Vol. 340: 109–119, 2007 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published June 18

Fine-scale genetic structuring in *Corallium rubrum*: evidence of inbreeding and limited effective larval dispersal

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ABSTRACT: The red coral *Corallium rubrum* has a long history of intensive exploitation. As a consequence, most populations have been overharvested, which may be leading to reduced levels of genetic diversity. *C. rubrum* is a gorgonian distributed in the Mediterranean Sea and along the neighbouring Atlantic coasts, with brooding larvae which disperse only over short distances. Such limited dispersal of larvae suggests that gene flow is restricted in this species, but no experimental evidence is yet available. In the present study, small spatial scale genetic structuring was analysed in *C. rubrum* samples collected in the Ligurian Sea using a hierarchical sampling design, including scales from 10s to 100s of metres. The genetic variation of each colony was analysed using 4 microsatellite loci. Significant deviations from Hardy-Weinberg equilibrium due to elevated heterozygote deficiencies were detected in all samples and were consistent with the occurrence of inbreeding and a Wahlund effect. Significant levels of genetic differentiation were found both between locations (F_{ST} = 0.209 ± 0.02) and among samples within each location (F_{ST} range = 0.025 to 0.082). Our results indicate the occurrence of significant genetic structuring at spatial scales of 10s of metres, supporting the hypothesis that planulae have a limited effective dispersal ability. The occurrence of structured breeding units and differences in genetic diversity among samples also suggest that strategies for sustainable management and conservation of red coral should be defined at a local scale.

KEY WORDS: Population structure · Microsatellites · Gene flow · Inbreeding · Wahlund effect · Larval dispersal · *Corallium rubrum*

INTRODUCTION

Scales of dispersal play a fundamental role in determining the dynamics of marine populations because they define the extent of breeding units as well as the level of connectivity between them. Broad dispersal generates high migration rates among populations, thereby counteracting the process by which genetic drift and natural selection produce genetic differentiation. In contrast, poor dispersal may enhance the occurrence of discrete breeding units that can become differentiated from each other (McFadden & Aydin 1996). Size and interconnectivity of genetic breeding units are important parameters to identify for conservation strategies, since small and isolated populations are vulnerable to inbreeding depression, which might reduce their evolutionary potential and increase their risk of extinction (Saccheri et al. 1998). In this context defining the 'larval neighbourhoods size' is a priority for marine invertebrates with a sessile adult stage, where dispersal relies on larval and/or gamete transport in the water column. Moreover, effective dispersal of larvae can be affected by physical barriers, lack of suitable habitats for settlement, larval viability and the stochasticity of reproductive success. These factors, either singly or in combination, appear crucial in determining the spatial scales of genetic structuring in marine invertebrates.

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Alcyonacea include sessile invertebrates showing a variety of reproductive systems (hermaphrodite or gonochoric; brooders or broadcasters). Broadcasters (corals that release eggs and sperm into the water) are thought to be good dispersers, whereas brooders (corals that release sperm, but brood the eggs) are considered to be poor dispersers (review in Carlon 1999). Several studies on brooding corals have shown high rates of mating between relatives (see Addison & Hart 2005 for a review) and high levels of genetic structuring over relatively small spatial scales (<5 km) as a result of restricted dispersal of gametes and larvae (McFadden & Aydin 1996, Gutierrez-Rodriguez & Lasker 2004). This is the case with e.g. *Pseudopterogorgia elisabethae*, a brooding Caribbean gorgonian coral, for which a significant proportion of the larvae settle in close vicinity of the maternal colony (Gutierrez-Rodriguez & Lasker 2004).

Our study focuses on the precious *Corallium rubrum*. The commercial importance of this species is mainly related to its solid red carbonate axial skeleton, highly valued for jewellery since antiquity. A long history of intensive exploitation along the Mediterranean coast has driven a dramatic shift in the size structure of red coral populations and led to the extinction of local commercial banks (Santangelo & Abbiati 2001, Garrabou & Harmelin 2002). Red coral is gonochoric, undergoes internal fertilisation and produces brooded planula larvae that, upon release, search actively for a suitable substrate for settlement and metamorphosis (Vighi 1972). Red coral planulae are indifferent to light and show negative geotropism, and laboratory experiments suggest that larvae do not spread very far from the parental colonies (Vighi 1972), although, currently, limited data support or refute this hypothesis. Red coral dwells on rocky bottoms in semi-dark areas, such as caves and smaller crevices, and on vertical cliffs and overhangs, from 10 to 200 m deep. Red coral distribution is not continuous along the Mediterranean coast as it is limited by the variability of rocky shores and by a combination of abiotic factors such as light, temperature, water turbidity and currents. At the small spatial scale, red coral settlements are characterised by high patchiness and varied density, mainly correlated with the heterogeneous nature of the substratum. Red coral, owing to its larval biology, life history and patchy distribution, appears to be a poor disperser, and its biological characteristics have been cited as promoters of small-scale genetic structuring (Abbiati et al. 1993, 1997, Garrabou & Harmelin 2002, Santangelo et al. 2003).

Genetic structuring in this species was first described using allozyme electrophoresis (Abbiati et al. 1993, 1997). These studies showed the occurrence of genetic differentiation among red coral populations at a spatial scale of a few kilometres and a lack of genetic structuring over shorter distances (about 200 m) (Abbiati et al. 1997). However, allozyme markers may have limited power in revealing small-scale genetic structure due to their low polymorphism and mutation rates. In contrast, microsatellite loci with their high level of polymorphisms, Mendelian inheritance and likely neutrality (but see Kashi & King 2006 for a discussion on the theoretical neutrality of microsatellites) may be useful molecular markers for examining the occurrence of genetic structuring at very small spatial scales (e.g. 10s of metres) in red coral. Moreover, microsatellites have proven to be one of the most informative genetic markers in Anthozoan population studies compared to mitochondrial DNA, which is highly conserved in these taxa (Costantini et al. 2003, Calderon et al. 2006).

