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Symbiont diversity is not involved in depth acclimation in the Mediterranean sea whip *Eunicella singularis*

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ABSTRACT: In symbiotic cnidarians, acclimation to depth and lower irradiance can involve physiological changes in the photosynthetic dinoflagellate endosymbiont, such as increased chlorophyll content, or qualitative modifications in the symbiont population in favour of better adapted strains. It has been argued that a lack of capacity to acquire new symbionts could limit the bathymetric distribution of the host species, or compromise its long-term survival in a changing environment. But is that always true? To address this question, we investigated the symbiont genetic diversity in *Eunicella singularis*, a Mediterranean sea whip species with a wide bathymetric distribution (10 to 50 m depth), which has recently suffered from mass mortalities after periods of abnormally high sea temperatures. We measured symbiont population densities and chlorophyll content in natural populations, and followed the response of the holobionts after reciprocal transplantations to deep and shallow depths. A total of 161 colonies were sampled at 2 depths (10 and 30 m) at 5 sites in the northwestern Mediterranean. All colonies harboured a single ribosomal *Symbiodinium* clade (A'), but a relatively high within-clade genetic diversity was found among and within colonies. This diversity was not structured by depth, even though the deeper colonies contained significantly lower population densities of symbionts and less chlorophyll. We did, however, reveal host-symbiont specificity among *E. singularis* and other Mediterranean cnidarian species. Transplantation experiments revealed a limit of plasticity for symbiont population density and chlorophyll content, which in turn questions the importance of the trophic role of *Symbiodinium* in *E. singularis*.

KEY WORDS: Algal-coral interactions · *Symbiodinium* · 18S rDNA · Genetic diversity · Depth distribution · Photoacclimation · *Eunicella singularis* · Mediterranean Sea

INTRODUCTION

Cnidarian–dinoflagellate symbioses, along with plant/mycorrhyzal associations and lichens, have been proposed to be non-classical mutualisms (Herre et al. 1999, Baker 2003, Hay et al. 2004). The flexibility in these systems (i.e. the evolutionary frequency at which the symbionts associate with a new host species) forms a sharp contrast with other trophic mutualisms such as the bacteria–insect relationships, which could in some cases be seen as frozen 'end of game' situations of a co-evolutionary arms race (Moran 1996). More recent results, however (Goulet 2006), seem to imply more specificity in the host–symbiont relationship in cnidarian symbiosis.

Many marine cnidarians (jellyfish, sea anemones, reef-building corals, and gorgonians) live in intimate endosymbiosis with photosynthetic dinoflagellates

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(commonly named zooxanthellae), which have been largely assigned to the genus *Symbiodinium.* These organisms form a mutualistic association, in which photosynthates and oxygen from the symbiont are transferred to the host and exchanged for shelter, inorganic carbon, and nitrogen from the host (Goodson et al. 2001, Furla et al. 2005). In tropical reef-building corals, the zooxanthellae provide up to 98% of the organic carbon needs of their animal hosts (Muscatine 1990). However, harbouring a photosynthetic organism in its cells, despite this obvious gain in autotrophy, also creates strong physiological constraints on the animal hosts. For example, the animal hosts need to adapt to daily transitions from quasi anoxia to hyperoxia in their tissues, according to the photosynthetic activity of their symbionts (Richier et al. 2003). This implies that both partners had to experience a lot of adaptive changes in order to live in such an intimate association, and this necessary co-evolution could have led these organisms to a frozen obligate mutualism. However, a high symbiont polymorphism and a relative lack of specificity in the host/symbiont association were revealed by molecular studies of tropical cnidarians (see Baker 2003, for review). The genus *Symbiodinium* can be divided into at least 8 divergent phylogenetic clades, designated A to I (Rowan et al. 1997, Pochon et al. 2005, Pochon & Gates 2010). These clades are found in association not only with various cnidarians, but also with phylogenetically diverse hosts, from soritid foraminiferans to molluscs (Baker 2003). Furthermore, some reef-building coral species can harbour genetically different symbionts, depending on their latitudinal or bathymetric location (Baker 2003). Several zooxanthella clades can even be found within the same coral colony in *Montastrea* spp. or *Acropora* spp., for example (Rowan et al. 1997, Chen et al. 2005a). The composition of this polymorphic symbiont population can vary in response to environmental change (see for example (Rowan et al. 1997, Baker 2001, Chen et al. 2005a).

On some occasions, this genetic diversity in *Symbiodinium* populations within the host has been linked to photoadaptive selection. A relatively clear depth zonation has been found *in vivo* in some coral species among *Symbiodinium* clades (Baker 2003, Iglesias-Prieto et al. 2004). As reef-building corals depend heavily on the photosynthetic activity of their symbionts, compensation mechanisms are required to maintain the supply of photosynthate. This photoacclimation seems to occur mainly through an increase in the cellular chlorophyll content of the symbionts or, over time, an increase in symbiont density or both (see Fitt & Cook 2001, for review). However, this photosynthetic plasticity seems to be limited, as the bathymetric distribution of tropical coral species appears to be constrained by the photosynthetic optima of their symbionts, as seen by Iglesias-Prieto et al. (2004) for *Pocillopora* and *Pavona* spp.

These observations (genetic diversity, limited plasticity, or observed zonation of the symbionts) in turn led to a reappraisal of another peculiarity of the coral symbiotic association: the relative ease with which it could break down. The disruption of the cnidarian–dinoflagellate association is a commonly observed phenomenon known as bleaching. Coral bleaching has mostly been publicised for the high mortality in tropical reefs that results from mass bleaching events (Hoegh-Guldberg 1999). But bleaching could also be seen as an adaptive strategy under changing environmental conditions for the host to exchange symbionts in order to establish a new symbiosis potentially better suited to the new conditions, the 'adaptive bleaching hypothesis' (Buddemeier & Fautin 1993). The ongoing debate on the validity of this hypothesis (Goulet 2006) is another testimony to the complexity of the dynamics of the cnidarian–dinoflagellate association.

Relatively few studies have focused so far on temperate zooxanthella diversity, but all have reported a relatively low symbiont diversity, either within (Bythell et al. 1997, LaJeunesse & Trench 2000, Chen et al. 2005b) or among host species (Savage et al. 2002). In host species distributed from tropical or subtropical to temperate areas, adaptation to lower temperatures and different irradiances has involved a switch to another zooxanthella clade such as in *Plesiastrea versipora* (Rodriguez-Lanetty et al. 2001), or a loss of *Symbiodinium* diversity such as in *Anthopleura elegantissima* that may eventually lead to a switch to an altogether different photosynthetic symbiont (LaJeunesse & Trench 2000). Similarly, it has been shown that in the northeastern Atlantic and the Mediterranean, a single derived 'temperate A' or A' ribosomal haplotype seems to be shared between all the host species sampled so far (Bythell et al. 1997, Savage et al. 2002, Barbrook et al. 2006, Visram et al. 2006). However, adaptation to the highly variable conditions of temperate or subtropical waters was also found to lead to a maintenance of symbiont polymorphism in some cases, either to face seasonal changes (Chen et al. 2005a) or to adapt to local light and temperature conditions (Bates 2000), and even to promote a high endemicity (Wicks et al. 2010).

