Title: Symbiont diversity is not involved in depth adaptation in the Mediterranean symbiotic sea whip *Eunicella singularis*.

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Abstract

In symbiotic cnidarians, acclimatization to depth and lower irradiance can involve physiological changes in the photosynthetic dinoflagellate endosymbiont, as a increased chlorophyll content, or qualitative modifications in the symbiont population, in favour of a better adapted strain. It has been argued that the lack of capacity to acquire new symbionts could in turn limit the bathymetric distribution of the species, and/or compromise its long term survival in a changing environment. But is that always true? To address this question, we investigated the symbiotic genetic diversity in *Eunicella singularis*, a Mediterranean sea whip species with a wide bathymetric distribution (from –10m to –50m) which has recently suffered from mass mortalities after periods of abnormally high sea temperatures. We measured symbiont densities and chlorophyll contents in natural populations, and followed the response of the holobionts after reciprocal transplantation at deep and shallow depths. 161 colonies were sampled at two depths (-10m and –30m) at five sites on the North Western Mediterranean coast. All the 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 if the deeper colonies contained significantly less symbionts and 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 density and chlorophyll content, which in turn, raises the question of the importance of the trophic role of *Symbiodinium* in *Eunicella singularis*.

Introduction

Cnidarian/dinoflagellate symbiosis, along with plant/mycorrhyzal associations and lichens, have been pointed to in the past decade as non classical mutualisms (Herre et al. 1999, Baker 2003, Hay et al. 2004). The flexibility in these systems (*i.e.* the frequency at which host shifts occur) forms a sharp contrast with other trophic mutualisms such as the bacteria/insect relationships, which could sometimes be seen as frozen "end of game" situations of a co evolutionary arms race (Moran 1996). More recent results however (e.g. Goulet, 2006), do raise again the question of 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 (commonly named zooxanthellae), which have been assigned in their vast majority to the genus *Symbiodinium.* These organisms form a mutualistic association, in which photosynthetates 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 host needs to adapt to daily transitions from quasi anoxia to hyperoxia in its tissues, according to the photosynthetic activity of its symbionts (Richier et al. 2003). The implied necessary co evolution could have led these organisms to a frozen obligate mutualism.

Unexpectedly, 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 a review). The genus *Symbiodinium* can be divided into at least eight divergent phylogenetic clades, named 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 very 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 position (Baker 2003) for review). Several zooxanthellar clades can even be found within the same coral colony in *Montastrea* sp. or *Acropora* sp. for example (Rowan et al. 1997, Chen et al. 2005a). The composition of this polymorphic symbiont population can eventually vary in response to environmental change (see for example Rowan et al. 1997, Baker 2001, Chen et al. 2005a).

In some occasion, this genetic diversity in *Symbiodinium* has been linked to photo adaptive differentiation. A relatively clear depth zonation has been found *in vivo* among the *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 in photosynthetate. This photo acclimation seems to occur mainly through an increase in the chlorophyll content of the symbionts or eventually an increase in symbiont density or both (see Fitt and Cook, 2001 for a review). However, this photosynthetic plasticity seems to be somehow 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 this symbiotic association: the relative ease with which it could breakdown. The separation of host and symbionts in cnidarian/dinoflagellate associations is a commonly observed phenomenon known as bleaching. Coral bleaching has mostly been publicised for the high mortality it provokes in tropical reefs during massive bleaching events (Hoegh-Guldberg 1999). But bleaching could also be seen as a bet hedging strategy, a way to end a no longer adapted association in the hope of finding a more

suitable partner, the. “adaptative bleaching hypothesis” (Buddemeier & Fautin 1993). The still on going debate on the validity of this hypothesis (Goulet 2006) is an other testimony to the complexity of the dynamics of the Cnidarian/Dinoflagellate association.