The main goal of the present study was to assess the small-scale genetic variation in populations of *Corallium rubrum* at 2 locations along the coast of the Ligurian Sea. Specifically, we used a hierarchical sampling design to test whether the population genetic structure differed among samples collected 10s to 100s of metres apart. Four microsatellite loci specifically developed for red coral were used (Costantini & Abbiati 2006).

MATERIALS AND METHODS

Sample collections and DNA extraction. The 2 study locations along the Ligurian coast of Italy (Fig. 1) were Calafuria (Cal; 43° 30′ N, 10° 20′ E) and San Fruttuoso (SanF; 44° 18′ N, 9° 10′ E). These locations were selected for their dense settlement of red coral *Corallium rubrum* (up to 1300 colonies m^{-2}) at relatively shallow depths (Santangelo & Abbiati 2001). The 2 locations were geographically isolated by about 200 km of wide stretches of sandy shore. At Calafuria, *C. rubrum* was distributed along a 10 km long cliff ~200 m offshore, from 25 to ~45 m depth. This cliff was characterised by small crevices where coral colonies densely settled on the vaults. In San Fruttuoso, the cliff slope reached about 45° and encompassed large crevices. Red coral colonies were found from 20 m in depth, with higher densities between 30 and 40 m (Santangelo & Abbiati 2001). At each location, 4 samples were collected according to the following sampling design; 3 samples (B, C, D) were located approximately 10 m apart from each other and 1 sample (A) was located approximately 100 m apart from Sample B (Fig. 1). From each of the 8 samples, branch fragments from 50 individual colonies were collected by SCUBA diving, within an area of 1 m^2 at an approximate depth of 25 m, and preserved in 80% ethanol at 4°C.

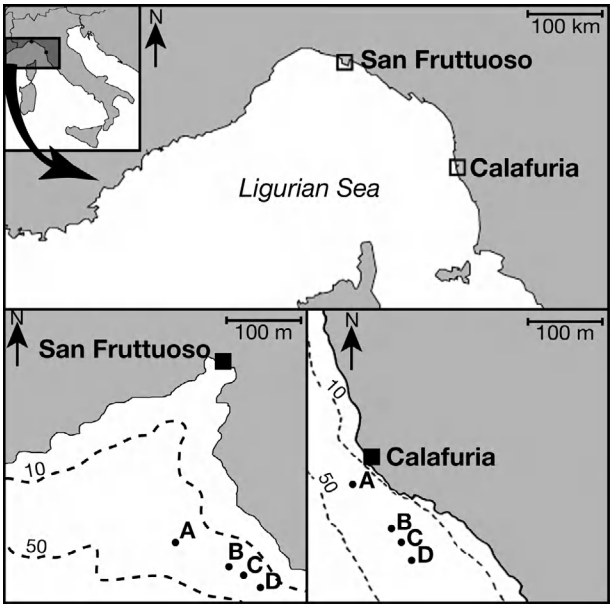

Fig. 1. Geographic positions of the 2 sampling locations (San Fruttuoso and Calafuria) along the Ligurian coast (Italy), and relative positions of sampling areas within locations at which *Corallium rubrum* colonies were collected. A, B, C and D: the 4 samples collected at each location; B, C and D are each separated by about 10 m along a linear transect and A is approximately 100 m distant from B along the same transect

Microsatellite loci analysis. Total genomic DNA was extracted from individual colonies (2 to 4 polyps colony^{-1}) according to the CTAB protocol (Winnepenninckx et al. 1993) and purified by standard phenol/chloroform/isoamyl alcohol (25:24:1) extractions. Four microsatellite loci (COR9, COR15, COR48, COR58), specifically developed for *Corallium rubrum*, were amplified according to the PCR protocol described by Costantini & Abbiati (2006). Because a high proportion of individuals failed to yield PCR products at the loci COR9 and COR58, primers were modified and redesigned based on the published sequences using PRIMER3 (available at http://www.genome.wi.mit.edu/genome_software/other/primer3.html) and Oligo 5.0 (www.basic.northwestern.edu/biotools/oligocalc.html) software. The sequences of the newly designed primers are given in Table 1. The new primer sets at COR9 and COR58 loci largely improved the amplification success rate (from 70% to nearly 95% PCR success over all samples for both loci).

Genotyping of individuals was conducted by allele sizing on an ABI310 Genetic Analyser, using 5'-FAM- and 5'-TAMRA-labelled (MWG Biotech) forward primers and ROX HD400 (Applied Biosystems) as an internal size standard.

Data analysis. Genetic diversity within samples was estimated as the mean number of alleles per locus (N_a), observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp}) using the GENETIX software package Version 4.05 (Belkhir et al. 2004). The genetic non-independence of the microsatellite loci (genotypic linkage disequilibria) was tested using GENEPOP Version 3.4 (Raymond & Rousset 1995) as implemented for online use (http://wbiomed.curtin.edu.au/genepop).

