.....-.-...---...A.----.........G.............--.............C....-----..---..--.....	......T...G..C........
22	.....-.-...---...A.----.........G.............--...........T....C....-----..---..--.....	.......T...G..C........
23	C....-.-...---...GC.G--C........G............CT.............-A...-----T..---..AC.....	.......G..GC........AC
24	.....-.-...---...A..---.........G.............--.............C....------.AG---..--.....	..........G..C........
25	..-T.-.-...---...A..---.........-.............--.............-....-----..---..--..... C.....	
26	...T.-.-...---...A.----.........-.............--.............C....-----..---..--.....	......T......C........
27	.....-.-...---...A.----.........-.............--.............-....------..C---..--.....	......T...............
28	.....-.T...-A-...A..---.........-.............--.............-....-----..---..--.....	..T.A..............
29	.....-.-...-A-...A..---.........-.............--.............-....-----..---..--.....	....A..............
30	.....-.T...---G..A.----..------------------------------...T.-....-----..---..--.....	..T...T..TAG..........
31	.....-.T...---...A.----..------------------------------...T.-....-----..---..--.....	..T...T..TAG..........
32	.....-.-...---...A.----..------------------------------...T.-....-----..---..--.....	......T..TAG..........
33	.....-.-...---...A.----.........-.............--.............-....-----..---..--.....	......T...C...........
34	.....-.-...-A-...A..---.........-.............--.............-...A-----..G---..--....G.	....A.....C...A.......
35	.....-.-...-A-...A..---.........-.............--.............-...A-----..G---..--.....	....A.........A.......
36	.....-.-...-A-...A..---.........-.............--.............-...A-----..---..--.....	....A.........A.......
37	.....-.-...---...A..---.........-.............--.............-...A-----..---..--.....	..............A.......
38	.....-.-...---...A..---.........-.............--.............-....-----..---..--....G.	....
39	.....-.-...---...A.----.........-.............--.............-....-----..---..--....G.	......T..............
40	.....-.-...---...A..---.........-.............--.G...........-....-----..---..--.....	...........G.........
41	.....-.-...---..AA..---.........-.............--.............-....-----..---..--.....	....
42	.....-.-.G.---...A..---.........-.............--.............-....-----..---..--.....	....
43	.....A.-...T--...A..GGG.........-.............--.............-....-----..---..--.....	.A.T...GG.............
44	.....-.-...T--...A..---.........-.............--.............-....-----..---..--.....	...T..................
45	.....-.-...---...A.----..------------------------------.G.T.-....-----..---..--.....	......T..TAG..........
46	.....-.-...--G...A..---.........-.............--.............-....-----..---..--.....	.....G................
47	.....-.-...---...A..---.........-.............--.............-...------T..---..--C....	..............C........
48	.....-.-...---.T.A..---.........-.............--.............-....-----..---..--.....	....
49	.....-.-...---...A..---.........-.............--.............-....-----..---..--G.....	....
50	.....-.-...---...A..---.........G.............--.............C....-----..---..--.....	..........G..C........
51	.....-.-...---...A..---.........G.............--.............C....AT---..---..--...C..	..........G..C.C......

804 **Table 4:** Analysis of molecular variance (AMOVA) among samples of *Corallium rubrum* using
805 ITS-1 dataset and microsatellite dataset. Red coral samples were grouped according to A) their sea
806 of origin (six groups: Balearic Sea, Sardinian Sea, Ligurian Sea, North and Southern Tyrrhenian
807 Sea, Adriatic Sea), B) the existing gaps in the distribution of the species (seven groups: Minorca;
808 Sardinia; Medes; Plane and San Fruttuoso; Calafuria, Argentario, Giannutri and Elba; Palinuro;
809 Korcula), C) the phylogenetic results (four groups: Minorca; Sardinia; Tuscan archipelago samples
810 and all the other samples) and D) the clustering analysis results (two groups: Minorca, Sardinia,
811 Medes, Plane, San Fruttuoso, Calafuria and Korcula; Argentario, Giannutri, Palinuro and Elba). * P
812 < 0.05, *** P < 0.001.
813

