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

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1 Title: GENETIC STRUCTURING OF THE TEMPERATE GORGONIAN CORAL *CORALLIUM*  
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3 MICROSATELLITES AND NUCLEAR SEQUENCES  
4

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13

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22 Running title: Genetic structuring of *Corallium rubrum*  
23

24 **Abstract**

25

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

46

47 **Introduction**

48

49 In the past decades, human-induced disturbance (e.g. habitat loss and fragmentation, global climate  
50 change, overexploitation and other effects due to fishing, pollution and tourism) has increased in  
51 marine coastal habitats (Airoldi & Back 2007). Owing primarily to this disturbance, biogenic reefs  
52 in tropical and temperate habitats have suffered long-term degradation affecting both species and  
53 genetic diversities (Hellberg *et al.* 2002, Hughes *et al.* 2002, Pandolfi *et al.* 2003, Bellwood *et al.*  
54 2004, Worm *et al.* 2006). Sustainable exploitation and conservation of marine species are among  
55 the priorities of modern bio-resource management (Gray 1997). In this context, estimating  
56 connectivity among populations is essential because dispersal is a key element warranting  
57 population resilience following disturbance (Palumbi 2003, Bellwood *et al.* 2004).

58

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

69

70 Within the Mediterranean Sea, the distribution of red coral is mainly restricted to the rocky shores  
71 of the western Mediterranean basin, though Chintiroglou *et al.* (1989) reported records along the  
72 Greek coasts of the North Aegean Sea. The distribution of the species shows major gaps related to  
73 the presence of stretches of sandy shores (e.g. Gulf of Valencia, Gulf of Lion, Versilia plain, Gulf of  
74 Gaeta). Moreover, red coral has a strong requirement towards its habitat, defined by the  
75 morphology and biogenic nature of the substratum, as well as the range of abiotic variables (e.g.  
76 light intensity, water temperature and turbidity, sediment loads, current regime) (Weinberg 1979).  
77 As a consequence, the distribution of *Corallium rubrum* is highly fragmented at various spatial  
78 scales.

79

80 Red coral is a gonocoric species, brooding lecithotrophic planulae (Weinberg 1979). In order to  
81 investigate the effective larval dispersal of red coral in the western Mediterranean Sea, early  
82 population genetics studies have been conducted using allozymes. They have shown the occurrence  
83 of significant genetic divergences among samples at a distance of tens of kilometres (Abbiati *et al.*  
84 1993). At shorter distances (about 200 m), no significant genetic structuring was observed using  
85 allozymes (Abbiati *et al.* 1997). However, allozyme markers, due to their low mutation rate and  
86 polymorphism may have a limited power in revealing a genetic structure at small spatial scales.  
87 Indeed, recently, Costantini *et al.* (2007) using four microsatellite loci specifically developed for *C.*  
88 *rubrum* (Costantini & Abbiati 2006) have found a strong genetic structuring at spatial scales of tens  
89 of meters among samples within two locations on the Ligurian Sea. These patterns are explained by  
90 the reduced swimming ability and geonegative behaviour of the planulae, together with a short  
91 larval duration (estimated from 4 to 12 days under laboratory conditions, Vighi 1972, Weinberg  
92 1979), which suggest that, once released, larvae settle in close vicinity of the parental colonies  
93 (Vighi 1972). Additionally, differences in genetic variability and in  $F_{ST}$  estimates between locations  
94 (Costantini *et al.* 2007) suggested that environmental features (e.g. habitat characteristics,

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

97  
98 In order to further investigate the spatial genetic structuring of *C. rubrum*, eleven populations within  
99 the distribution range of the species in the North Western Mediterranean Sea have been analysed.  
100 Since mitochondrial DNA did not reveal intraspecific variation in red coral (Costantini *et al.* 2003),  
101 likely due to its slow evolutionary rate as observed in anthozoans (Romano & Palumbi 1997,  
102 Shearer *et al.* 2002, Hellberg 2006), in this study, two nuclear molecular markers with differing  
103 levels of polymorphism have been used. Allele frequencies at five microsatellite loci have been  
104 analysed in *C. rubrum* samples. Since microsatellites previously revealed genetic structuring at very  
105 small scales (Costantini *et al.* 2007), sequences of the internal transcribed spacer 1 region (ITS-1) of  
106 the nuclear ribosomal DNA were also analysed in order to test for homoplasy at microsatellite loci  
107 over large scales. The results of this study may help understanding how small scale processes may  
108 influence patterns over larger spatial scales in *Corallium rubrum* populations, as well as being  
109 useful to conservation and sustainable management strategies of the species at the Mediterranean  
110 scale.

## 111 112 **Materials and methods**

### 113 114 *Sample collections and molecular analysis*

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

120  
121 Total genomic DNA was extracted from 2-4 polyps per individual colonies using a cetyltrimethyl  
122 ammonium bromide (CTAB) protocol (Winnepennickx *et al.* 1993) with standard phenol-  
123 chloroform-isoamyl alcohol (25:24:1) extraction. Five microsatellite loci (COR9, COR15, COR48  
124 and COR58, used in Costantini *et al.* (2007), and COR46) specifically developed for *Corallium*  
125 *rubrum* were amplified (Costantini & Abbiati 2006). Genotyping was carried out on an ABI 310  
126 Genetic Analyser, using forward primers labelled with 6-FAM, HEX or TAMRA (MWG Biotech)  
127 and ROX HD400 (Applied Biosystems) as internal size-standards. Allele sizing was performed  
128 using GeneScan Analysis Software version 2.02 (Applied Biosystems).