Relatively few studies have focused so far on temperate zooxanthellae diversity. They all detected a reduced symbiont diversity, either within host species (Bythell et al. 1997, LaJeunesse & Trench 2000a, Chen et al. 2005b) or among (Savage et al. 2002). In host species distributed from tropical or subtropical to temperate areas, adaptation to lower temperatures has involved a switch to another zooxanthellar clade as in *Plesiastrea versipora* (Rodriguez-Lanetty et al. 2001) or a loss of *Symbiodinium* diversity in *Anthopleura elegantissima,* eventually leading to a switch to an altogether different photosynthetic symbiont (Lajeunesse & Trench 2000b). Along the same line, it has been shown that in the North eastern 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 waters has also led 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).

Until the present study, no full-sized 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 sea (Carpine & Grasshoff 1975). This species has a very wide bathymetric distribution (Carpine & Grasshoff 1975, Weinberg 1979a, Linares et al. 2008) and must be adapted to very different light and temperature regimes. Moreover, this species suffered from catastrophic (though geographically restricted) mortality, following high 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 photoacclimatization does depend on symbiotic diversity in a temperate sea symbiosis. We thus analysed *Symbiodinium* diversity within and among populations, and within colonies of *E. singularis*, at two different depths (-10m and –30m). 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 belongs to the class Anthozoa, subclass Octocorallia and order Gorgonacea. *E. singularis* is common through all the Western Mediterranean basin, with a patchy distribution. It usually has a wide bathymetric distribution, from 5 to 50 meters deep, with the highest population densities between 15 and 30 meters deep (Weinberg, 1979 but see Linares *et al.* 2009). Exposure to temperatures higher than 24-26°C is lethal to *E. singularis* within days (Weinberg, 1979a). *E. singularis* is a gonochoric brooding species; fertilization occurs on the female colonies, which then release mobile zooxanthelate larvae, the planulae. This release occurs once a year between June and July (Weinberg, 1991).

Sample Collection

Individual colonies were sampled from five locations on the North Western Mediterranean coast, from the Gulf of Genoa (Italy) to the Balearic Island of Menorca (Spain) at each of two depths, around 10 and 30 meters, except in Menorca (see Fig. 1). For each location, the set of individuals sampled at 10 m will be referred to as the "shallow" population, whereas the other set will form the "deep" population. Branches of 10 to 15cm long were clipped from 20 individual colonies for both populations at each location, except at Portofino and Menorca (see Fig. 1). A total of 161 colonies were sampled for this study from March to July 2003. 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, the other part was frozen in dry ice for the biological measurements. They were later stored respectively at room temperature and at -80°C.

Symbiont genetic diversity

Total DNA extraction

Total genomic DNA was purified from 200mg of the ethanol conserved fragments, following a standard phenol/chloroform extraction preceded by a 3 hours digestion in 5mM EDTA, 10mM Tris (pH 7.5) and 0.5% SDS with 0.5 $\mu g.l^{-1}$ proteinase K (Finnzymes). 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)

Zooxanthellar clade can be 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 and Powers (1991). The PCR mix composition was 1µM of each primer, 0.8 mM dNTPs, 2mM $MgCl_2$, 1X PCR Taq Platinum® reaction buffer and 1.5 units of Taq Platinum® (Invitrogen), to which was added 4 to 12µl of the 1/100 dilution of the total genomic DNA extracts, and MilliQ water up to a total reaction volume of 25µl. PCR conditions were 2 min at 94°C, followed by 40 cycles of 45 sec 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 95°C, 45 cycles of 45 sec at 92°C, 45 sec at 63°C, 1 min 30 sec 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 by ethidium bromide colouration of a 2% agarose electrophoresis gel in 2X 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.* (Santos et al. 2003), a marker which has been used in tropical symbioses to detect within clade variation. Amplification products were separated on 1mm thick 8% polyacrylamide gels run at 80mA for 2 hours in 2xTAE buffer. Amplified DNA fragment sizes were estimated as described above.