Until the present study, no in depth molecular study of *Symbiodinium* diversity had been performed

for any Mediterranean host species. We therefore explored the symbiont diversity in natural populations of *Eunicella singularis*, the only symbiotic gorgonian in the Mediterranean (Carpine & Grasshoff 1975). This species has a very wide bathymetric distribution (Carpine & Grasshoff 1975, Weinberg 1979a, Linares et al. 2008) requiring adaptation to very different light and temperature regimes. Moreover, this species suffered from catastrophic (though geographically restricted) mortality, following sea temperature rises in the past decade (Cerrano et al. 2000, Perez et al. 2000, Garrabou et al. 2009), an occurrence reminiscent of the tropical bleaching phenomenon. *E. singularis* is thus an appropriate model to see if photoacclimation does depend on symbiont diversity in a temperate marine symbiosis. We analysed *Symbiodinium* diversity within and among populations, and within colonies of *E. singularis*, at 2 different depths (10 and 30 m). We also analysed within-colony *Symbiodinium* density and chlorophyll concentrations both in natural settings and after reciprocal transplantations in these shallow and deep populations.

MATERIALS AND METHODS

Biological model

Eunicella singularis (class Anthozoa, subclass Octocorallia, order Gorgonacea) is common throughout the western Mediterranean basin, with a patchy distribution. It has a wide bathymetric distribution, from 5 to 50 m depth, with the highest population densities between 15 and 30 m (Weinberg 1979a, but see Linares et al. 2008). Exposure to temperatures higher than 24 to 26°C is lethal to *E. singularis* within days (Weinberg 1979a). *E. singularis* is a gonochoric brooding species; fertilization occurs in the female colonies, which then release mobile zooxanthellate planula larvae. This release occurs once a year between June and July (Weinberg 1979b).

Sample collection

Individual colonies (n = 161) were sampled from 5 locations on the northwestern Mediterranean coast, from the Gulf of Genoa (Italy) to the Balearic Island of Menorca (Spain) in March–July 2003. Samples were collected from 2 depths, around 10 and 30 m at each site, except in Menorca, where only a 10 m depth population was sampled (see Fig. 1). For each location, the sets of individuals sampled will be referred to as the 'shallow' (10 m) or 'deep' (30 m) population. Branches 10 to 15 cm long were clipped from 20 individual colonies from both depths at each location, except at Portofino (only 13 shallow and 17 deep colonies) and Menorca (only 10 shallow colonies) (see Fig. 1). After collection, samples were rapidly dried on paper and cut in two: one part was preserved in 70% ethanol for the genetic analysis of the symbionts, and the other part was frozen in dry ice for symbiont density and chlorophyll content analysis. Samples were stored at room temperature or –80°C, respectively.

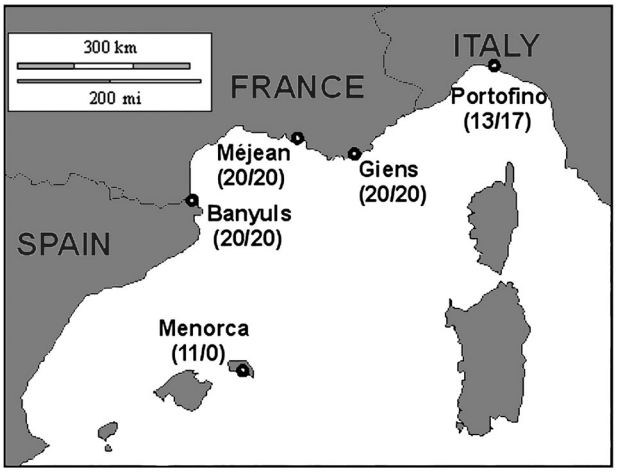

Fig. 1. Sampling sites (O) of *Eunicella singularis* in the Northwestern Mediterranean Sea. The name of each location is followed in brackets by the number of colonies sampled in shallow (10 m) and deep (30 m) waters, respectively. At Menorca a single population was sampled at 10 m

Symbiont genetic diversity

Total DNA extraction. Total genomic DNA was purified from 200 mg of the ethanol-preserved fragments, following a standard phenol–chloroform extraction preceded by digestion in 5 mM EDTA, 10 mM Tris (pH 7.5), and 0.5% SDS with 0.5 µg l^{-1} proteinase K (Finnzymes) for 3 h. DNA was then precipitated in ethanol and resuspended in Ultrapure MilliQ water. A 1:100 dilution of the extracts was used for the subsequent PCR amplifications. DNA extracts and dilutions were stored at –20°C.

Clade identification (small and large ribosomal subunits PCR-RFLP). Zooxanthella clade was determined by PCR RFLP of both the nuclear small and large ribosomal subunits (Rowan & Powers 1991, Savage et al. 2002, Baker 2003). PCR amplification of

the nuclear small ribosomal subunit (SSU) was performed following McNally et al. (1994), using the PCR primers ss3z and ss5 from Rowan & Powers (1991). The PCR mix composition was 1 µM of each primer, 0.8 mM dNTPs, 2 mM $MgCl_2$, 1× PCR *Taq* Platinum reaction buffer and 1.5 units of *Taq* Platinum (Invitrogen), to which was added 4 to 12 µl of the 1:100 dilution of the total genomic DNA extracts, and MilliQ water up to a total reaction volume of 25 µl. PCR conditions were 2 min at 94°C, followed by 40 cycles of 45 s at 92°C, 1 min at 53°C, and 2 min at 72°C, and a final elongation of 7 min at 72°C. One half of the PCR product was then digested by *Taq*I restriction enzyme and the other half by *Dpn*II, following manufacturer's recommendations (New England Biolabs).

PCR amplification of the nuclear large ribosomal subunit (LSU) was performed using the Ls1-3 and Ls1-5 primers from Wilcox (1998) and the same reaction mix as for the SSU PCR. PCR conditions were 3 min at 95°C, 45 cycles of 45 s at 92°C, 45 s at 63°C, 1 min 30 s at 72°C, and finally 7 min at 72°C. PCR was followed by a *Dde*I digestion, following the manufacturer's recommendations (New England Biolabs).

Restriction products were visualised using ethidium bromide staining of a 2% agarose electrophoresis gel in 2× TAE buffer (90 min migration at 50 mA). Restriction fragment sizes were estimated for each gel from a molecular weight marker (1kb+; Invitrogen) using the Genetools software (Syngene).

Within-clade diversity analysis. Three different markers of within-clade diversity were used: LSU PCR-RFLP, size variation of the amplified chloroplast 23S ribosomal subunit, and LSU sequence variation. LSU PCR-RFLP was performed as described for the clade identification, except that DNA restriction was performed with *Dpn*II (New England Biolabs) instead of *Dde*I.

We also screened for length variation in the plastid sequence coding for Domain V of the chloroplast large subunit (cp23S) following Santos et al. (2003). Amplification products were separated on 1 mm thick 8% polyacrylamide gels run at 80 mA for 2 h in 2× TAE buffer. Amplified DNA fragment sizes were estimated as described for Clade identification.