129  
130 Polymerase chain reaction (PCR) amplifications of the ITS-1 region were carried out using the  
131 primers ITS1-new-F 5'-TCGTAACAAGGTTTCCGTA-3' and ITS1-new-R 5'-  
132 TAACGGTGGATCTCTTGGCT-3' (Costantini 2001). Each 25 µl PCR reaction contained  
133 approximately 20 ng DNA, 1X PCR buffer (Promega), 2 mM MgCl<sub>2</sub>, 0.5 µM of each primer, 0.4  
134 mM dNTPs and 1 U of *Taq* polymerase (Promega). Amplifications were performed on a GeneAMP  
135 PCR System 2700 (Applied Biosystems) as follows: an initial denaturation at 95°C for 3 min, 30  
136 cycles including 95°C for 30s, 57°C for 30s and 72°C for 60s. A final extension at 72°C for 7 min  
137 was added. Amplified fragments were purified with an ExoSAP-IT kit (Amersham Pharmacia) and  
138 cycle sequenced in both directions using an ABI Prism Big Dye Terminator Cycle Sequencing kit  
139 (Applied Biosystems). Sequences were run on an ABI 310 Genetic Analyser (Applied Biosystems).  
140 Given that the cloning of PCR products is costly and laborious, and, additionally, artefacts can be  
141 produced during the cloning step due to *in vitro* recombination upon transformation of bacterial  
142 cells with heteroduplex DNA (Zhang & Hewitt 2003; Vollmer & Palumbi 2004), all ITS-1

143 fragments were directly sequenced without prior cloning. In order to avoid the term “haplotype”,  
144 which represents an haploid component of a given sequence, the term “sequence type” was used to  
145 refer to every distinct type of ITS-1 sequence detected, as proposed by Worheide *et al.* (2002).  
146

#### 147 *Genetic variability*

148 Microsatellite diversity within samples was estimated using El Mousadik & Petit (1996) allelic  
149 richness ( $A$ ) in FSTAT version 2.9.3.2 (Goudet 2001), and observed ( $H_{obs}$ ) and Nei 's (1987)  
150 unbiased expected heterozygosity ( $H_{exp}$ ) in GENETIX software package version 4.03 (Belkhir *et al.*  
151 2004). All loci were tested for linkage disequilibrium using GENEPOP version 3.4 (Raymond &  
152 Rousset 1995) as implemented for online use (<http://genepop.curtin.edu.au/>). Single and multilocus  
153  $F_{IS}$  were estimated using Weir & Cockerham's (1984) fixation index and, because most of  $F_{IS}$  were  
154 positive ( $H_{obs} < H_{exp}$ ), deviations from Hardy-Weinberg equilibrium (HWE) were tested using  
155 Fisher's exact test, using the null hypothesis  $H_0 =$  no heterozygote deficiency, with the level of  
156 significance determined by a Markov-chain randomization (1000 dememorizations, 100 batches,  
157 and 1000 iterations per batch) in GENEPOP. Significance levels for multiple comparisons of loci  
158 across samples were adjusted using a standard Bonferroni correction (Rice 1989). The presence of  
159 null alleles was examined by estimating null allele frequencies for each locus and sample following  
160 the Expectation Maximization (EM) algorithm of Dempster *et al.* (1977) using FREENA (Chapuis &  
161 Estoup 2007).  
162

163 ITS-1 sequences were edited and aligned manually using BIOEDIT version 7.0.4 (Hall 1999).

164 Genetic diversity within samples was estimated using sequence type diversity ( $h$ , Nei 1987) and  
165 nucleotidic diversity ( $\pi$ , Nei 1987) using ARLEQUIN ver 3.1 (Excoffier *et al.* 2005).

166 Since ITS regions have frequent insertions/deletions (indels) that may be phylogenetically  
167 informative (Vogler & DeSalle 1994) and may originate from single evolutionary events regardless  
168 of their size (Girabet & Wheeler 1999), alignment gaps were treated using a conservative approach.  
169 Here, each indel with a different start and/or end position (i.e. not point mutations), which all  
170 sequence type either have or lack, was recoded as a new single base and added at the end of the  
171 sequence (see Table 3), and gaps were further treated as missing nucleotides.  
172

173 Hierarchical likelihood ratio tests were used to determine the best-fit model of nucleotide  
174 substitution using the program MRMODELTEST version 2.2 (Nylander 2004). The Jukes & Cantor  
175 (JC) model (equal nucleotide frequencies and equal rate of nucleotide substitution between  
176 nucleotides) was supported by the likelihood ratio test ( $P < 0.000001$ ) and was then used in  
177 following analysis. Phylogenetic trees of the relationships among sequence types were constructed  
178 using Maximum parsimony (MP) in PAUP 4.0b10 (Swofford 2002) and Bayesian inference (BI)  
179 using MRBAYES 3.1 (Ronquist & Huelsenbeck 2003). Maximum parsimony analysis was performed  
180 under the heuristic search option employing random stepwise addition, 10 random additions and  
181 TBR branch swapping. Nodal support was measured using a non parametric bootstrap method with  
182 100 pseudo replications. BI analysis was performed using one cold chain and three incrementally  
183 heated chains with  $T = 0.1$ . Starting trees for each chain were set as random and all settings were  
184 default values of MRBAYES unless stated. Each Metropolis-coupled Markov chain Monte Carlo  
185 (MCMCMC) was run for 5 million generations, with trees sampled every 1000 generations, and the  
186 first million generations were discarded. Results were summarized as 50% majority rule consensus  
187 trees including compatible groupings and branch lengths. Posteriors probabilities were used to asses  
188 clade support. Trees were displayed using the software TREEVIEW version 1.6.0 (Page 1996).  
189