		ITS - 1			Microsatellites		
	Source of variation	**d.f.**	**Variance components**	**%**	**d.f.**	**Variance components**	**%**
(A)	Six basins						
	Among basins	5	1.187	8.66	5	0.059	3.12
	Among samples within basins	5	0.872	40.42***	5	0.300	15.95***
	Within samples	220	1.098	50.93***	729	1.521	80.93***
(B)	seven groups						
	Among groups	6	0.483	21.77	6	0.049	2.62
	Among samples within groups	4	0.637	28.71***	4	0.309	16.45***
	Within samples	220	1.098	49.52***	729	1.521	80.93***
(C)	Four groups						
	Among groups	3	0.813	33.19***	3	0.06	3.27
	Among samples within groups	7	0.538	21.96***	7	0.31	16.31***
	Within samples	220	1.098	44.85***	729	1.52	80.41***
(D)	Two groups						
	Among groups	1	0.061	2.82	1	0.065	3.42*
	Among samples within groups	9	1.002	46.36***	9	0.315	16.59***
	Within samples	220	1.098	50.82***	729	1.521	79.99***

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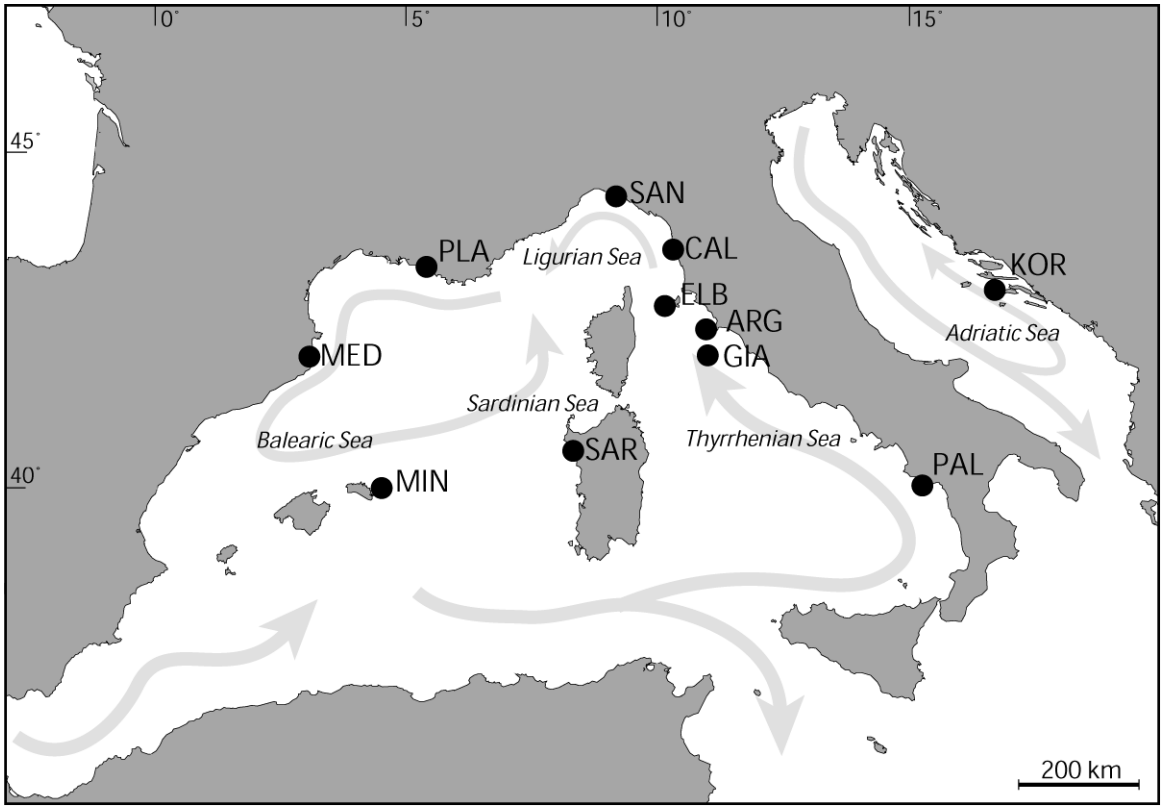
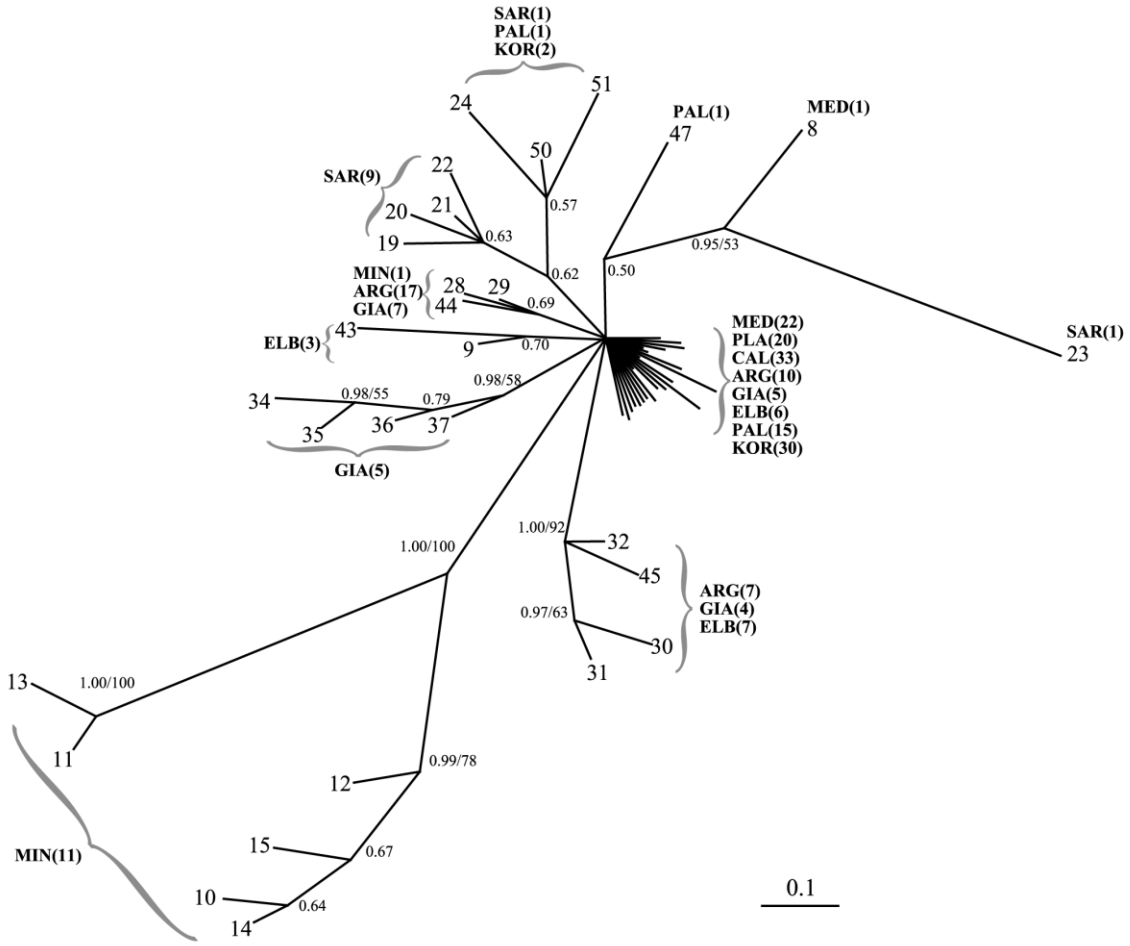