To detect more 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 recommendations. At least three positive

clones per amplification were sequenced in direct and forward orientation. 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 zooxanthellar strain had been previously assigned by PCR RFLP to the "Tropical A" clade (data not shown). All sequencing reactions were performed by Macrogen Inc. (South Korea)

Data analysis

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

Sequences were proof read and edited using Bioedit software (Park 2001). Already 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 (gene bank accessions : *Symbiodinium sp.* ex *Cereus pedonculatus* 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 using Multalin at « http://bioinfo.genotoul.fr/multalin/multalin.html » (Corpet 1988).

These sequences were 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 more than 98% conservation among the 23S data from Fields and 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.

A 95% statistical parsimony network (maximum connection steps equal to 10) (Posada & Crandall 2001) was 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 and Beaumont (2007) after alternative branching resolution according to Pfenninger and Posada (2002). We performed a nested clade analysis (NCA) with Geodis 2.0 (Posada et al. 2000) to determine if the three origins of the zooxanthellae, i.e. from shallow or deep colonies or from non *E. singularis* hosts, were randomly distributed in the phylogeny. As we only had interest in testing the null hypothesis of random distribution of the zooxanthellar lineages among these three compartments, 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 among the two depths. The same analysis was however also performed to detect any geographic structure of the LSU diversity, by pooling the shallow and deep populations at each location and using in Geodis a matrix of geographic distances between the locations. For both shallow vs. deep and geographic locations 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 density and chlorophyll concentration

For each population, at each location, we measured total holobiont chlorophyll content and zooxanthellar density for 7 colonies. For each colony, 200mg of frozen tissue were ground in liquid nitrogen. Subsequent manipulations were performed at 4°C. Ground tissue was suspended in 0.75mL 0.5 M potassium phosphate buffer (pH 7) and filtered by centrifugation (2 min at 600 g) through a 100µm 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. 100µL of the filtrate were used for zooxanthellar density measurements and 1mL for chlorophyll extraction.

Colony surface area estimation

The surface of a set of 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 this surface S (in cm^2) and the frozen colony weight W (in mg). We then used this regression to estimate the surface of each colony fragment here used from its frozen weight.

Zooxanthellar density

100µL of the filtrate were deposited on a modified Neubauer haemocytometer, and zooxanthellae counting on this cell was repeated four times for each sample under x20 magnification. Zooxanthellar density was estimated per unit surface of colony.

Chlorophyll concentration

Chlorophyll *a* and *c2* contents were estimated by spectrometry after two successive acetone extractions. The initial 1mL of sample filtrate was spun at 13000g for 3 minutes 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 hours. This was followed by a 20 minutes centrifugation at 13000g. The acetone supernatant was collected in the dark and the pellet was extracted a second time in 1mL 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 chlorophyll *a* and *c2* concentrations in µg per mL of acetone according to Jeffrey & Humphrey (1975). For each sample, the absolute quantities of chlorophyll were normalised per unit surface area of colony or per zooxanthella.

Reciprocal transplantation

A total of 20 colonies (10 "shallow" colonies at 10-15 meters deep and 10 "deep" colonies at 30-35 meters deep) were sampled at Portofino (Italy) in April 2004. Each colony was cut in four 15 to 20 cm long fragments. Two transplantation grids were used: one at -15 meters ("shallow" transplantation), the other at -35 meters ("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 four fragments of five shallow and five deep colonies were placed on each grid, which thus bore both transplanted and control colonies. One fragment of each colony was collected just after the transplantation (D+0) and at each of the three following times: 11 days (D+11), 20 days (D+20) and six months (D+6m) after transplantation. The fragments were frozen in dry ice upon collection and stored at –80°C until analysis.

Genetic identification of the zooxanthellar clade by LSU PCR-RFLP, symbiont densities and chlorophyll content were determined as above for all samples.

Statistical Analysis

Population differentiation within and among sites for zooxanthellar density and chlorophyll content was analysed through Kruskall Wallis non parametric ANOVA. The effects through time of the reciprocal transplantation were analysed through repeated measures two factors ANOVA. All the statistical analyses were performed on Statistica v6 (Statsoft, 2001), as were the Fisher exact tests and Mann Whitney U tests.