#### 190 *Clustering analysis*

191 The number of clusters detected among samples,  $K$ , was estimated by employing a Bayesian  
192 approach implemented in the program STRUCTURE version 2.2 (Pritchard *et al.* 2000, Falush *et al.*  
193 2003, 2007). Each individual was assigned to probable common clusters based on the similarity of  
194 their multilocus genotypes at five microsatellite loci. Mean and variance of log likelihoods of the  
195 number of clusters for  $K=1$  to  $K=12$  were inferred from multilocus genotypes by running  
196 STRUCTURE 5 times with 500 000 repetitions each (burn in = 50 000 iterations) under the admixture  
197 ancestry model and the assumption of correlated allele frequencies among samples as suggested in  
198 Falush *et al.* (2003). The mean membership of each individual described the likelihood of that  
199 individuals belonging to the respective clusters. Following the recommendations of Evanno *et al.*  
200 (2005), we calculated the ad hoc statistic  $\Delta K$  based on the rate of change in the log likelihood of  
201 data between consecutive  $K$ -values. Due to the presence of null alleles, the clustering analysis was  
202 conducted on the original dataset (not in HWE), using the option of null alleles coded as recessive  
203 alleles described in Falush *et al.* (2007). Indeed, we could not use a dataset adjusted for the presence  
204 of null alleles such as the so-called INA method described in Chapuis & Estoup (2007) since this  
205 procedure consider only one null allele common to all populations, which may biased the  
206 assignment tests (Chapuis & Estoup 2007, Chapuis personal communication).  
207

### 208 *Population structure analysis*

209 Genetic divergence among samples at microsatellite loci was estimated in FREENA using the  $F_{ST}$   
210 estimates of Weir (1996) and following the so-called ENA method described in (Chapuis & Estoup  
211 2007) which provides unbiased  $F_{ST}$  estimates, computed excluding null alleles. Pairwise  $F_{ST}$  among  
212 samples were also estimated using the original dataset (not corrected for nulls). Genotypic  
213 differentiation among samples was tested with an exact test (Markov chain parameters: 1000  
214 dememorizations, followed by 1000 batches of 1000 iterations per batch) with the original dataset,  
215 and the  $P$ -value of the log-likelihood (G) based on the exact test (Goudet *et al.* 1996) was estimated  
216 in GENEPOP. For the ITS-1 sequences dataset, genetic differentiation for each pairwise sample was  
217 estimated using  $\Phi$  statistics ( $\Phi_{ST}$  based on haplotype frequencies and molecular divergence, using  
218 JC model) and its significance determined using a permutation test (10 000 permutations) in  
219 ARLEQUIN. Sequential Bonferroni corrections (Rice 1989) for multiple comparisons were applied.  
220

221 For both microsatellite and ITS-1 sequences datasets, isolation by distance model between samples  
222 was tested through a Mantel test (Mantel 1967) computed using the ISOLDE program implemented  
223 in GENEPOP. A significant correlation between genetic differentiation estimates and the logarithm of  
224 the geographic distances among samples was tested using 1000 permutations within the original  
225 matrices.  
226

227 An analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) implemented in ARLEQUIN was  
228 conducted to examine the partition of the genetic variance among red coral samples. With this  
229 purpose, red coral samples were grouped A) according to their sea of origin (six groups: Balearic  
230 Sea, Sardinian Sea, Ligurian Sea, North and Southern Tyrrhenian Sea, Adriatic Sea), B) based on  
231 the existing gaps in the distribution of the species (seven groups: Minorca; Sardinia; Medes; Plane  
232 and San Fruttuoso; Calafuria, Argentario, Giannutri and Elba; Palinuro; Korcula), C) *a priori*, based  
233 on the ITS-1 phylogenetic tree (four groups: Minorca; Sardinia; Tuscan archipelago samples and all  
234 the other samples, see results), and D) *a priori*, based on the results of the clustering analysis (two  
235 groups: Medes, Minorca, Plane, Sardinia, San Fruttuoso, Calafuria and Korcula; Argentario,  
236 Giannutri, Elba and Palinuro).  
237

## 238 **Results**

239 *Microsatellite loci variability and null alleles*

240 Over all samples, locus COR15 exhibited the lowest variation (9 alleles in total), while COR9 had  
241 the largest number of alleles (33). Overall, observed heterozygosities ranged from 0.26 (in COR9  
242 and COR46) to 0.45 (in COR15 and COR48) and expected heterozygosities ranged from 0.66 (in  
243 COR15) to 0.93 (in COR9). No linkage disequilibrium was detected among loci (all  $P > 0.05$  after  
244 Bonferroni corrections) and all loci were therefore considered genetically independent. *Corallium*  
245 *rubrum* samples showed considerable variation in their allelic richness over all five microsatellite  
246 loci, ranging from 5.1 for Giannutri to 10.0 for Minorca (Table 1). Significant multilocus deviations  
247 from Hardy-Weinberg expectations (HWE) were observed in all samples (Table 1). Multilocus  
248 estimates of  $F_{IS}$  ranged from 0.15 to 0.71, showing in all cases heterozygote deficiencies.  
249 Significant deviations from HWE were also observed in nearly all samples for each locus, with the  
250 exception of COR15, the less polymorphic one. Assuming HWE, estimated null allele frequencies  
251 ( $R$ ) ranged among loci from 0 to 0.78 (Table 1). Within each locus, the number of expected null  
252 homozygotes within samples based on HWE ( $N \cdot R^2$ ) was significantly higher than the average  
253 number of observed null homozygotes for COR9, COR46 and COR48 (paired t-test,  $P < 0.001$ ,  $P =$   
254  $0.006$  and  $P = 0.012$  respectively). Over all samples, the average number of expected null  
255 homozygotes per locus was significantly higher than the average number of observed null  
256 homozygotes (paired t-test,  $P = 0.035$ ).