Since significant differences in genetic diversity were found among samples (see 'Results'), evidence of recent changes in effective population size was tested using BOTTLENECK 1.2.02 (Piry et al. 1999). This test detects significant differences between the gene diversity (H_E; Nei 1987) and the expected equilibrium heterozygosity (H_{Eq}) calculated through simulations from the observed number of alleles at each locus, under various mutation models (Luikart & Cornuet 1998). A significant excess or deficiency of H_E compared to H_{Eq} can be interpreted as a signature of recent change in population size (Luikart & Cornuet 1998). H_{Eq} was calculated under the stepwise mutation model (SMM), the infinite allele model (IAM) and the 2-phased mutation model (TPM), with 95% single-step mutations as suggested by Luikart & Cornuet (1998). A Wilcoxon signed-rank test was used to test the null hypothesis of no difference between H_E and H_{Eq}.

Single- and multilocus F_{IS} values were estimated using Weir and Cockerham's f (Weir & Cockerham 1984), and significant departures from Hardy-Weinberg equilibrium (HWE) were tested using the exact test implemented in GENEPOP, with specified Markov chain parameters of 1000 dememorisations, followed by 100 batches of 1000 iterations batch^{-1}. We further conducted a series of tests in order to identify the cause of heterozygote deficits observed in all samples (see 'Results'), which may have different non-exclusive techni-

Table 1. Repeat motif, primer sequences, $MgCl_2$ concentrations, annealing temperature (T_a) and number of cycles for the amplification of COR9 and COR58 loci in *Corallium rubrum*. F: forward; R: reverse

Locus	Repeat motif	Primer sequence	$MgCl_2$ (mM)	T_a (°C)	No. of cycles	Accession No.
COR9	$(CA)_{13}$	F: TGCGAGATGCGTAAAGTCTN R: AGCGGCATCACTTTGGTCN	2.5	54	35	AY726758
COR58	$(TTG)_{22}$	F: CCACCCTGCCACGTATTTAT R: TCAGAGGTACGCTTCGGAGT	2.5	57	30	AY726764

cal and biological causes such as the presence of null alleles, inbreeding (mating between relatives) and/or Wahlund effect (mixing of differentiated gene pools).

To test for the occurrence of null alleles (i.e. alleles that failed to amplify because of mutation in primer sites), the expected allele frequency of a presumed null allele (r), within each sample and for each locus, was estimated under the hypothesis that null alleles are the only cause for the observed heterozygote deficits, according to Brookfield's (1996) estimator, where $r = (H_{exp} - H_{obs})/(H_{obs} + H_{exp})$. Using the null allele frequency estimates expected under HWE, the frequencies of null homozygotes (the homozygote for a null allele) within each sample were calculated. Finally, the expected number of null homozygotes (based on HWE) was compared to the observed number of null allele homozygotes (i.e. non-amplifying individuals), in each sample for each locus, to test whether nulls alone explained observed heterozygote deficiencies. The program MICRO-CHECKER (Van Oosterhout et al. 2004) was used to adjust the individual genotypes in order to account for the bias resulting from the presence of null alleles. The genotypes were adjusted by (1) estimating the number of expected homozygotes according to HWE and (2) replacing the 'false' homozygotes by genotypes made of 1 known allele and 1 null allele.

When individuals differ in their inbreeding history, inbred individuals are more homozygous over all loci than expected based on their single-locus genotype frequency. The hypothesis that observed heterozygote deficiencies may be explained by the presence of inbreeding within samples was tested using the original dataset, by comparing the distribution of individual multi-locus heterozygosity (MLH; number of heterozygous loci per individual calculated by counting the number of individuals heterozygous at 0, 1, 2, 3, or 4 loci) to its expected distribution under random mating using a permutation procedure (Castric et al. 2002, Hoarau et al. 2005). In our case, 5 classes were defined. Into Class 0 was calculated the frequency of individual colony homozygotes for all 4 loci, and into Class 4, the frequency of individual colonies that were heterozygotes for all 4 loci. Null amplifications were considered as homozygotes for a null allele. For each sample, the MLH distribution expected under random mating (MLH_{exp}) was obtained from 100 randomised pseudo-samples in which alleles were randomly associated within individuals using GENETIX. As inbred individuals appear more homozygous over all loci than expected under the hypothesis of random mating, inbreeding occurrence in our dataset should lead to lower MLH values than expected and a shift in the distribution towards lower MLH values. Significance was tested within each class by comparing the observed and expected MLH of the 100 pseudo-samples, and p-values were estimated as the probability of observing smaller or higher values than expected from the randomisation.

To test for relatedness between individuals and to obtain indirect estimates of gene dispersal distance parameters (e.g. neighbourhood size) in the context of isolation-by-distance processes (Hardy & Vekemans 1999), the program SPAGEDI 3.0 (Hardy & Vekemans 2002) was used. Moran's I relationship coefficients for diploid multilocus genotypes (Hardy & Vekemans 1999) were computed within geographical distance classes, following the hierarchical sampling design. Spatial autocorrelation methods have the advantage of allowing inference of spatial genetic structure independently from the often violated assumptions of classic F-statistics, such as absence of selection and mutation or complete random migration of a constant number of individuals between the subpopulations (Hardy & Vekemans 1999). Significant positive autocorrelation implies that individuals within a particular distance class are more genetically similar than individuals randomly taken from any distance class. The first x-intercept of the correlogram, which gives the average distance at which the similarity of any 2 sites is equal to the region-wide similarity expected by chance alone, is termed the 'genetic patch size' (Sokal & Wartenberg 1983). The significance of relatedness between individuals in each geographic distance class was obtained by comparing the observed coefficient to the distribution of the statistic under the null hypothesis of no spatial structure generated using 10 000 resamplings of the data, permuting spatial location among distance groups.