10°
5°
10°
15°
45°
40°
SAN
PLA
Ligurian Sea
CAL
ELB
ARG
GIA
KOR
Adriatic Sea
MED
Sardinian Sea
Balearic Sea
SAR
Thyrrhenian Sea
PAL
MIN
200 km

Figure 1



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826
827 **Figure 2**
828

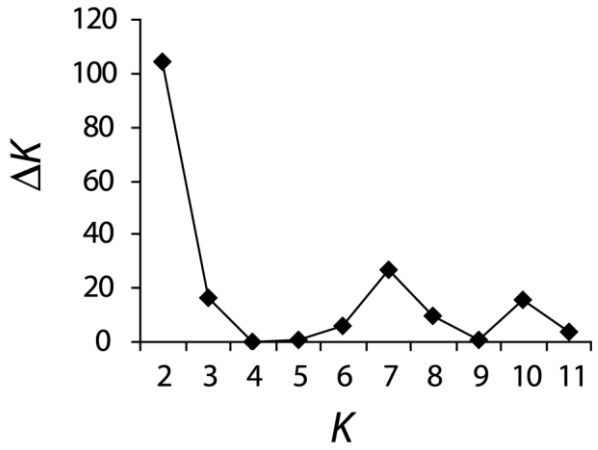
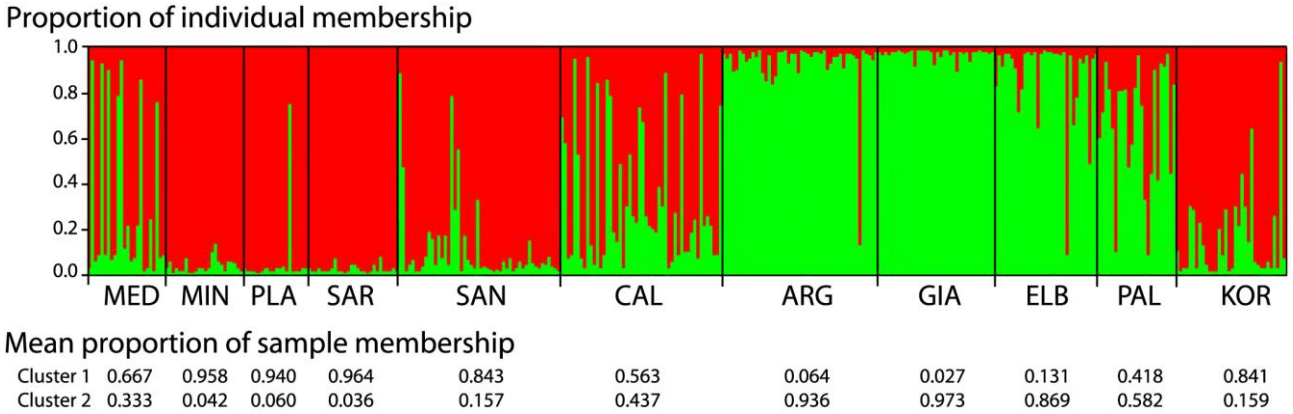


Figure 3



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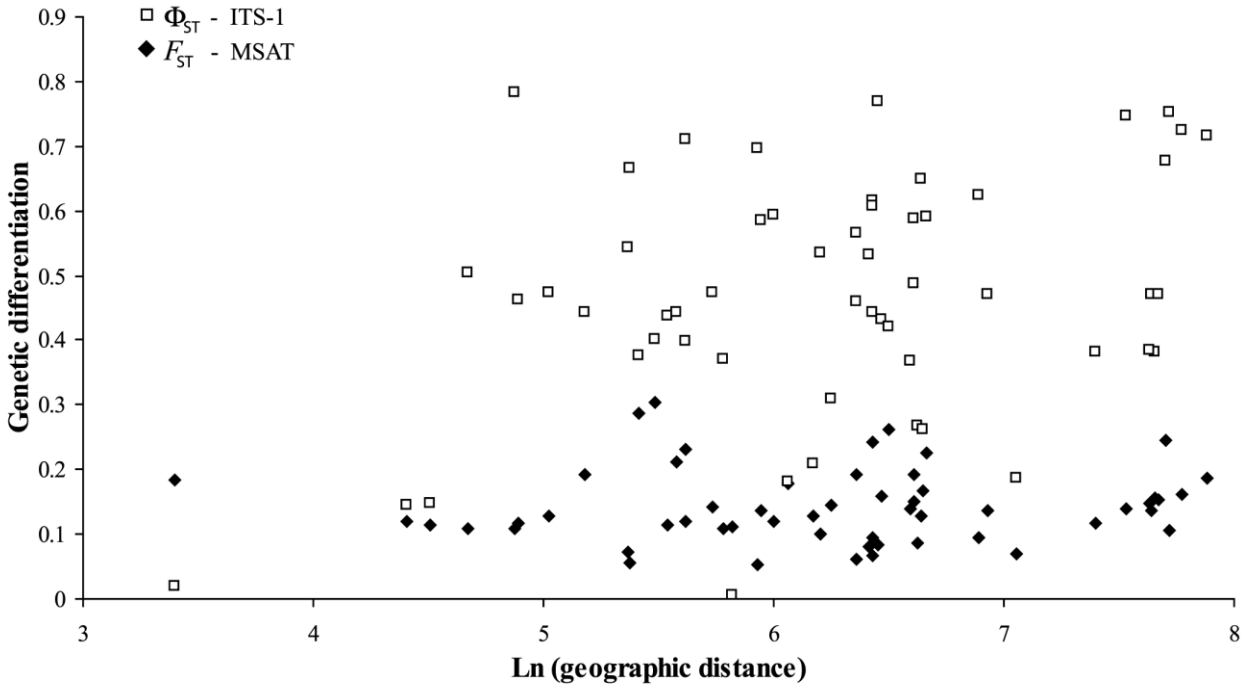
835 **Figure 4**

836



837

838 **Figure 5**

839