257

258 *ITS-1 sequence variation and phylogenetic analysis*

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

265

266 Across all 231 individuals, the length of the amplified ITS-1 fragment varied among individuals  
267 between 228 and 266 bp due to the presence of numerous indels (Table 3). Single base pair indels  
268 were mostly associated with the presence of homopolymers but not with microsatellite repeat  
269 regions as observed in ITS1 for *Acropora* (van Oppen *et al.* 2000, Vollmer & Palumbi 2004).  
270 Including all gaps, the alignment of all individual sequences consisted of 278 positions of which 87  
271 were variable, corresponding to 35 nucleotide substitutions and 52 indels. The sequence alignment  
272 (Table 3) showed two highly variable blocks of indels and minor stutters. The first large indel, from  
273 158 to 188 bp, correspond to a 31 bp deletion that was observed only in the Tuscany archipelago  
274 samples (Argentario, Giannutri and Elba; sequence types 30, 31, 32, and 45, Table 2). The second  
275 highly variable region consist of two smaller indels, situated between 210 to 222 bp and between  
276 244 and 246 bp, that were found only in Minorca sample (sequence types from 10 to 15, Table 2).  
277 Out of the 51 different sequence types revealed, nine were found in more than one sample  
278 (sequence types 1-5-6-17-29-32-42-49-50, Table 2). Sequence type 5 was the most widespread  
279 (seven samples out of 11), though not the most abundant one (12%; Table 2). The most abundant  
280 sequence type (sequence type 17, 18.6%) was found only in Plane and Calafuria. The remaining  
281 sequence types were unique to a single location and often found in a single individual (28 singletons  
282 in total). Genetic variation across locations varied considerably, with a sequence type diversity ( $h$ )  
283 ranging from 0.273 in Calafuria to 0.890 in Minorca, and a nucleotide diversity ( $\pi$ ) ranging from  
284 0.003 in San Fruttuoso to 0.071 in Elba (Table 2).

285

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



287 Inference (BI), produced similar trees for the 51 ITS-1 sequence types so that only the BI consensus  
288 unrooted tree is presented, with both BI posterior probability values and bootstrap values greater  
289 than 50% for MP (Figure 2). Four highly supported clades (with BI posterior probability >0.95)  
290 could be identified: one consisting of all but one Minorca sequence types (10, 11, 12, 13, 14, 15),  
291 one containing sequence types found exclusively in the Tuscany archipelago samples (Giannutri,  
292 Elba and Argentario; 30, 31, 32, 45); one containing sequence types found exclusively in Giannutri  
293 (34, 35, 36, 37); and one including two sequences found in Sardinia (23) and Medes (8) (Figure 2).

294

### 295 *Population genetic analysis*

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

301

302 High levels of genetic differentiation were found among Mediterranean Sea samples for both  
303 microsatellite loci and ITS-1 sequences datasets (Figure 5). Genetic differentiation estimates  
304 between microsatellites loci and ITS-1 sequences varied greatly (Figure 5) and were not  
305 significantly correlated ( $P = 0.87$ ). For the microsatellite dataset, pairwise  $F_{ST}$  estimates based on  
306 the ENA method ranged from 0.053 (Minorca vs. Planes) to 0.305 (Giannutri vs. Sardinia; data not  
307 shown) and all pairwise comparisons were highly significant ( $P < 0.001$ ). Though these pairwise  
308  $F_{ST}$  estimates were significantly smaller than estimated from the original dataset (paired t-test,  $P <$   
309 0.001), they were significantly correlated (Pearson  $r = 0.974$ ,  $P < 0.001$ ).

310 For the ITS-1 sequence dataset,  $\Phi_{ST}$  ranged from 0.004 (Calafuria vs. Plane) to 0.789 (Calafuria vs.  
311 San Fruttuoso) and two out of the 55 pairwise  $\Phi_{ST}$  estimates were not statistically significant after  
312 sequential Bonferroni correction (Calafuria vs. Plane, and Giannutri vs. Argentario; data not  
313 shown). For both datasets (microsatellite and ITS-1), genetic differentiation estimates were not  
314 significantly correlated with the logarithm of geographic distances (Figure 5;  $P = 0.273$  for  
315 microsatellites,  $P = 0.136$  for ITS-1)

316

317 The AMOVAs conducted among red coral samples using ITS-1 sequence dataset showed a  
318 significant differentiation among samples within all defined groups ( $P < 0.001$  for all four analyses,  
319 Table 4). A significant variance was observed among groups when grouping samples *a priori* based  
320 on the phylogenetic results, representing 33% of the total nuclear variance (i.e. four groups:  
321 Minorca vs. Tuscany archipelago vs. Sardinia vs. all others;  $V_a = 0.813$ ,  $P = 0.002$ , Table 4). Based  
322 on the existing gaps in the distribution of the species (i.e. seven groups) or based on the results of  
323 the clustering analysis, the nuclear variance attributed among the groups was not significant ( $V_a =$   
324 0.483,  $P = 0.109$ ;  $V_a = 0.061$ ,  $P = 0.120$ , respectively; Table 4). Using the microsatellite dataset, the  
325 nuclear variance distributed among groups was not significant except for the grouping based on the  
326 clustering analysis ( $V_a = 0.065$ ,  $P = 0.020$ ) and more than 80% of the total variance was observed  
327 within samples whatever the grouping (Table 4).

328

## 329 **Discussion**

330

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

335 whereas in most animal groups the mitochondrial genome provides an appropriate tool for such  
336 investigations, the use of mitochondrial DNA in Anthozoa is limited due to its extremely low  
337 evolution rates (Romano & Palumbi 1997, Shearer *et al.* 2002, Hellberg 2006, Calderon *et al.*  
338 2006). In particular, in *Corallium rubrum*, no sequence variability was observed in the  
339 mitochondrial COI and 16S genes (Costantini *et al.* 2003, Calderon *et al.* 2006). Consequently, for  
340 anthozoans, nuclear markers have been mostly used for intraspecific studies (van Oppen & Gates  
341 2006), with microsatellites and ITS-1 sequences being nowadays predominant (Rodriguez-Lanetty  
342 & Hoegh-Guldberg 2002, Maier *et al.* 2005, Magalon *et al.* 2005, Costantini *et al.* 2007).