To test for the existence of subgroups within each sample (Wahlund effect), a partitioning method implemented in the program PartitionML (available from www.univ-montp2.fr/~genetix/partitionml.htm) was used as described in Castric et al. (2002). PartitionML searches for the best possible partition of a sample into independent panmictic clusters and simultaneously assigns individuals to the identified clusters using a maximum-likelihood (ML) criterion. By testing an artificial matrix along a Markov chain, this method is able to find the best partition of the whole sample into an *a priori* number (k) of subgroups, under the best panmixia and linkage equilibrium. Under the null hypothesis of k source populations, the optimal number of subgroups to be retained can be obtained by incrementing k until the null hypothesis is not rejected by the 'k + 1 versus k' likelihood ratio test. For this, a χ^2 test is successively performed between k + 1 and k, with the degree of freedom equal to the sum of the number of alleles of each locus minus the number of loci.

The genetic divergence among samples was determined from the original dataset using Weir & Cockeram's (1984) F_{ST} estimator in the ARLEQUIN Version 2.0 software (Schneider et al. 2000). The significance of the estimator was determined using bootstrap resampling with 10 000 permutations. Pairwise F_{ST} estimates were used to calculate the relationship between genetic differentiation and geographical distance by regressing $F_{ST}/(1 - F_{ST})$ values against the natural logarithm of the geographical distance between sample pairs using the Mantel test with 10 000 permutations, as implemented in GENEPOP. The test was conducted both among all samples and among samples within each location separately. The microsatellite data were subjected to a hierarchical analysis of molecular variance (AMOVA), using allele frequencies as those implemented in ARLEQUIN. Three hierarchical levels were considered: between locations, between samples within locations and within samples. When necessary, sequential Bonferroni corrections for multiple comparisons were applied using an experiment-wise significance level of 0.05.

RESULTS

Microsatellite loci variation

A total of 398 *Corallium rubrum* colonies were genotyped. The 4 microsatellite loci analysed were polymorphic in all samples. Tests of genetic disequilibrium between loci within samples indicated no significant association of alleles (all $p > 0.05$), confirming that all loci can be treated as independent markers.

Over all samples, the number of alleles per locus ranged from 10 (in COR15) to 29 (in COR9 and COR58). Within samples, over all loci, the mean number of alleles ranged between 7.2 (in SanFD) and 12 (in CalA). Overall, observed heterozygosities ranged between 0.20 (in COR58) and 0.43 (in COR15) and expected heterozygosities ranged between 0.56 (in COR15) and 0.77 (in COR9). Estimates of multilocus expected heterozygosity within samples ranged from 0.48 (in SanFD) to 0.80 in (CalA) (Table 2). Calafuria samples showed significantly higher numbers of alleles and expected heterozygosities than San Fruttuoso samples (Student's t-test: $p = 0.037$ for N_a; $p = 0.008$ for H_{exp}). In order to explain such differences in gene diversity, the original dataset, as well as the dataset adjusted according to the presence of null alleles, was used to test for recent population expansion. Both datasets gave similar results. Recent changes in effective population sizes were detected through heterozygote excesses in CalA and CalB under the IAM model and in CalB under the TPM model for the original dataset (all $p = 0.031$). Moreover, CalA and CalB showed evidence of heterozygote excesses under the IAM model for the adjusted dataset (all $p = 0.031$). None of the San Fruttuoso samples showed evidence of recent changes in effective population sizes.

Hardy-Weinberg equilibrium

All loci showed significant deviations from Hardy-Weinberg expected genotype frequencies in almost all samples (28 significant comparisons out of 32 after standard Bonferroni corrections). All multilocus estimates of F_{IS} were significantly different from zero ($p < 0.001$) and ranged from 0.38 to 0.60 (Table 2), reflecting heterozygote deficiencies in all analysed samples. A few specimens, even after repeated attempts, did not yield PCR products at some loci, while they successfully amplified at the other loci, strongly suggesting the presence of null alleles. Estimated null allele frequencies (r) per sample were high and ranged from 0.11 to 0.73 for COR9, from 0 to 0.35 for COR15, from 0.19 to 0.46 for COR48 and from 0.28 to 0.73 for COR58 (Table 2). On average, these estimates generated expected numbers of null homozygotes exceeding the observed number of non-amplified individuals (COR9: N_{00}[exp] = 12.2 and N_{00}[obs] = 4; COR15: N_{00}[exp] = 1.9 and N_{00}[obs] = 1.2; COR48: N_{00}[exp] = 6.7 and N_{00}[obs] = 2.5; COR58: N_{00}[exp] = 16 and N_{00}[obs] = 2.9). Allelic frequencies at each locus for each sample from original and adjusted datasets are available upon request.

In all samples, the results of the test assessing whether homozygote genotypes were represented randomly in the dataset showed significantly ($p < 0.001$) higher and lower frequencies than expected for the first 2 and for the last 2 MLH classes, respectively, leading to a shift in the distribution towards lower MLH values (Fig. 2). These results indicate that individuals were more homozygous at all 4 loci than expected under the null hypothesis of random mating.

Significantly positive Moran's I relationship coefficients were detected between individuals within samples (0 m class) at both locations ($I_{Cal} = 0.0182$, $p < 0.001$; $I_{SanF} = 0.0712$, $p < 0.001$), indicating that neighbouring individuals had a higher genetic relatedness than random pairs of individuals issued from all samples. The autocorrelogram (Fig. 3) suggested an estimated patch size for *Corallium rubrum* of <10 m, as shown by the negative, non-significant values of Moran's I coefficient in the 10 m class ($I_{Cal} = -0.0016$, $p > 0.05$; $I_{SanF} = -0.0015$, $p > 0.05$).