To further analyse the levels of polymorphism, we sequenced the D1-D3 domains of the nuclear LSU for one colony per PCR RFLP/cpDNA haplotype per population. PCR amplifications were performed as for the PCR RFLP analysis. PCR products were then cloned using the pGEM-T Easy Vector System II cloning kit (Promega) following the manufacturer's instructions. At least 3 positive clones per amplification were sequenced on both strands. This resulted in sequencing 56 clones from 8 deep colonies and 10 shallow colonies. As a control for cloning artefacts, we also cloned and sequenced in the same manner the LSU of a *Symbiodinium* sp. culture that was originally extracted from *Galaxea fascicularis*, a tropical reef-building coral. This zooxanthella strain had been previously assigned by PCR RFLP to the 'Tropical A' clade (data not shown). All sequencing reactions were performed by Macrogen.

Data analysis. Population differentiation for haplotype frequencies was assessed by calculating Wright fixation indices using Arlequin version 3.0 software (Excoffier et al. 2005) within a hierarchical sampling scheme (shallow and deep populations at each of the 4 main locations and only a shallow population for Menorca).

Sequences were proofread and edited using Bioedit software (Hall 1999). Previously published sequences of A' zooxanthellae from temperate symbiotic sea anemones and scleractinian corals (Savage et al. 2002, Barbrook et al. 2006, Visram et al. 2006) were added for comparison to the alignment (GenBank accessions: *Symbiodinium* sp. ex *Cereus pedunculatus* AY074945, AY588469; ex *Anemonia* sp. AY074939, AY074940, AY074973, AY074974, AY074975, AY074976, AY074977; ex *Balanophyllia europaea* AY588471; ex *Caryophyllia smithi* AY588472; ex *Cladocora caespitosa* AY588473). All these sequences were aligned together using Multalin at http://bioinfo.genotoul.fr/multalin/multalin.html (Corpet 1988).

The *Symbiodinium* alignment was then hand-aligned to a reference dinoflagellate LSU sequence of *Prorocentrum micans* from the CRW database at http://www.rna.ccbb.utexas.edu/ (Cannone et al. 2002), in order to identify mutations in the dataset that hit highly conserved regions (positions with >98% conservation among the 23S data from Fields & Gutell 1996).

Nucleotide diversity (π) and the proportion of different nucleotides were calculated with Mega4 (Kumar et al. 2004), excluding indels from pairwise comparisons. Standard errors were obtained by 500 bootstrap replications, which allowed *t*-test comparisons of these measures among deep and shallow populations.

We performed a nested clade analysis (NCA) with Geodis 2.0 (Posada et al. 2000) to determine if the 3 origins of the zooxanthellae, i.e. from shallow or deep colonies, or from hosts other than *Eunicella singularis*, were randomly distributed among zooxanthella lineages. A 95% statistical parsimony network (maximum connection steps equal to 10) (Posada & Cran-

dall 2001) was therefore built from the aligned sequences using TCS (Clement et al. 2000), with the gaps treated as a fifth state. Nested clades were defined following Panchal & Beaumont (2007) after alternative branching resolution according to Pfenninger & Posada (2002). As we only had interest in testing the null hypothesis of random distribution of the zooxanthella lineages among these 3 compartments, and did not intend to build a phylogeographic scenario for A' *Symbiodinium*, we arbitrarily fixed the distance value between shallow, deep, and non *E. singularis* hosts at 100, and we did not refer to the inference key of the NCA. This analysis was performed either on the whole dataset or on *E. singularis* symbionts only to test the random distribution of lineages between the 2 depths. The same analysis was also performed to detect any geographic structure of the LSU diversity, by pooling the shallow and deep populations at each location and using a matrix of geographic distances between the locations in Geodis. For both shallow vs. deep and geographic location comparisons, an alternative nesting process was also performed considering contiguous indels along a branch as a single mutational event. These analyses were finally repeated after excluding sequences bearing mutations in conserved regions of the LSU, which could represent potential ribosomal pseudogenes and, hence, intragenomic variation.

Symbiont population density and chlorophyll concentration

For each population, at each location, we measured total holobiont chlorophyll content and zooxanthella population density within the tissues for 7 colonies. For each colony, 200 mg of frozen tissue was ground in liquid nitrogen. Subsequent manipulations were performed at 4°C. Ground tissue was suspended in 0.75 ml 0.5 M potassium phosphate buffer (pH 7) and filtered by centrifugation (2 min at 600 × *g*) through a 100 µm nylon mesh to eliminate most spicules and fragments of proteinaceous axis. To prevent any loss of zooxanthellae, the skeleton fragments were rinsed with 0.75 ml buffer and centrifuged again through the mesh in the same conditions and both filtrates were finally pooled. 100 µl of the filtrate were used for zooxanthella population density measurements and 1 ml for chlorophyll extraction.

Colony surface area estimation. The weight W (mg) and the surface area S (cm^2) of a set of frozen colony fragments was measured by the aluminium foil method (Marsh Jr. 1970) and a very strong linear correlation ($S = 0.0136\,W + 0.4432$, $R^2 = 0.91$) was found between S and W. We then used this regression to estimate the surface of each colony fragment from its frozen weight.

Zooxanthella population density. For each sample, 100 µl of the filtrate was deposited once on a modified Neubauer haemocytometer, and zooxanthellae were counted 4 times on this cell under 20× magnification. Zooxanthella density was calculated per unit surface area of colony.

Chlorophyll concentration. Chlorophyll *a* (chl *a*) and chl c_2 contents were estimated by spectrophotometry after 2 successive acetone extractions. The initial 1 ml of sample filtrate was centrifuged at 13000 × *g* for 3 min to pellet the zooxanthellae. After elimination of the supernatant, the pellet was resuspended in 1 ml of pure acetone and left in the dark at 4°C under constant stirring for 24 h. This was followed by centrifugation at 13000 × *g* for 20 min. The acetone supernatant was stored in the dark and the pellet was re-extracted in a further 1 ml acetone, following the same protocol. The optical densities of the first and second extracts were measured on a spectrophotometer (UVmc2; Saphas) first at 750 nm to check for the turbidity of the sample and then at 630 and 663 nm. The optical densities of the first and second extracts were then added to calculate chl *a* and chl c_2 concentrations in µg per ml of acetone according to Jeffrey & Humphrey (1975). For each sample, the chlorophyll content was normalised per unit surface area of colony or per zooxanthella.

Reciprocal transplantation

A total of 20 colonies (10 shallow colonies at 10 to 15 m depth and 10 deep colonies at 30 to 35 m depth) were sampled at Portofino (Italy) in April 2004. Each colony was cut into 4 fragments of 15 to 20 cm in length. Two transplantation grids were used, one at 15 m (shallow transplantation), the other at 35 m (deep transplantation) at the same location. Each grid consisted of a wooden square frame weighted with concrete, onto which were screwed colony fragments put on holders. The 4 fragments of 5 shallow and 5 deep colonies were placed on each grid, which thus bore both transplanted and control colonies. One fragment of each colony was collected just after transplantation (D0), 11 d (D11) and 20 d (D20) after transplantation. The fragments were frozen in dry ice upon collection and stored at −80°C until analysis as described in the 'Symbiont population density and chlorophyll concentration' subsection.