344 The main result of our study is that *Corallium rubrum* samples in the Mediterranean Sea are highly  
345 genetically heterogeneous. However, the two assessed nuclear markers provide different genetic  
346 divergence estimates among samples. Polymorphism levels found in *C. rubrum* samples using  
347 microsatellites ( $H_{exp} = 0.59 - 0.79$ ) are comparable with those observed in the gorgonian  
348 *Pseudopterogorgia elisabethae* ( $H_{exp} = 0.09 - 0.78$ , Gutierrez-Rodriguez & Lasker 2004) and in the  
349 range of those observed for scleractinian corals (Maier *et al.* 2005,  $H_{exp} = 0.73 \pm 0.04$ ; Magalon *et al.*  
350 2005,  $H_{exp} = 0.75 \pm 0.01$ ). However, they are much higher than those obtained with allozyme for  
351 the same species ( $H_{exp} = 0.073 - 0.104$ , Abbiati *et al.* 1993), as expected from the higher mutation  
352 rates and polymorphism of microsatellite loci compared to allozyme loci (Estoup & Angers 1998).  
353 Heterozygote deficits (strong positive deviations from HWE) were detected for all samples and at  
354 almost all microsatellite loci. Heterozygosity deficits have frequently been observed in marine  
355 invertebrates (Duran *et al.* 2004a; Addison & Hart 2004), including in many shallow-water  
356 Anthozoa (Ayre & Hughes 2000; Ridgway *et al.* 2001; Le Goff-Vitry *et al.* 2004). In *Corallium*  
357 *rubrum*, the heterozygote deficit observed may result from technical factors, such as the presence of  
358 null alleles, or biological factors, such as inbreeding, Wahlund effect or selection. In our case, as in  
359 Costantini *et al.* (2007), non amplifying individuals (null homozygotes) were found in all samples  
360 suggesting the occurrence of null alleles. However, observed frequency of null homozygotes were  
361 significantly smaller than expected if the heterozygote deficiencies were generated only by null  
362 alleles under HWE. In a previous study, Costantini *et al.* (2007) showed that heterozygosity  
363 deficiencies found in four proximate (less than 100 meters) samples of red coral collected both  
364 within San Fruttuoso and Calafuria were explained by the combined occurrence of null alleles  
365 (detected by the presence of non amplifying individuals), inbreeding (more inbred individuals were  
366 found than under the hypothesis of random mating) and Wahlund effect (revealed using a  
367 maximum-likelihood partitioning method). The heterozygote deficiencies observed in the present  
368 analysis further suggest that the latter two biological processes co-occur within all Mediterranean  
369 populations of red coral.

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

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

392  
393 Despite the intragenomic variation of the ITS-1 and the overestimated genetic differentiation among  
394 samples resulting from our method to handle it, some clear patterns are drawn from the  
395 phylogenetic analyses. Minorca and Sardinia samples both showed a high genetic variability (0.8 –  
396 0.9 sequence type diversity, 1.3 - 3.3% nucleotide diversity) with seven and six private sequence  
397 types respectively. Phylogenetic reconstructions showed that Minorca sample appear the most  
398 divergent, as expected from its particular insular situation and the complex atmospheric and  
399 hydrological systems observed in the Balearic Islands (La Violette *et al.* 1990). Sardinia appears  
400 less divergent from the coastal samples compared to Minorca, as expected from its closer location  
401 to Corsica and Ligurian coastlines. However, for both islands, we could not propose hypothesis  
402 explaining patterns of genetic divergence, neither date the origin of divergence. Indeed, ITS-1  
403 region is a nuclear marker experiencing recombination and, to our knowledge, no proper  
404 evolutionary rate has been defined in Anthozoa for this marker.

405  
406 *Corallium rubrum* analysis revealed a high degree of genetic differentiation between samples  
407 throughout the North Western Mediterranean Sea, with apparent genetic homogeneity within the  
408 Tuscan archipelago (revealed using ITS-1 sequences, though microsatellite revealed significant  
409 differentiation). Microsatellites and ITS-1 sequences showed no correlation between genetic  
410 differentiation and geographical distance among the analysed samples. In some cases, for ITS-1  
411 sequence data, small geographical distances were associated with relatively small  $F_{ST}$  estimates,  
412 such as was found when comparing the Argentario and Giannutri samples using ITS-1. In other  
413 cases,  $F_{ST}$  estimates were large despite geographical proximity, such as was found when comparing  
414 the Calafuria and Elba samples for both markers. Such lack of correlation between pairwise  $F_{ST}$   
415 estimates and geographical distances may support an island model of dispersal, where larvae move  
416 from one area to another with equal probability, rather than an isolation by distance model, where  
417 the probability of dispersal between sites declines with increasing geographical distance.

418  
419 While microsatellite data showed no correlation between  $F_{ST}$  estimates and geographic distances  
420 ranging from 30 to 2650 km, Costantini *et al.* (2007) have shown the occurrence of significant  
421 genetic structuring at spatial scales of ten meters with a significant isolation by distance at the  
422 spatial scale of less than one kilometer, suggesting that larval dispersal in red coral is highly  
423 restricted (likely few meters). Therefore, due to their high variability and polymorphism,  
424 microsatellites likely appear useless in revealing a clear genetic structuring pattern at the  
425 Mediterranean scale. Indeed, microsatellites and ITS-1 are non-coding nuclear markers that should  
426 reveal the same patterns under neutrality. However, microsatellites based  $F_{ST}$  estimates appeared  
427 much smaller as compared to ITS-based estimates (Figure 5). This discrepancy is likely due to allele  
428 size homoplasy typical of microsatellites (identity by state but not by descent) resulting from their  
429 high mutation rates and allele size range constraints (Goldstein *et al.* 1995, Slatkin 1995). Similar  
430 results have been obtained when using loci differing in heterozygosities (O'Reilly *et al.* 2004) or