The null hypothesis $k = 1$ (i.e. all colonies belong to a random mating population) was rejected for all samples ($p < 0.001$). The most likely numbers of clusters

Table 2. *Corallium rubrum*. Genetic diversity at 4 microsatellite loci in each and over all *C. rubrum* samples. Cal: Calafuria; SanF: San Fruttuoso; n: total number of sampled individuals; N: total number of genotypes per loci; N_a: number of alleles per locus; H_{obs}: observed heterozygosity; H_{exp}: expected heterozygosity according to Hardy-Weinberg equilibrium (HWE); F_{IS}: Weir & Cockerham's (1984) estimate fixation index; r: null allele frequency as estimated using Eq. (3) from Brookfield (1996); N_{00}(exp): expected number of null homozygotes based on r. F_{IS} values in **bold** type indicate significant deviations from HWE after standard Bonferroni correction of the 5% threshold ($p < 0.001$)

Locus Parameters	CalA (n = 50)	CalB (n = 49)	CalC (n = 50)	CalD (n = 50)	SanFA (n = 50)	SanFB (n = 50)	SanFC (n = 50)	SanFD (n = 48)	Overall (n = 397)
COR9									
N	47	46	46	45	46	46	45	44	
N_a	16	19	15	15	11	13	11	12	29
H_{obs}	0.21	0.24	0.17	0.13	0.26	0.46	0.58	0.48	0.32
H_{exp}	0.89	0.9	0.91	0.85	0.62	0.57	0.76	0.68	0.77
F_{IS}	**0.77**	**0.74**	**0.81**	**0.85**	**0.58**	0.2	**0.25**	**0.31**	**0.56**
r	0.62	0.58	0.69	0.73	0.41	0.11	0.13	0.17	0.43
N_{00}(exp)	19	16	24	27	8	1	1	1	
COR15									
N	50	49	46	50	47	49	49	47	
N_a	5	4	6	7	3	3	3	4	10
H_{obs}	0.68	0.59	0.37	0.4	0.36	0.55	0.22	0.23	0.43
H_{exp}	0.67	0.7	0.69	0.68	0.53	0.53	0.46	0.26	0.56
F_{IS}	−0.001	**0.16**	**0.47**	**0.42**	**0.33**	−0.02	**0.52**	0.13	**0.25**
r	0	0.09	0.30	0.26	0.15	0	0.35	0.06	0.15
N_{00}(exp)	0	0	5	3	1	0	6	0	
COR48									
N	48	46	45	49	47	47	48	47	
N_a	8	9	8	14	8	10	12	8	21
H_{obs}	0.27	0.24	0.53	0.49	0.25	0.28	0.52	0.42	0.38
H_{exp}	0.73	0.79	0.78	0.73	0.66	0.71	0.8	0.74	0.74
F_{IS}	**0.64**	**0.70**	**0.33**	**0.34**	**0.62**	**0.62**	**0.36**	**0.43**	**0.51**
r	0.46	0.53	0.19	0.20	0.45	0.43	0.21	0.28	0.34
N_{00}(exp)	11	14	2	2	10	9	2	4	
COR58									
N	46	48	45	44	45	50	49	47	
N_a	19	7	8	8	8	14	11	5	29
H_{obs}	0.50	0.19	0.13	0.29	0.20	0.12	0.14	0.06	0.20
H_{exp}	0.89	0.70	0.59	0.67	0.76	0.76	0.66	0.21	0.66
F_{IS}	**0.45**	**0.74**	**0.78**	**0.57**	**0.74**	**0.84**	**0.79**	**0.71**	**0.70**
r	0.28	0.58	0.64	0.40	0.58	0.73	0.65	0.56	0.55
N_{00}(exp)	4	16	20	8	17	27	21	15	
Multilocus									
N_a	12	9.7	9.2	11	7.5	10	9.2	7.2	
H_{obs}	0.41	0.31	0.30	0.33	0.27	0.35	0.36	0.30	
H_{exp}	0.80	0.77	0.74	0.73	0.64	0.64	0.67	0.48	
F_{IS}	**0.49**	**0.60**	**0.60**	**0.58**	**0.59**	**0.46**	**0.46**	**0.38**	

identified within samples were $k = 2$ for CalA, CalD and all San Fruttuoso samples and $k = 3$ for CalB and CalC ($p < 0.001$), making, in total, 18 clusters with reassigned colonies. None of the 18 clusters were at HWE (data not shown).

Genetic differentiation between samples

F_{ST} estimates were found to be homogeneous across all 4 microsatellite loci (global single locus F_{ST} values were 0.15 for COR9, 0.16 for COR15, 0.15 for COR48 and 0.12 for COR58 using original data; all $p < 0.001$) and indicated strong genetic structuring over all samples (multilocus $F_{ST} = 0.144$, $p < 0.001$). Global estimates of F_{ST} within Calafuria ($F_{ST} = 0.021$, $p < 0.001$) were lower than those observed within San Fruttuoso ($F_{ST} = 0.073$, $p < 0.001$). The AMOVA revealed that 16.62% ($p = 0.022$) of the total genetic variation occurred among locations, 4.23% ($p < 0.001$) was attributable to variation among samples within locations and 79.15% ($p < 0.001$) occurred among individuals within samples.