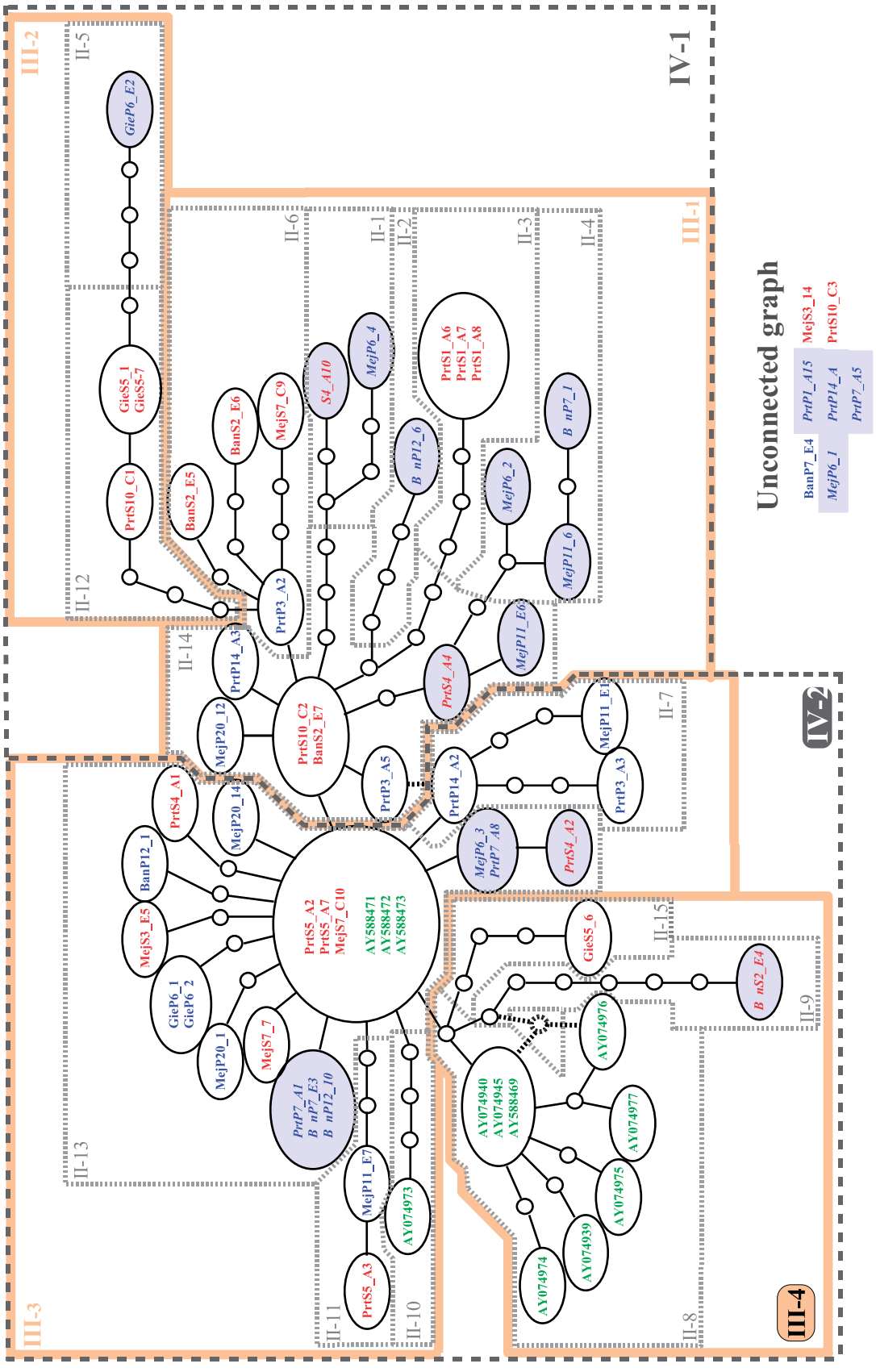

Fig. 2. Maximum parsimony network of the zooxanthellae nuclear large ribosomal subunit and nested clade analysis (NCA) results. A 95% confidence parsimony network was obtained with TCS version 2.1. Contiguous indels are represented as single mutational events. The sequences in red were obtained from shallow colonies, the ones in blue from deep colonies. The non-*Eunicella* sequences from GenBank are designated by their accession number in green. The shaded sequences bore mutations on highly conserved positions. The unconnected graph regroups the sequences that were excluded from the main network, as they were >10 mutational steps away from their potential closest neighbour. Reticulations in the network that were resolved are represented in dotted lines. All the clades identified for NCA are drawn and numbered, but these numbers are only shown for 'two steps' and higher-level nested clades. The name labels of the clades within which the distribution of the shallow, deep, or non-*Eunicella* symbionts were significantly non-random are shaded

Statistical analysis

Population differentiation within and among sites for zooxanthella population density and chlorophyll content was analysed by Kruskal-Wallis non-parametric ANOVA. The effects through time of the reciprocal transplantation were analysed by repeated measures 2-factor ANOVA. Equal distribution of potentially paralogous rDNA variants among deep and shallow colonies was tested by Fisher's exact test. Differences in nucleotide diversities and chlorophyll contents among shallow and deep colonies, as well as differences in nucleotide diversities within and among colonies were tested by Mann-Whitney *U*-tests. All the statistical analyses were performed using Statistica version 6 (Statsoft 2001).

RESULTS

Symbiont genetic diversity

Clade identification and within-clade diversity. As determined from the SSU and LSU PCR RFLP (Fig. 2), all the zooxanthellae in the sampled gorgonians belonged to the 'temperate A' or A' subclade, as defined by Savage et al. (2002).

A single major zooxanthella haplotype, as defined by LSU PCR RFLP and cp23S size variation, was found in all the populations, in 133 of the 161 colonies (haplotype *At1;* Table 1). Relatively few colonies displayed another profile: 2 at Méjean (cp23S size variation in haplotypes *At3* and *At4*), 2 at Banyuls (LSU PCR RFLP pattern, haplotype *At2*) and 24 at Portofino (on 2 different LSU PCR RFLP patterns, haplotype *At5* and *At6*) (Table 1). This resulted in a highly significant overall F_{ST} value of 0.57, entirely due to high frequency of private alleles in the Portofino populations. At this molecular level, no differentiation was found among shallow and deep populations within sites.

High within-colony diversity. Despite this low diversity, Cp23S variation in Méjean showed that a single gorgonian colony could harbour different zooxanthellae genotypes (haplotype *At4*; Table 1). This result was confirmed by the analysis of LSU sequence variation. A 628 bp long fragment of the LSU was amplified and cloned from one colony for each haplotype in each population, and 3 clones were sequenced for each PCR product. These 3 LSU clones were found to be identical for only one colony in the whole set of samples, surprisingly at the location displaying the highest diversity, Portofino. The mean within-colony nucleotide divergence ($8.01 \times 10^{-3} \pm 1.05 \times 10^{-3}$) was not significantly different from the overall proportion of different nucleotides in the complete data set ($8.55 \times 10^{-3} \pm 1.17 \times 10^{-3}$) ($U_{58,1427} = 0.64$, $p = 0.52$). However, as 45 haplotypes were identified from a total of 56 sequenced clones (Fig. 2), identical LSU sequences could be found in different colonies.