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

433  
434 Overall, the AMOVAs conducted on the ITS-1 sequences indicated a partitioning of the total  
435 genetic variability in four groups: one main group gathering samples belonging to the  
436 western/Ligurian Sea (Medes, Plane, San Fruttuoso, Calafuria samples), a second main group of  
437 samples from the Tyrrhenian/eastern Seas (Tuscany archipelago, Palinuro and Korcula samples),  
438 and two other groups representing an island sample each: Minorca and Sardinia respectively. Using  
439 microsatellites data, a subdivision in two clusters identified with a clustering analysis method was  
440 confirmed by the AMOVA. However, samples within clusters were not homogeneous (high genetic  
441 differentiation for all pairwise comparisons was found), and these results are likely biased by  
442 microsatellite size homoplasy (see previously). Strong patterns of genetic divergence over the North  
443 Western Mediterranean Sea have already been reported for other marine invertebrates, and mainly  
444 result from large or medium-scale hydrodynamic process or by geomorphologic characteristics of  
445 Mediterranean Sea. Duran *et al.* (2004a, 2004b), using both microsatellite loci and ITS sequences,  
446 showed that Mediterranean populations of the marine sponge *Crambe crambe* are highly divergent  
447 due to the restricted gene flow and follow the isolation by distance model. In this sessile  
448 invertebrates, currents and behaviour of the lecithotrophic larvae have a strong influence on the  
449 effective larval dispersal, as it may be the case for red coral. Similarly, Lejeune & Chevaldonné  
450 (2006) have found high genetic structuring at the Mediterranean scale in two brooding cave-  
451 dwelling mysids. While restricted gene flow with isolation by distance explains the patterns  
452 observed at the finest scale, habitat disjunction (natural fragmentation) and coastal geomorphology  
453 strongly influence population structuring since, in these mysids, dispersal is only possible at the  
454 adult stage.

455  
456 The results obtained in this study on population genetics of *Corallium rubrum* in the Mediterranean  
457 Sea showed the occurrence of a strong large scale structuring and the saturation of the microsatellite  
458 loci as compared to the ITS-1 sequences. Lack of clear patterns at the Mediterranean scale together  
459 with the results on small scale structuring from Costantini *et al.* (2007) have major implications for  
460 the conservation and the sustainable management of this species. Indeed, populations of *C. rubrum*  
461 are genetically differentiated at the small scale so that management plans have to be developed at  
462 the local scale. Our findings also suggest that, for species with restricted dispersal, absence of clear  
463 patterns of genetic structuring over large spatial scales derived from highly polymorphic markers  
464 such as microsatellite loci should be interpreted with caution. In fact, chaotic genetic structure at  
465 large scales may reveal underlying small scale genetic structuring. Use of different markers (e.g.  
466 nuclear or mitochondrial sequences) together with analyses at small spatial scales may help  
467 understanding the effective larval dispersal and prevent erroneous interpretation of the genetic  
468 patterns observed.

469  
470 These results, together with studies on demography (e.g. Santangelo & Abbiati 2001; Garrabou &  
471 Harmelin 2002; Torrents *et al.* 2005) and reproductive structure (Santangelo *et al.* 2003) have  
472 important consequences for red coral populations resilience. Indeed, an increase of natural and  
473 anthropogenic source of mortality such as over harvesting (Santangelo & Abbiati 2001) or water  
474 temperature anomalies (Cerrano *et al.* 2000; Garrabou *et al.* 2001) could lead to the disappearance  
475 of red coral in shallow water habitats, not only along the Mediterranean continental coasts (Torrents  
476 *et al.* 2005) but also along the Mediterranean islands. Further studies that include additional  
477 Mediterranean and Atlantic regions, as well as deep sea and peripheral populations of red coral are

478 needed to identify bio-geographical discontinuities and refugia populations of this highly valuable  
479 species.  
480

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675

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688

#### 689 **Figure Legends:**

690

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

699

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

704

705 **Figure 3:** Values of  $\Delta K$ , calculated as in Evanno *et al.* (2005) and based on the log likelihood of the  
706 data given by STRUCTURE version 2.2 for each number of cluster assumed ( $K$ ).  
707

708 **Figure 4:** Results of the clustering analysis conducted in STRUCTURE 2.2. In the bar plot, each of  
709 the 370 individuals is represented by a vertical bar indicating its estimated proportion of  
710 membership to each cluster (represented by different colours). Under the bar plot is indicated the  
711 mean proportion of membership of each sample to each cluster.  
712

713 **Figure 5:** Relationship between genetic differentiation estimates and the logarithm of geographical  
714 distance among *Corallium rubrum* samples for both microsatellite and ITS-1 markers.  
715

#### 716 **Authors Information Box**

717 This paper was part of the PhD work of Dr Federica Costantini supervised by Prof. Marco Abbiati,  
718 which aimed to focus on population genetics and molecular systematic of marine invertebrates in  
719 coastal habitats, mainly for biodiversity conservation purposes. Dr Cécile Fauvelot is a molecular  
720 ecologist who has worked on both coral reef fish and rainforest butterfly population genetics and  
721 contributed to this work during her post doctoral position in Ravenna.  
722  
723

724 **Table 1:** Summary of genetic diversity at five microsatellite loci from *Corallium rubrum* samples;  
725 n: number of sampled individuals; N: number of genotypes per loci; A: allelic richness based on 16  
726 ind.;  $H_{obs}$ : observed heterozygosity;  $H_{exp}$ : expected heterozygosity;  $F_{IS}$ : Weir and Cockerham's  
727 (1984) estimate of Wright's (1951) fixation index (bold type indicate significant deviations from  
728 HWE after standard Bonferroni correction);  $R$ : null allele frequency based on Dempster *et al.*  
729 (1977) estimator.