Pairwise multilocus estimates of F_{ST} were generally high (range: 0.01 to 0.30) and significantly different from zero for all pairwise comparisons after sequential

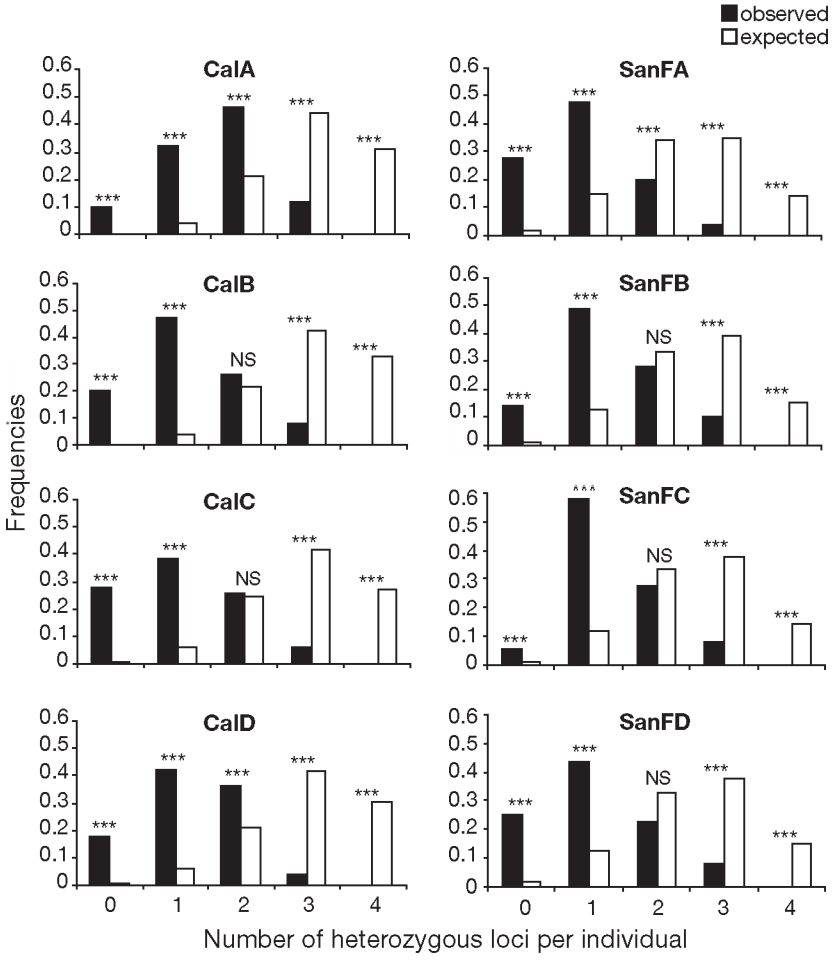

Fig. 2. *Corallium rubrum*. Distributions of observed and expected multi-locus heterozygosity (MLH) frequencies estimated within samples. Five classes were defined according to the number of heterozygote loci per individual, ranging from Class 0, which includes individual colony homozygotes for all 4 loci, to Class 4, which includes individual colony heterozygotes for all 4 loci. Significance was tested within each class by comparing the observed and expected MLH of the 100 pseudo-samples, and p-values were estimated as the probability of observing lower or higher values than expected from randomisation. NS: not significant; ***$p < 0.001$

Bonferroni corrections (Table 3). A statistically significant correlation was observed between genetic divergences and geographical distances when considering all samples ($p = 0.01$). However, no significant correlation was observed within each location separately ($p_{Cal} = 0.08$; $p_{SanF} = 0.12$).

DISCUSSION

Inferring the spatial scales at which genetic variation occurs is a key issue in genetic studies on marine sessile invertebrates. However, genetic variation at the scale of metres has rarely been analysed, despite the theoretical expectations of its importance (Palumbi 2004). Using microsatellite markers and a replicated, hierarchical sampling design, we have demonstrated that in the red coral *Corallium rubrum* significant genetic structure exists at spatial scales as small as a few metres. The 2 main results of this study are (1) *C. rubrum* populations are characterised by heterozygote deficiency and (2) a significant genetic structuring occurs among samples 10 m apart.

Microsatellite genetic variability

The multilocus polymorphism found in *Corallium rubrum* using microsatellites (H_{exp} between 0.48 and 0.80, with a mean of 0.68 ± 0.09) was within the range of values reported for other marine invertebrates, such as sponges (H_{exp} = 0.605; Duran et al. 2004) and scleractinian corals (H_{exp} = 0.75; Magalon et al. 2005), using the same molecular markers. Conversely, the microsatellite H_{exp} values were much higher than those obtained for the same species using allozymes (mean H_{exp} = 0.088; Abbiati et al. 1993). This is an expected result given the higher rates of polymorphism of microsatellites relative to allozymes.

San Fruttuoso samples of *Corallium rubrum* showed a significantly lower genetic diversity compared to Calafuria samples, which may suggest differences in evolutionary history between the locations, e.g. San Frut-

Table 3. *Corallium rubrum*. Pairwise multilocus estimates of F_{ST} (Weir & Cockerham 1984) between all red coral samples; all $p < 0.001$ and all are statistically significant after sequential Bonferroni correction

	CalA	CalB	CalC	CalD	SanFA	SanFB	SanFC
CalB	0.023						
CalC	0.041	0.010					
CalD	0.038	0.014	0.023				
SanFA	0.180	0.175	0.193	0.209			
SanFB	0.176	0.180	0.203	0.200	0.053		
SanFC	0.157	0.168	0.175	0.192	0.072	0.041	
SanFD	0.266	0.277	0.295	0.300	0.138	0.116	0.070