This sequence diversity could not be attributed to PCR or cloning artefacts. As a control, we sequenced 5 clones obtained from 3 independent PCR amplifications of a cultured 'tropical A' strain from *Galaxea fascicularis*: only 2 substitutions were found, for a proportion of different nucleotides of $7 \times 10^{-4} \pm 4 \times 10^{-4}$. Considering the level of within-colony polymorphism we observed, heteroplasmy, at least at the colony level, seems widespread.

Table 1. Distribution of the LSU and cp23S haplotypes. Absolute frequencies of the different haplotypes in each population (S: shallow; D: deep). The number of sequenced LSU clones are given in parentheses for each haplotype in each population. The total numbers of colonies sampled and sequenced per population are given on the bottom line. LSU: nuclear ribosomal large subunit PCR RFLP restriction patterns. 1: *Dpn*II:520/270bp *Dde*I 360/70bp; 2: *Dpn*II:790bp *Dde*I 360/70bp; 3: *Dpn*II:520/270bp *Dde*I 350/180/80bp; 4: *Dpn*II:520/270bp *Dde*I 350/180/100/50bp Cp23S. Length variants of the plastidial 23S ribosomal DNA: 1:191bp, 2:183bp

Haplotypes	LSU	cp23S	Sampling location									Total
			Menorca	Banyuls		Mejean		Giens		Portofino		
			S	S	D	S	D	S	D	S	D	
At1	1	1	11(1)	20(4)	18(3)	18(3)	20(10)	20(3)	20(3)	1(3)	5(6)	133(36)
At2	2	1			2(3)							2(3)
At3	1	2				1(3)						1(3)
At4	1	1+2				1						1
At5	3	1								10(6)	9(1)	19(7)
At6	4	1								2(3)	3(3)	5(6)
Totals			11(1)	20(4)	20(6)	20(6)	20(10)	20(3)	20(3)	13(12)	17(10)	161(55)

Thornhill et al. (2007) argued that rDNA sequence divergence could be observed due to the presence of paralogous non-functional rDNA copies. Such non-functional copies could be identified by assessing the stability of their secondary structure. Unfortunately, almost half of the LSU domains sequenced here were highly variable and contained no consensus conserved secondary structure. On the other hand, a series of conserved nucleotides along this sequence does exist (Fields & Gutell 1996, Cannone et al. 2002). After comparison with a consensus alignment of LSU sequences (Fields & Gutell 1996), 20 of our cloned sequences (for 17 haplotypes out of 45) were found to bear at least one mutation on positions that are >98% conserved among prokaryote and eukaryote LSU sequences (Fig. 2). More precisely, 17 clones (for 14 haplotypes) bore 1 or 2 point mutations or indels in these conserved regions. One of the GenBank sequences included in the analysis that had been obtained from *Anemonia sulcata* var. *rufescens* symbionts (AY074973; Savage et al. 2002) also bore 2 such point mutations (alignment available upon request). These 20 potentially paralogous sequences were not randomly distributed between the depths, the deep populations containing 3× as many mutants as the shallow ones (5 and 15 mutants, respectively, for 26 shallow and 29 deep clones; Fisher exact test $p = 0.012$). Even if such mutations are not proof that the clones bearing them were obtained from paralogous, non-functional copies of the ribosomal genes, all further analyses were nevertheless conducted either with or without these deviant sequences, in order to avoid any confusion due to possible intragenomic variation.

Host-specific rather than depth-specific symbiont lineages. The same general topology was obtained for the statistical parsimony network for the LSU sequence (Fig. 2) when taking into account the whole dataset, only the gorgonian symbionts, or after the exclusion of the potentially paralogous haplotypes. No differentiation was detected by nested clade analysis among deep or shallow zooxanthellae, regardless of the data set or analysis option. No geographic structure of LSU diversity was detected among locations.

The only symbiont sequences that were not randomly distributed in the parsimony network were the ones belonging to non gorgonian A′ strains. NCA detected that 2 clades (clades IV-2 and III-1 in Fig. 2) contained a significantly non-random distribution of the haplotypes: the clade IV-2 contained all the 12 non-*Eunicella* sequences (and 24 out of the 48 *Eunicella singularis* symbiont sequences in the network), and the single clade III-1, included within IV-2, contained all but one actinarian symbionts (and 6 out of the 7 *Anemonia* sp. symbionts) and 2 *Eunicella* symbionts. Another clade nested within IV-2 (clade I-22), containing the most frequent *Eunicella* symbiont; and the symbionts of the scleractinians *Cladocora caespitosa*, *Balanophyllia europaea,* and *Caryophyllia smithi*, also showed a significantly non-random distribution of the haplotypes, but this result did not hold when the potentially paralogous sequences were removed from the analysis. As the distances used in this analysis were arbitrary, this NCA result is better interpreted as a marked differentiation with very little mixing between *Eunicella* zooxanthellae and those from other Mediterranean hosts, especially those from *Actinaria* species.

More diverse deep symbiotic populations. The only genetic difference between the shallow and deep symbiont populations was a higher mean nucleotide diversity in deep populations ($6.7 \times 10^{-3} \pm 1.3 \times 10^{-3}$ among 26 shallow clones vs. $10.6 \times 10^{-3} \pm 1.3 \times 10^{-3}$ among 29 deep clones, $U_{435,378} = 64100$, $p < 0.001$). This difference persisted when the potentially paralogous sequences were taken out, the mean diversities dropping to $5.2 \times 10^{-3} \pm 1.2 \times 10^{-3}$ among 21 shallow colonies versus $6.9 \times 10^{-3} \pm 1.5 \times 10^{-3}$ among 14 deep colonies ($U_{231,91} = 9156$, $p = 0.07$). However, this difference between shallow and deep clones disappeared when the most divergent location (Portofino) was taken out of the analysis.

Symbiont density and chlorophyll concentration

Symbiont density and chlorophyll concentration varied greatly among locations (Fig. 3a,b), probably reflecting the differing local conditions. However, at each location, colonies from deep populations harboured significantly less zooxanthellae than did shallow colonies (Fig. 3a). The deep colonies also contained less chlorophyll per cm^2 of colony (Fig. 3b), be it chl *a* or chl c_2 (data not shown). The quantity of chlorophyll per zooxanthella cell did not vary significantly among the 2 depth populations (Fig. 4) for 3 of the 4 locations. Only in Méjean did the chlorophyll content per zooxanthella cell increase significantly with depth, for chl *a* ($4.48 \pm 0.40\ 10^{-7}$ µg zooxanthellae^{-1} in deep and $7.52 \pm 1.00\ 10^{-7}$ µg zooxanthellae^{-1} in shallow colonies; $U_{7,7} = 5$, $p = 0.013$) but not chl c_2 ($1.11 \pm 0.16\ 10^{-7}$ µg zooxanthellae^{-1} in deep and $1.92 \pm 0.32\ 10^{-7}$ µg zooxanthellae^{-1} in shallow colonies; $U_{7,7} = 10$, $p = 0.064$). This higher chlorophyll content per zooxanthella was not enough to compensate for the reduction in zooxanthellae when normalised per colony (Fig. 3b).