	MED	MIN	PLA	SAR	SAN	CAL	ARG	GIA	ELB	PAL	KOR
<i>n</i>	24	24	20	28	50	50	48	36	32	24	34
<b>COR9</b>											
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
$F_{IS}$	<b>0.56</b>	<b>0.71</b>	<b>0.76</b>	<b>0.64</b>	<b>0.58</b>	<b>0.77</b>	<b>0.75</b>	<b>0.60</b>	<b>0.52</b>	<b>0.71</b>	<b>0.83</b>
$R$	0.29	0.42	0.42	0.35	0.32	0.40	0.40	0.30	0.29	0.34	0.40
<b>COR15</b>											
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	<b>0.28</b>	-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
<b>COR46</b>											
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
$F_{IS}$	<b>0.70</b>	<b>0.44</b>	<b>0.79</b>	<b>1</b>	<b>0.88</b>	<b>0.94</b>	<b>0.55</b>	<b>0.83</b>	<b>0.57</b>	0.07	<b>0.64</b>
$R$	0.39	0.28	0.42	0.71	0.78	0.52	0.29	0.32	0.33	0.03	0.41
<b>COR48</b>											
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
$F_{IS}$	<b>0.51</b>	-0.03	<b>0.60</b>	<b>0.80</b>	<b>0.62</b>	<b>0.64</b>	<b>0.50</b>	0.04	<b>0.67</b>	-0.11	<b>0.30</b>
$R$	0.34	0.05	0.35	0.47	0.32	0.31	0.23	0.03	0.38	0.00	0.12
<b>COR58</b>											
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
$F_{IS}$	<b>0.54</b>	<b>0.51</b>	<b>0.65</b>	<b>1</b>	<b>0.74</b>	<b>0.45</b>	0.09	<b>0.54</b>	0.20	<b>0.26</b>	<b>0.50</b>
$R$	0.28	0.30	0.37	0.26	0.41	0.28	0.11	0.29	0.21	0.25	0.27
<b>Multilocus</b>											
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
$F_{IS}$	<b>0.51</b>	<b>0.34</b>	<b>0.70</b>	<b>0.71</b>	<b>0.66</b>	<b>0.57</b>	<b>0.46</b>	<b>0.42</b>	<b>0.46</b>	<b>0.15</b>	<b>0.53</b>

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733 **Table 2:** Distribution of sequence types and genetic diversity in *Corallium rubrum* samples;  
734 number of individuals amplified per sample (N), number of individuals per sample with analysed  
735 sequence (n), total number of sequence type (H), sequence type diversity (*h*, Nei 1987), nucleotide  
736 diversity ( $\pi$ , □□Nei 1987).  
737

	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
<i>h</i>	0.747	0.89	0.442	0.8	0.39	0.273	0.747	0.862	0.794	0.853	0.341	0.92
$\pi$	0.01	0.033	0.005	0.013	0.003	0.005	0.046	0.044	0.071	0.012	0.006	0.046



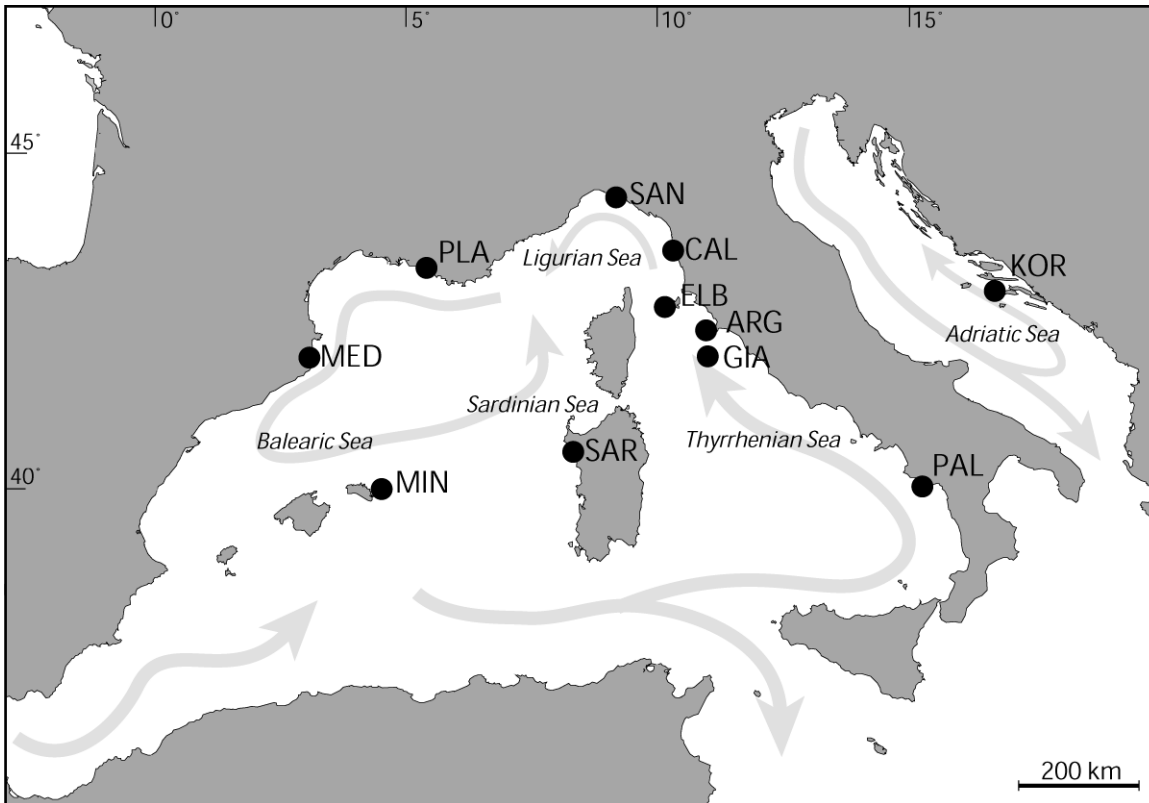


804 **Table 4:** Analysis of molecular variance (AMOVA) among samples of *Corallium rubrum* using  
805 ITS-1 dataset and microsatellite dataset. Red coral samples were grouped according to A) their sea  
806 of origin (six groups: Balearic Sea, Sardinian Sea, Ligurian Sea, North and Southern Tyrrhenian  
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