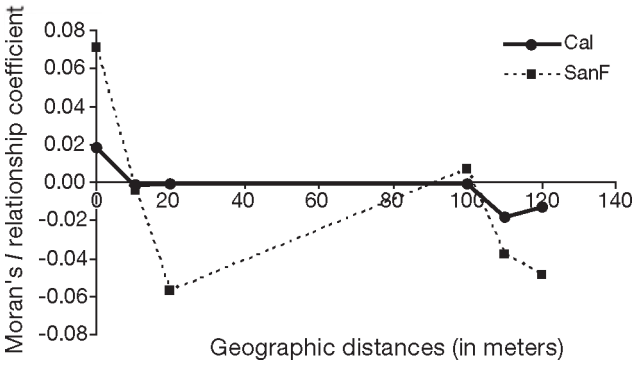

Fig. 3. *Corallium rubrum.* Spatial autocorrelograms based on Moran's *I* coefficients estimated over all microsatellite loci for Calafuria (Cal) and San Fruttuoso (SanF) locations. Significant Moran's *I* coefficients were only observed in the 0 m class for both locations ($p < 0.01$)

tuoso being affected by a recent bottleneck (reduction in census population size) or being genetically isolated. The bottleneck test did not support the hypothesis of a recent reduction in effective population size in San Fruttuoso samples. This result was consistent using both the original dataset and a dataset adjusted for the presence of null alleles. Nevertheless, the hypothesis that differences in genetic diversity are related to a past bottleneck cannot be rejected with full confidence because of the limited number of loci (not in Hardy-Weinberg equilibrium) that were analysed; a bottleneck is in fact most likely to be detectable when using 8 to 10 polymorphic microsatellite loci (Luikart & Cornuet 1998). Differences in habitat characteristics at the 2 study locations could also account for the differences in genetic variability observed within San Fruttuoso and Calafuria samples of *C. rubrum*. The size and spatial arrangement of habitat, indeed, may be of great importance for the ecology and population structure of species with limited dispersal range (Palumbi 2004). The habitat in San Fruttuoso was characterised by the presence of fewer large crevices compared to Calafuria, which could affect both fine-scale larval settlement and post-settlement mortality rates (e.g. different protection from disturbance or predation).

Heterozygote deficiency

Strong deviations from Hardy-Weinberg equilibrium were detected for all samples and emphasised by high positive F_{IS} estimates. Heterozygote deficiencies may be the result of (1) technical factors, such as the presence of non-amplifying or null alleles, whereby some of the apparent homozygotes might actually be heterozygotes with 1 visible and 1 null allele, and/or (2) biological factors, e.g. mixing of differentiated gene pools (Wahlund effect) or high levels of consanguineous mating (inbreeding). In our study non-amplifying individuals (null homozygotes) were found in all samples and for all loci, but their number was significantly smaller than expected if the heterozygote deficiencies were generated only by null alleles. Moreover, heterozygosity deficiencies have already been observed in a previous allozymatic study on Mediterranean red coral populations (Abbiati et al. 1993, 1997). Therefore, it is unlikely that the observed positive F_{IS} estimates were caused only by the presence of null alleles, and biological features of the species probably contributed to the observed heterozygote deficits.

While the presence of null alleles overestimates the number of single-locus homozygote genotypes, the possibility that an individual colony carries more null alleles at all genotypes than another is unlikely. Indeed, under the random mating hypothesis, false homozygote genotypes (i.e. a genotype that in reality is a heterozygote with a null allele) should be randomly distributed among individuals when no linkage disequilibrium is observed among loci. Occurrence of inbred individuals in all samples was shown by individual MLH analysis, suggesting that inbreeding contributes to the observed heterozygote deficiencies. Deficiencies of heterozygotes have been described in many shallow-water Anthozoa populations, and it has been hypothesised that heterozygote deficiencies were a consequence of localised recruitment (via restricted dispersal of gametes or larvae) and inbreeding (Gutierrez-Rodriguez & Lasker 2004, Magalon et al. 2005). Observed heterozygote deficiencies in red coral samples, reported from allozyme studies, have been attributed to restricted genetic mixing and a predominance of local recruitment (Abbiati et al. 1993, 1997). Our study confirms previous findings and is in accordance with the reproductive biology and larval ecology of red coral. Indeed, in red coral, embryo development takes place within the female polyps for about 30 d, before release of planula larvae (Vighi 1972). Moreover, reduced swimming ability and geonegative behaviour of the planulae suggest that, once released, larvae settle in close vicinity of the parental colonies (Vighi 1972). Therefore, reproduction events in red coral are more likely to involve related individuals compared to species with broadcasting development.

Admixture of cohorts with different allele frequencies, resulting from spatially or temporally distinct recruitment events of larval cohorts (Johnson & Black 1984), may also account for the observed heterozygote deficiencies. Lack of significant relatedness between individuals collected 10 m apart and high genetic divergences observed between samples both suggest limited gene flow at the 10 m scale. Moreover, the

maximum-likelihood partitioning method revealed the occurrence of subpopulations within each sample. Both these findings support the presence of a spatial Wahlund effect within samples. The Wahlund effect may result from the chaotic recruitment of cohorts with different geographical/parental origins. The source population of settling larvae may vary from year to year, depending on the oceanographic and environmental conditions. Evidence of genetic variation among settling larvae has been documented in marine invertebrates, such as limpets (Johnson & Black 1984) and polychaetes (Virgilio et al. 2006). In those studies, extremely large variance in individual reproductive success resulted in only a small portion of the adult population contributing to the yearly recruitment. This may also be the case for red coral, which shows large variation in demographic features and in larval production (Santangelo et al. 2003). Moreover, spatiotemporal variation in oceanographic conditions may produce limited windows for larval survival, further amplifying the effect of variations in reproductive success. According to such a hypothesis, natural populations may show detectable genetic heterogeneity among cohorts, because only a small proportion of the adults contributes to maintaining the population. A temporal Wahlund effect is an alternative explanation, whereby each sample may consist of several breeding subunits issued from individuals reproducing at different times over the year. Temporal variation of the genetic structure of populations should be investigated in order to test for significant temporal structuring within populations.