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' sub clade, as defined by Savage *et al* (2002).

A single major zooxanthellar 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 in Méjean (cp23S size variation in haplotypes *At3 and At4*), 2 in Banyuls (LSU PCR RFLP pattern, haplotype *At2*) and 24 in Portofino (on two 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 (haplotype *At4*, Table 1). This result was confirmed by the analysis of the LSU sequence variation. A 628bp long fragment of the LSU was amplified and cloned from one colony for each haplotype in each population and three bacterial clones were sequenced for each PCR product. These three 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 ($8.01\times10^{-3}\pm1.05\times10^{-3}$) was not different from the overall proportion of different nucleotides in the complete data set ($8.55\times10^{-3}\pm1.17\times10^{-3}$) ($U_{58,1427} = 0.64$, $P = 0.52$). 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 five clones obtained from three independent PCR amplifications of a cultured "tropical A" strain from *Galaxea fascicularis* : only two substitutions were found, for a proportion of different nucleotides of $7\times10^{-4} \pm 4\times10^{-4}$. Considering the level of within colony polymorphism we observed, heteroplasmy, at least at the colony level, seems widespread.

Thornhill *et al.* (2007) argued that rDNA sequence divergence could be acknowledged by the presence of paralogous non functional rDNA copies. Such non functional copies could be identified by assessing the stability of their secondary structure. Unfortunately, a good half of the LSU domain sequenced here is highly variable and contains no consensus conserved secondary structure. On the other hand, a list 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 halotypes out of 45) were found to bear at least one mutation on positions that are more than 98% conserved among prokaryote and eukaryote LSU sequences (fig. 2). More precisely, 17 clones (for 14 haplotypes) bore one or two

point mutations/indels on 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 two such point mutations (alignment available upon request). These 20 potentially paralogous sequences were not randomly distributed between the depths, the deep populations containing three times as many mutants than the shallow ones (5 for 15 mutants for respectively 26 shallow and 29 deep clones, Fisher exact test P = 0.012). Even if such mutations *per se* are not proof that the clones bearing them were obtained from paralogous, non functional copies of the ribosomal genes, all further analyses have nevertheless been conducted either with or without these deviant sequences, in order to avoid any confusion due to eventual intragenomic variation.

Host specific rather than depth specific symbiont lineages

Figure 2 shows a statistical parsimony network obtained by TCS (Clement et al. 2000) for the LSU sequence of *E. singularis* symbionts and other A' symbionts, with contiguous indels counted as single mutational events. The same general topology was obtained when taking into account only gorgonian symbionts, or after exclusion of the potentially paralogous haplotypes. No differentiation was detected by nested clade analysis among deep or shallow zooxanthellae, whatever the data set or analysis option. No geographic structure of LSU diversity was detected among locations, mainly because of a lack of statistical power due to the small sample size per location.

The only symbionts sequences that were not randomly distributed in the parsimony network were the ones belonging to non gorgonian A' strains (Fig. 2), as detected by NCA: The clade IV-2 contained all the non *Eunicella* sequences (and also a good half of the *E. singularis* symbionts) and the clade III-1, included within IV-2, contained quasi all the *Anemonia sp.* symbionts for two *Eunicella* symbionts (Fig.2). Another clade nested within IV-2 (clade I-22 on Fig. 2), containing the most frequent *Eunicella* symbiont and the symbionts of *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 taken out. 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 net difference between shallow and deep symbiotic population resided in a higher mean nucleotide diversity in deep populations ($6.7\times10^{-3}\pm1.3\times10^{-3}$ among 26 shallow clones vs. $10.6\times10^{-3}\pm1.3\times10^{-3}$ among 29 deep clones, $U_{435,378} = 64099.5$, $P < 1.10^{-6}$). This difference persisted when the potentially paralogous sequences were taken out, the mean diversities dropping to respectively $5.2\times10^{-3}\pm1.2\times10^{-3}$ among 21 shallow colonies vs. $6.9\times10^{-3}\pm1.5\times10^{-3}$ 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) were taken out.