Source of variation	ITS - 1			Microsatellites		
	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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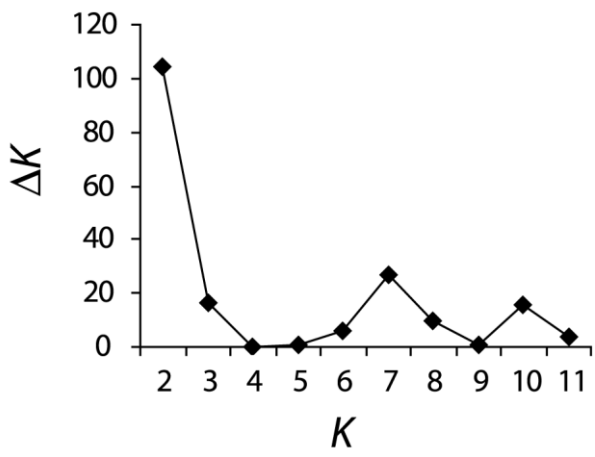
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**Figure 1**





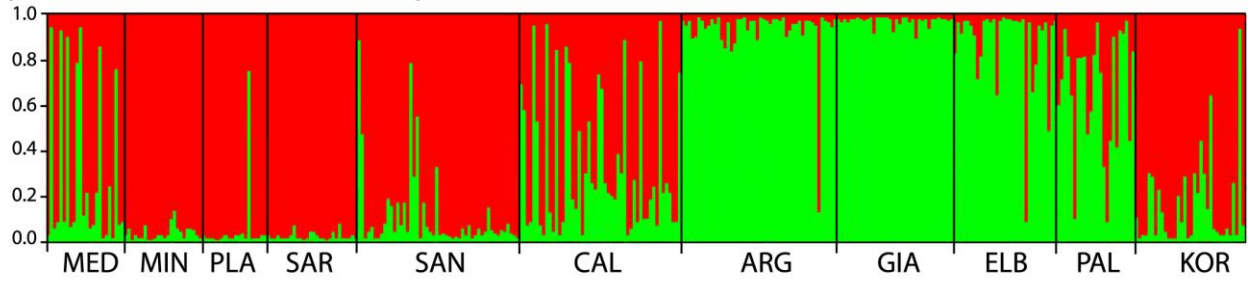
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831 **Figure 3**

832

Proportion of individual membership



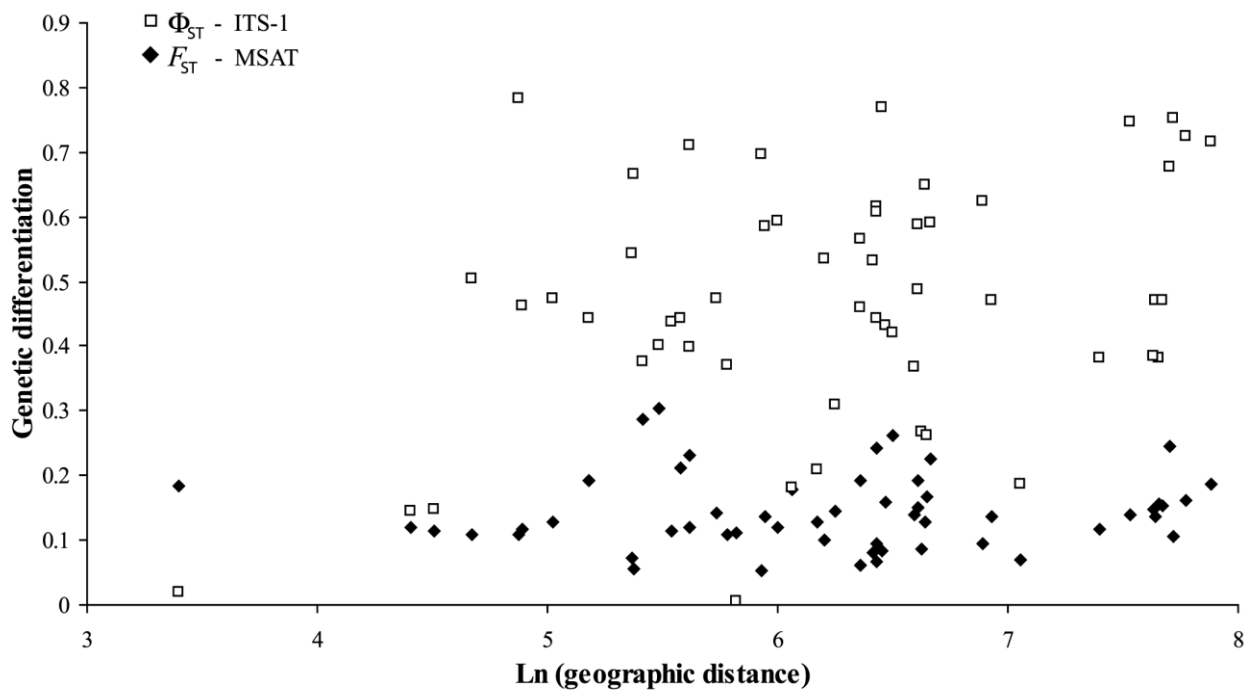
Mean proportion of sample membership

833	Cluster 1	0.667	0.958	0.940	0.964	0.843	0.563	0.064	0.027	0.131	0.418	0.841
	Cluster 2	0.333	0.042	0.060	0.036	0.157	0.437	0.936	0.973	0.869	0.582	0.159

834

835 **Figure 4**

836



837

838 **Figure 5**

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