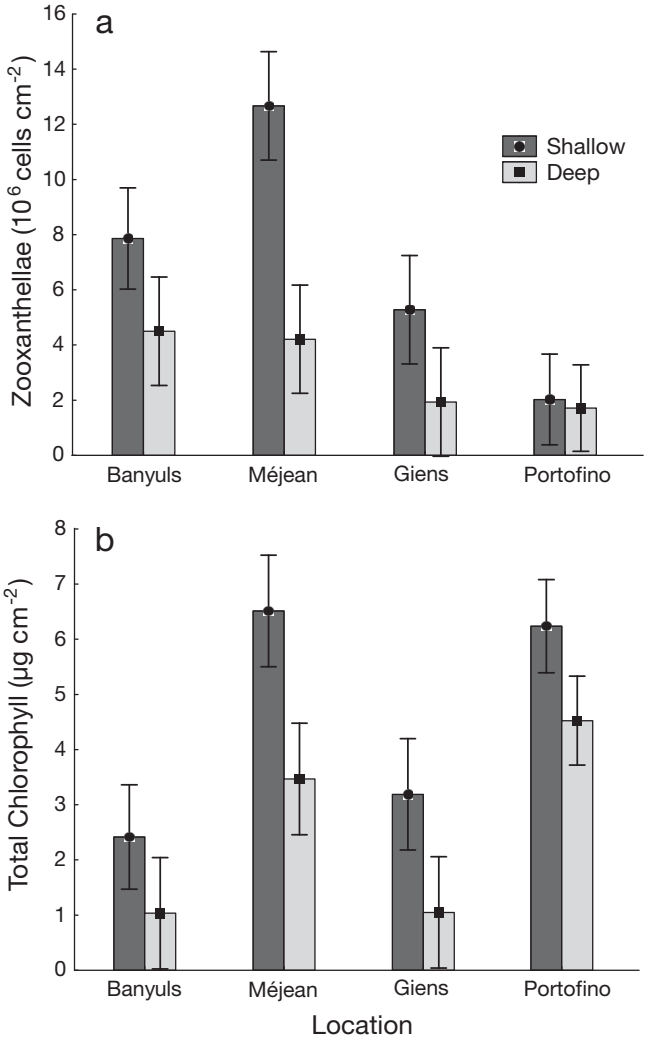

Fig. 3. Zooxanthellae population densities and total chlorophyll concentration for each population at each location (means ± SE). As only 7 colonies from each population at each location were analysed, the homogeneity of the shallow and deep populations was tested by a Kruskal-Wallis (KW) non-parametric analysis of variance, considering each location as an independent replicate. (a) Number of zooxanthellae per cm^2 of colony surface; shallow vs. deep colonies KW = 7.393, p = 0.0065. (b) Total chlorophyll concentration (µg cm^{-2} of colony surface); shallow vs. deep colonies KW = 7.801, p = 0.0052

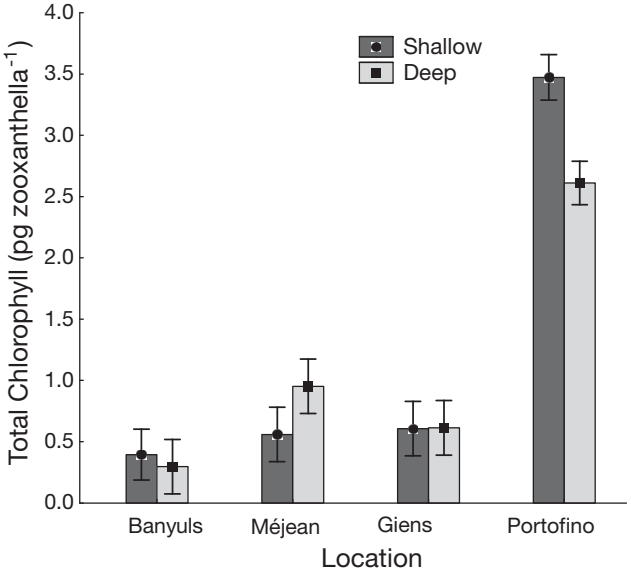

Fig. 4. Total chlorophyll concentration per zooxanthella for each population at each location (total chlorophyll content divided by number of zooxanthellae per cm^2 of colony surface; means ± SE, n = 7 colonies for each population at each location); shallow vs. deep colonies KW = 0.221, p = 0.6384

Reciprocal transplantation

The control samples that were manipulated but replaced at their depths of origin harboured significantly less zooxanthellae and chlorophyll in deep than in shallow colonies (Fig. 5). However, 11 d after transplantation both shallow-to-deep and deep-to-shallow transplanted colonies became intermediate compared to controls for chlorophyll content per cm^2 of colony (Fig. 5). They kept this intermediate status for 20 d after transplantation. No mortality or bleaching was observed during the experiment. The same pattern was observed for symbiont density. By 11 d after transplantation, the shallow-to-deep and deep-to-shallow transplanted colonies contained densities of 1.3 ± 0.2 and 1.7 ± 0.1 10^6 zooxanthellae cm^{-2}, respectively, while densities were 2.2 ± 0.3 for control shallow colonies and 1.0 ± 0.2 10^6 zooxanthellae cm^{-2} for control deep colonies. The transplantation had no significant effect on the quantity of chlorophyll per zooxanthella (which was not different among the shallow and deep control colonies). No change in symbiont genetic diversity was observed after the reciprocal transplantation either, as could be determined with nuclear LSU PCR RFLP.

DISCUSSION

Symbiont genetic diversity

A single clade and no clear depth differentiation. All the 161 specimens of *Eunicella singularis* analysed in this study, whatever the depth or location, harboured the same *Symbiodinium* clade, as identified by diagnostic nuclear SSU and LSU PCR-RFLP. This clade was identical to the A′ clade previously identified in Mediterranean Sea and north