Symbiont density and chlorophyll concentration

As shown in Figures 3a and 3b, symbiont density and chlorophyll concentration varied greatly among locations, 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 (Fig. 3b), be it chlorophyll *a* or *c2* (data not shown).

The quantity of chlorophyll per zooxanthella did not vary significantly among the two depth populations (Fig. 4) for three of the four locations. Only in Méjean did the chlorophyll content per zooxanthella increase significantly with depth, for *a* ($4.48 \pm 0.40\ 10^{-7}$ µg.zoox.$^{-1}$ in deep and $7.52 \pm 1.00\ 10^{-7}$ µg.zoox.$^{-1}$ in shallow colonies, $U_{7,7} = 5$, $P = 0.013$) but not *c2* ($1.11 \pm 0.16\ 10^{-7}$ µg.zoox.$^{-1}$ in deep and $1.92 \pm 0.32\ 10^{-7}$ µg.zoox.$^{-1}$ in shallow colonies, $U_{7,7} = 10$, $P = 0.064$) chlorophyll. This

rise in chlorophyll content per zooxanthella was not high enough to compensate for the diminution of zooxanthellae (fig. 3b).

Reciprocal transplantation

The control individuals that were transplanted at their depths of origin showed the same trend during the whole experiment, *i.e.* significantly less zooxanthellae and chlorophyll (Fig. 5) in deep than in shallow colonies. However, as soon as 11 days after transplantation both surface-to-depth and depth-to-surface transplanted colonies became intermediate compared to controls for their chlorophyll content (Fig. 5). They kept this intermediate status for 20 days after transplantation. No mortality nor bleaching was observed during the experiment. The same pattern was observed for the symbiont density. As soon as 11 days after transplantation, the surface-to-depth and depth-to-surface transplanted colonies contained densities of 1.3 ± 0.2 and 1.7 ± 0.1 10^6 zooxanthellae.cm^{-2} respectively, when these 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

This study is the first detailed analysis of symbiont diversity within a Mediterranean cnidarian. We analyzed the symbiont diversity within and among natural populations, and within colonies of *Eunicella singularis*, at two different depths. We also analysed within colony zooxanthellar density and chlorophyll concentrations both in natural settings and after reciprocal transplantations between deep and shallow populations. Within *E. singularis* we detected only one *Symbiodinium* clade at the LSU PCR RFLP level (temperate A or A') but high genetic diversity at the sequence level within that clade. This high genetic diversity was nevertheless not structured by depth. Unexpectedly, the symbiont density and chlorophyll content decrease with depth. Finally reciprocal transplantation experiments showed that depth adaptation still occurs in *E. singularis*

Symbiont genetic diversity

A single clade and no clear depth differentiation

All the 161 specimens of *E. 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 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 able to colonize a wide diversity of hosts (from hydrozoan to hexacorals) (Visram et al. 2006). A similar loss in cladal diversity at high latitudes was found in other oceans (Chen et al. 2005a, Chen et al. 2005b), but this reduction is apparently more drastic in the Mediterranean sea, as the A' *Symbiodinium* clade seems to be the sole symbiont of an overwhelming majority of species in that region. This overall lack of symbiont diversity can obviously explain the absence of diversity at the clade level among the *E. singularis* colonies sampled in this study. It should however be noted that

heteroplasmic colonies are not found in tropical octocorals either, even in places where *Symbiodinium* diversity exists (Van Oppen et al. 2005). Whether the homoplasmic status of *E. singularis* is intrinsic to symbiotic octocorals or due to the quasi absence of an alternative symbiont in the Mediterranean basin is still an open question, but in any case this observation puts *E. singularis* with the vast majority of homosplasmic cnidarians (*i.e.* harbouring a single *Symbiodinium* clade) (Goulet 2006).