Fine-scale genetic structuring

Our results indicated high degrees of genetic structuring among *Corallium rubrum* samples at spatial scales of 10s of metres. The 4 microsatellite loci that were used contributed significantly to the overall genetic differentiation. Moreover, sample size (N = 50 for each sample) is considered adequate to produce accurate estimates of F_{ST} with highly variable loci (Ruzzante 1998). Our results are similar to those from other sessile marine invertebrates known to have limited larval dispersal, such as the brooding soft coral *Alcyonium* sp. (McFadden & Aydin 1996), *Clavularia koellikeri* (Bastidas et al. 2002) and the viviparous coral *Pocillopora meandrina* (Magalon et al. 2005). All these results are consistent with the hypothesis that brooders are more likely to exhibit restricted gene flow (Carlon 1999). Nevertheless, a study by Miller (1998), using spatial autocorrelation analyses, estimated a patch size of <10 m in a broadcast-spawning antipatharian coral, suggesting that other ecological and evolutionary factors may affect gene flow and genetic differentiation in Alcyonacea species.

A previous study on red coral (Abbiati et al. 1993) showed the occurrence of marked genetic differentiation between samples collected at 2 locations about 200 km apart. F_{ST} values based on allozymes were consistent with our estimates. This differentiation is likely to be related to the occurrence of barriers to gene flow, such as the geographic distance and the presence of a wide sandy shore between the 2 sampling locations, resulting in a major gap in the distribution of red coral.

In contrast to our study, genetic structuring among red coral samples collected over a distance scale of about 200 m has not been detected (Abbiati et al. 1997). Differences in polymorphism between microsatellites and allozymes may account for this inconsistency, since higher levels of polymorphism at microsatellite loci result in the higher power of statistical tests to detect population differentiation (Estoup & Angers 1998). Reduced gene flow at a scale of 10s of metres likely originates from the restricted dispersal capacities of gametes and larvae. Localised recruitment in several species, which has often been attributed to larval retention processes caused by physical and behavioural mechanisms. Differences in F_{ST} estimates within locations ($F_{ST\ within\ Cal} = 0.025 \pm 0.002$; $F_{ST\ within\ SanF} = 0.082 \pm 0.006$) may be related to variations in geomorphologic and hydrodynamic features, such as crevice size and shape, current flow, or other local features. For example, at Calafuria, *Corallium rubrum* is distributed over a wide cliff characterised by nearly vertical walls scattered with small crevices and overhangs, while, at San Fruttuoso, the cliff slopes down at an inclination of about 45° and an area of large boulders is succeeded by larger crevices. These geomorphologic differences, as well as differences in small-scale habitat heterogeneity, may lead to microscale regimes of currents, further affecting patterns of dispersion/retention of planulae. Furthermore, as already stated, biological processes (e.g. pre- and post-settlement mortality and the stochasticity of reproductive success) may differ between the 2 locations.

We illustrated the occurrence of significant genetic structuring at spatial scales of 10s of metres in *Corallium rubrum*, supporting the hypothesis of limited dispersal ability of planulae. Furthermore, evidence of inbreeding, the Wahlund effect and high genetic variance within samples suggest that the effective larval dispersal range may be <10 m. An accurate paternity analysis between neighbouring individuals is required to corroborate these results, and microscale studies would clarify the underlying ecological and evolutionary factors influencing the genetic structuring of red coral. Nevertheless, the conclusions of the present

genetic study and previous demographic and ecological studies (e.g. Santangelo & Abbiati 2001, Garrabou & Harmelin 2002, Torrents et al. 2005) provide insights for the conservation of Mediterranean red coral populations. The occurrence of structured breeding units and differences in genetic diversity among locations indicate that a rotating harvesting scheme of red coral resources at a pan-Mediterranean scale, as recently recommended (Caddy 1993), will be detrimental for the conservation of the species. Instead, strategies for sustainable management and conservation of red coral should be defined at a local scale. Moreover, the 'boom and burst' harvesting strategy applied in many countries should be avoided, since it may increase risks of local extinction, especially in shallow-water habitats.

Acknowledgements. This study is part of the doctoral thesis of F.C. at the University of Bologna, Italy. We thank the administrative authority of the Marine Protected Area of Portofino and the Reparto Operativo Aeronavale Guardia di Finanza of Genova for their logistical support during the dives. We also thank M. Carrera, F. Bulleri and M. Ponti for their help in the field. We are grateful to F. Garoia for his valuable support during the different stages of this study. We thank M. Virgilio, K. Peijnenburg, R. Toonen, for their comments on earlier versions of this manuscript. We further thank L. Airoldi and 3 anonymous referees for constructive comments. This paper was written under the EU funded Research Contract EVK3-CT-2001-00048 (EUMAR) and has benefited from funding by the AMS project (Artificial Marine Structures: Multifunctional Tool for Research and Environmental Management in the Mediterranean and Red Sea, Italian Ministry of the Environment and Territory). This publication is contribution number MPS-07014 of the EU Network of Excellence MarBEF.

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Editorial responsibility: Matthias Seaman (Assistant Editor-in-Chief), Oldendorf/Luhe, Germany

Submitted: September 15, 2006; Accepted: December 14, 2006
Proofs received from author(s): June 6, 2007