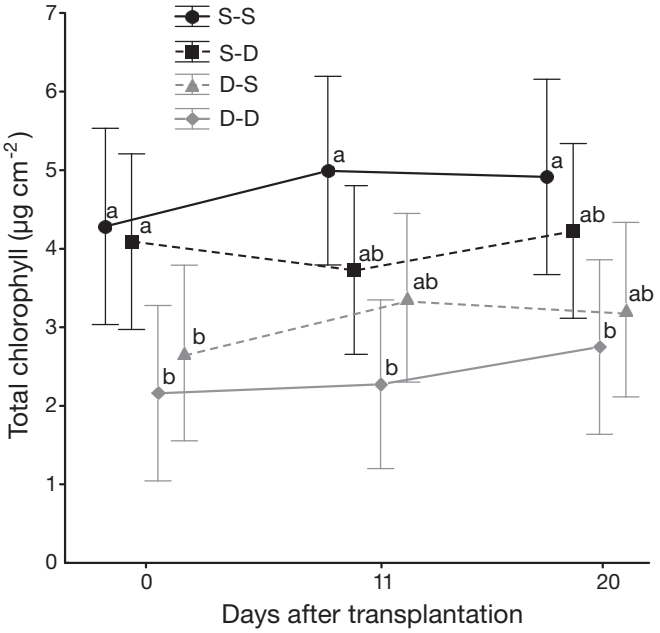

Fig. 5. Total chlorophyll concentration in transplanted colonies. The total chlorophyll concentration (µg cm^{-2} of colony) was estimated for each of the control and transplanted colonies. Vertical bars denote 95% confidence intervals. Two transplantation grids (shallow and deep) were set up. Five colonies per modality, i.e. shallow population to shallow grid (S-S), deep population to deep grid (D-D), shallow population to deep grid (S-D), and deep population to shallow grid (D-S), were thus analysed, on the day of the transplantation (D0), Day 11 (D11), and 20 d later (D20). The data were analysed following a 2-factor repeated measures ANOVA: Time after transplantation, $F = 1.97$, $p = 0.144$. Transplantation modality, $F = 3.75$, $p = 0.015$, Time × Transplantation, $F = 0.83$, $p = 0.675$. The letters indicate significantly different values for each time value (post-hoc test, $p < 0.05$)

Atlantic anemones by Savage et al. (2002). According to the LSU sequences deposited in EMBL (Savage et al. 2002, Barbrook et al. 2006, Visram et al. 2006), this clade is present in all but one of the symbiotic cnidarian species sampled in the Mediterranean (*Bunodeopsis strumosa*), and is clearly able to colonize a wide diversity of hosts (from hydrozoans to hexacorals) (Visram et al. 2006). A similar loss in clade diversity at high latitudes was found in other oceans (Chen et al. 2005a,b), but this reduction is apparently more drastic in the Mediterranean, as the A′ *Symbiodinium* clade seems to be the sole symbiont of an overwhelming majority of species in the region. Since only a single clade was detected in the present study, all individual colonies were also homoplasmic at the SSU and LSU PCR-RFLP level (i.e. harboured a single *Symbiodinium* clade). It should however be noted that heteroplasmic colonies are not found in tropical octocorals either, even in places where significant *Symbiodinium* diversity exists (Van Oppen et al. 2005). Whether the homoplasmic status of *E. singularis* is intrinsic to symbiotic octocorals or due to the apparent absence of an alternative symbiont in the Mediterranean is still an open question; but in any case this observation means that *E. singularis* appears to be homoplasmic, along with the vast majority of symbiotic cnidarians (Goulet 2006).

Host-specific rather than depth-specific symbiont strains. While adaptation to different light and temperature regimes along the depth gradient was not associated with a change in symbiont clade in this species, likely due to the presence of a single clade, we may have expected to see environmentally relevant, within-clade genetic diversity among this A′ clade, as has been reported among C clade DGGE haplotypes by Ulstrup & Van Oppen (2003) on the Great Barrier Reef. Within-clade LSU RFLP and cp23S diversity were relatively low in our sample. The only diverging populations were the Portofino populations, which accounted for most of the LSU PCR RFLP variation. In Portofino, 24 out of 30 colonies contained private (i.e. only found at this site) LSU variants, equally distributed among both shallow and deep populations (Table 1). Apart from this, no clear geographic or bathymetric differentiation pattern emerged from our analysis, as a single LSU/cp23S haplotype was present in >80% of the colonies sampled, and all the other rare variants were limited to a single population within a single location. An even stronger local differentiation has been found in the host for a brooding cnidarian *Seriatopora hystrix* (Underwood et al. 2007), and notably among populations of another Mediterranean octocoral, *Corallium rubrum* (Abbiati et al. 1993, Costantini et al. 2007). As in these other species, a limited dispersal of planulae was used to explain this strong differentiation. It is then not surprising to find some population differentiation among the vertically transmitted symbionts of *Eunicella singularis* (Weinberg 1979b). It should also be noted that, to our knowledge, *E. singularis* is rare between Giens and Portofino, as only a few isolated colonies have been observed along this stretch of coast. The Portofino populations could then represent a geographically differentiated genetic stock, which could be verified by further sampling along the Italian coast. Either way, the low variability found at this PCR RFLP level precluded any further analysis of population structure for the zooxanthellae of this species.

The analysis of LSU sequence variation showed that within-clade diversity could be found among and within locations, within deep or shallow popula-

tions, and even within colonies. This relatively high amount of sequence variation has also been found in tropical corals using a similar molecular approach with ITS2 sequences (Apprill & Gates 2007). As for these tropical zooxanthella ITS2 sequences, the observed LSU sequence diversity within our sample could not be attributed to cloning or PCR artefacts since: (1) the different haplotypes identified differed by more than a single point mutation; (2) some of them were found in more than one colony; and (3) no such PCR or cloning artefacts were detected in our control clones of a cultured tropical clade-A zooxanthella strain. Another source of sequence variation in our sample could be intragenomic sequence divergence among rDNA genes. Such intragenomic divergence can occur in *Symbiodinium* (Thornhill et al. 2007), and could thus lead to misleading paralogous comparisons and wrongly inflate within- and among-colony polymorphism estimates. However, intragenomic divergence is unlikely to explain our results, since the inference was unchanged after the exclusion of the 20 clones potentially affected (as they bore point mutations on highly conserved positions).

If symbionts were only vertically transmitted in *Eunicella singularis,* as proposed by Weinberg (1979a,b), one would have expected a lack of within-colony symbiont diversity. Strictly vertically transmitted symbionts would be expected to show similar genetic variation to the cytoplasmic organelles (mitochondria and plastids) that suffer repetitive bottlenecks at each ovule formation and thus tend towards an homogeneous, non-polymorphic, homoplasmic state in most organisms (Birky et al. 1989, Atlan & Couvet 1993). Mutations occurring during the development of the colony could still create some diversity in the large zooxanthella population, but in that case less diversity would be expected within than among colonies. Otherwise, some form of horizontal transfer of symbionts (or, for cytoplasmic organelles, biparental inheritance) has to be invoked to explain the diversity pattern we observe (Roze et al. 2005). Since we show a significant within-colony diversity, horizontal transfer of symbionts among colonies might be frequent. This interpretation is not contradictory to the fact that *E. singularis* planulae bear zooxanthellae (Weinberg 1979b), as horizontal transfer can occur in adult colonies on top of vertical transmission through ovules.

This observed within-colony symbiont diversity, proposed here to be mediated by horizontal transfer of the symbiont, potentially creates an additional level of selection for appropriate symbionts, as occurs with organelles within heteroplasmic organisms (Birky et al. 1989, Otto & Orive 1995, Otto & Hastings 1998). This opportunity for genetic selection within an individual colony would have been expected to promote differentiation of the zooxanthellae along the irradiance and temperature gradient with depth, as seen among the symbionts of tropical coral species (Baker 2001, Iglesias-Prieto et al. 2004). The systematically lower density of zooxanthellae observed in *Eunicella singularis* deep colonies (Fig. 3a), and the dynamic change of this density after colony transplantation (Fig. 5), is consistent with such a qualitative change of the symbiont population. However, no symbiont genetic differentiation could be detected along the depth gradient. Indeed, the nested clade analysis revealed that the LSU sequence diversity was randomly distributed among deep and shallow populations (Fig. 2). Therefore, *E. singularis* does not seem to adapt to depth by switching to a more appropriate symbiont.