Host specific rather than depth specific symbiont lineages

Adaptation to different light and temperature regimes along the depth gradient did not imply a change in symbiont clade in this species. But such an adaptation, if it occurred, could still have induced some genetic differentiation among locally adapted symbiont lineages, within this A' clade, as was seen among C clade DGGE haplotypes by Ulstrup and van Oppen (2003) on the Great Barrier Reef. Within clade LSU RFLP and cp23S diversity were relatively low in our sample. Apart from the Portofino populations, which concentrated most of the LSU PCR RFLP variation, no clear geographic or bathymetric differentiation pattern emerged from our analysis, as a single LSU/cp23S haplotype was present in more than 80% of the colonies sampled, and all the other rare variants were limited to a single population within a single location. The only diverging populations were the Portofino populations, where 24 out of 30 colonies contained private LSU variants, specific to Portofino, equally distributed among both shallow and deep populations (Table 1). An even stronger local differentiation has been found in 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 *a priori* vertically transmitted symbionts of *E. 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 differentiated vicariant genetic stock, which could be verified by further sampling along the Italian coasts. Eitherway, 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 populations 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 zooxanthellar ITS2 sequences, the observed LSU sequence diversity within our sample could not be attributed to cloning or PCR artefacts: the different haplotypes identified differed by more than a single point mutation, some of them were found in more than one colony, and no such PCR and cloning artefacts were detected in our control clones of a cultured tropical A clade 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, this is not the case with our results, as they still hold in essence after the exclusion of the 20 clones potentially issued from such sequences (as they bore point mutations on highly conserved positions).

Moreover, whatever the set of included sequences, our results showed that several zooxanthellar strains, as identified by their LSU sequences, did co exist within nearly all the *E. singularis* colonies analysed here.

If symbionts were only vertically transmitted in *Eunicella singularis* (as proposed by Weinberg, 1979), one would have expected a severe lack of within colony symbiont diversity, especially in this context of low genetic variability. Strictly vertically transmitted symbionts will

behave, from a genetic point of view, as the cytoplasmic organelles (mitochondria, plastids,…), that suffer repetitive bottlenecks at each ovule formation and thus end in an homogeneous, non polymorphic, homoplasmic state in most organisms (Birky Jr et al. 1989, Atlan & Couvet 1993). As with cytoplasmic genomes, mutations occurring during the development of the colony could recreate some diversity in the huge zooxanthellar population, but in that case less diversity is 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). Our results thus show that horizontal transfer of symbionts among colonies might be frequent. This interpretation is not contradictory to the previous observations, as horizontal transfer can occur in adult colonies on top of vertical transmission through ovules. After all, experimental horizontal transfer of heterologous zooxanthellae, even belonging to different clades, has already succeeded in species displaying vertical transmission of symbionts (Zurel et al. 2008). That such events occur naturally in *E. singularis* could be confirmed by comparing mother colony and planula symbiont populations or temporal variation of *Symbiodinium* populations in adult colonies.

This observed within colony symbiont diversity, eventually amplified by horizontal transfer of the symbiont, potentially creates an additional level of selection for the best symbiont within the individual host, as occurs with organelles within heteroplasmic organisms (Birky Jr et al. 1989, Otto & Orive 1995, Otto & Hastings 1998). This within individual selection could have favoured a differentiation of the zooxanthellae along the depth luminosity and temperature gradient, as seen among the symbionts of tropical coral species (Baker 2001, Iglesias-Prieto et al. 2004). The systematically lower density of zooxanthellae observed in *E. singularis* deep colonies (Fig. 3a), and the dynamic change of this density after colony transplantation (Fig. 5) could have been 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).