The only significant non-random distribution in the NCA was obtained when non *Eunicella* symbionts were included. This can be interpreted as a non-random distribution of *Symbiodinium* A′ clade genetic diversity among the different animal hosts. More precisely, all but one of the sea anemone, *Anemonia* sp. symbionts (clade III-4 in Fig. 2) diverged from a group of symbionts associated with the sea whip *Eunicella singularis* and the symbionts of 3 scleractinian corals, *Cladocora caespitosa*, *Balanophyllia europaea,* and *Caryophyllia smithii* (clade IV-2 in Fig. 2). There is therefore some genetic differentiation within A′ zooxanthellae in the Mediterranean Sea, which is driven by the animal host rather than by the environment, but the lack of genetic differentiation by depth is not forced by a lack of diversity in the available symbionts. Alternatively, there may be a limit to the horizontal transfer of zooxanthellae, which is at least partially host-specific. It should be noted, however, that 2 *E. singularis* symbionts (from Giens and Banyuls shallow populations) are grouped with the *Anemonia* symbionts, that an *Anemonia* symbiont is grouped with *E. singularis,* and that the 3 scleractinian coral symbionts are identical to an *E. singularis* symbiont. Moreover, some *E. singularis* symbionts had more divergent LSU sequences than the ones from symbionts from other species, to the point of not being included in the parsimony network (Fig. 2). Similar to tropical cnidarian–dinoflagellate associations, the emerging picture of host–symbiont specificity among Mediterranean species is a complex one, implying multiple differentiations below the clade level (Baker 2003), and probably some degree of horizontal symbiont transfer.

More diverse deep populations. The only genetic difference detected among shallow and deep populations was that ribosomal nucleotide diversity was significantly higher in the latter. This could be due to higher effective population sizes in these populations. Even though within-colony zooxanthella population density is lower in these populations, the population density of *Eunicella singularis* colonies is usually higher in depths >20 m (Weinberg 1979a, but see Linares et al. 2008). Moreover, *E. singularis* is thermosensitive (Weinberg 1979a) and its planulae are phototropic (Weinberg 1979b). Differences in stress sensitivity have been found among populations from different depths either experimentally (Ferrier-Pages et al. 2009) or in natural settings as seen, for example, in the summer of 2003, when surface populations were affected by a temperature rise that did not affect deeper colonies (Garrabou et al. 2009). It is possible that the relatively stable, more productive deep populations may therefore act as sinks supplying propagules to the shorter-lived, temperature-challenged shallow populations. The diversity pattern expected in this case would be consistent with our observations, i.e. no lineage differentiation between deep and shallow populations but reduced neutral genetic diversity in the shallow populations. The ongoing development of more appropriate genetic diversity markers (such as microsatellite loci) will allow a clarification of the migration patterns among these populations.

Symbiont density and chlorophyll concentration

Although no genetic differentiation was detected between depths in the present study, there was significantly lower tissue density of zooxanthellae in deeper colonies, although the chlorophyll content per cell of the zooxanthellae did not vary significantly. Tropical reef-building corals depend heavily on their photosynthetic symbionts to satisfy their needs for organic carbon (Muscatine 1990). As such, adaptation to lower light regimes at deeper locations happens in most cases through an increase in chlorophyll content, usually through an increase of chlorophyll content per zooxanthella (Fitt & Cook 2001). This also occurs in the temperate coral *Cladocora caespitosa* harbouring the A′ *Symbiodinium* (Rodolfo-Metalpa et al. 2008). In contrast, Bythell et al. (1997) did not observe this increase but rather a stability in chlorophyll content among intertidal and subtidal individuals from the English Channel in their study of a clonal, shallow (9 m deep at the most) population of *Anemonia viridis*. These anemones harboured zooxanthellae from the same A′ clade, but probably slightly different from the *Eunicella singularis* symbionts, according to our genetic analysis. This apparent absence of photoacclimation has partly led to the conclusion that temperate symbiotic anemones depend less on the photosynthetic activity of their zooxanthellae than do their tropical counterparts (Muller-Parker & Davy 2001). Our results seem to extend this conclusion to the temperate octocoral *E. singularis* as well.

Who profits from the association in *Eunicella singularis*?

The transplantation results show that, in the absence of clear genetic differentiation in the symbionts, the holobionts were not plastic enough to fully respond to the change in bathymetry. The density of symbionts, and the concentration in chlorophyll, in the transplanted colonies did not reach the values observed in non-transplanted controls 20 d after transplantation. In *Cladocora caespitosa*, a Mediterranean coral able to photoacclimatize, the density of A′ symbionts was of the same order of magnitude, but with a 5 to 10× higher chlorophyll content (both at the colony or zooxanthella cell level) (Rodolfo-Metalpa et al. 2008). In a more contentious way, as we observed still relatively high symbiont densities in deep colonies and, in comparison with *C. caespitosa*, relatively low chlorophyll contents, we could suggest a change in focus: if deep colonies fare so well with such a symbiotic population, why do the zooxanthellae proliferate so much in shallow colonies? It should not be forgotten that some *Symbiodinium* strains can still be experimentally selected to switch to a more parasitic way of life (Sachs & Wilcox 2006), and that all the *Symbiodinium* clades may not be as good mutualists as they first seem (Stat et al. 2008). A dedicated study of the metabolism of the holobiont is still needed to determine how much *Eunicella singularis* depends on its symbionts, and notably to determine if deep colonies are really more heterotrophic than the shallow ones.

Being able to change its zooxanthella population in a variable environment has been presented as a major adaptive mechanism for symbiotic cnidarians (Baker 2001). Another cnidarian even switches to *Chlorella,* an altogether different photosynthetic unicellular organism, along the temperate waters of the American Pacific coast (LaJeunesse & Trench 2000). In the Mediterranean Sea, the roles seem to be

reversed, as a single *Symbiodinium* clade invaded all the locally available hosts (Savage et al. 2002, Visram et al. 2006). Each of these hosts apparently faces the changing environment on its own, without even the possibility of switching to a better adapted symbiont, at least at the clade level. This is the case for *Eunicella singularis*, as it does not seem to change its symbiont population with depth, but rather harbours a lower density of the same symbionts with depth. The consequence of catastrophic temperature rise in this species was not bleaching but mass mortality (Cerrano et al. 2000, Perez et al. 2000, Garrabou et al. 2009). Are these 2 facts linked, i.e. is this mortality a direct consequence of the impossibility to change to an altogether different symbiont, as it has been proposed for other cnidarian species (Goulet 2007)? Considering that genetic polymorphism was found within the *Symbiodinium* strains of *E. singularis*, that this polymorphism was present within nearly all the colonies sampled so far, and that horizontal transfer of the symbionts does seem to occur frequently, there seem to be a lot of opportunities for adaptive selection for the symbiont population at the within-clade level. On the other hand, nothing is known yet of the genetic differentiation of the animal host. The real question about these mass mortalities should then be whose limits of plasticity are reached first, the symbiont's or the host's? This question has yet to be answered.

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