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 *Anemonia* sp. symbionts were split from a group of symbionts from *E. singularis* and three scleractinian corals (*Cladocora caespitosa*, *Balanophyllia europaea* and *Caryophyllia smithii*) symbionts (clade IV-2 in Fig. 2). This means that there is some genetic differentiation within A' zooxanthellae in the Mediterranean sea, which is driven by the animal host rather than by the environment. This result also shows that there is a limitation to the eventual horizontal transfer of zooxanthellae, which must be at least partially host specific. It should be noted however that two *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 three scleractinian corals symbionts are identical to a basal *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 to the parsimony network (Fig. 2). As for the 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 dose of horizontal transfer.

<u>More diverse deep populations</u>

From a genetic point of view, the only difference among shallow and deep populations is that ribosomal nucleotide diversity is significantly higher in the latter. This higher diversity in deep populations could directly be due to higher effective population sizes in these populations. Even if

within colony zooxanthellar density is lower in these populations, the density of *E. singularis* colonies itself is usually higher below –20 meters (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 for example, in the summer of 2003, surface populations were affected by a temperature rise, that did not affect deeper colonies (Garrabou et al. 2009). One could thus imagine a well/sink migration pattern between usually stable, productive deep populations and possibly short 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

In *E. singularis*, adaptation to depth conditions does not happen through a change in zooxanthellar lineage, as defined by LSU sequence variation. The NCA even indicated that within clade *Symbiodinium* genetic diversity is randomly distributed among shallow and deep populations. However, there are significantly less zooxanthellae in deep colonies (Fig 3a). This in turn leads to a lower chlorophyll concentration in deep colonies (Fig. 3b), as the chlorophyll content of the zooxanthellae is globally invariant along the depth gradient (Fig. 4). 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 of the holobiont, usually through an increase of chlorophyll content per zooxanthellae (Fitt & Cook 2001, Rodolfo-Metalpa et al. 2008). Such a phenomenon seems also to occur in the temperate coral *Cladocora caespitosa* harbouring the A'

Symbidinium (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 probably clonal, shallow (9 meters deep at the most) population of *Anemonia viridis*. These anemones harboured zooxanthellae from the same A' clade, but probably slightly different from the *E. singularis* symbionts according to our genetic analysis,. This apparent absence of photo acclimation has led anyway to the conclusion that temperate symbiotic anemones depend less on the photosynthetic activity of their zooxanthellae than their tropical counterparts (Muller-Parker & Davy 2001). Our results seem to extend this conclusion to the temperate octocoral *E. singularis* as well. Even if we did not compare directly the photosynthetic activity among deep and shallow colonies, at each location studied, colonies from -30m contained significantly less chlorophyll than colonies at -15m. As irradiance rapidly drops with depth, especially in temperate waters (see as an indication Muller-Parker & Davy, 2001), this lower chlorophyll content could actually reflect a lower photosynthetic activity in deep colonies, and is hence probably a sign of a more heterotrophic regime for these colonies.

Reciprocal transplantation

Transplanted shallow colonies decreased their symbiont densities, whereas transplanted deep ones increased theirs. Symbiont density, and hence chlorophyll content (as the chlorophyll content per zooxanthella did not vary in this experiment either), seems thus to be conditioned at least partly by the environment (Fig. 5). This adaptive response could explain the observed high variability in symbiont density among locations, as conditions of irradiance can be different in each location. The fact that transplanted shallow colonies lose zooxanthellae in lower light conditions is more indicative of a higher maintenance cost of the symbionts in such conditions, as suggested by Mueller Parker and Davy (2001), than of a photoadaptation mechanism.

Is *Eunicella singularis* built on a totally mutualistic relationship?

We did not find any symbiont genetic differentiation among deep and shallow populations of *E. singularis*, despite the systematically lower symbiont densities in the deep populations. 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 twenty days after transplantation. It should be noted that in *Cladocora*, a Mediterranean coral able to photo acclimatize , the density of A' symbionts was of the same order of magnitude, but with a 5 to 10 times higher chlorophyll content (both at the colony or zooxanthellae 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 *Cladocora*, 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 *E. 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 zooxanthellar 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 2000b). 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 *E. singularis*, as it doesn't 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 two 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 still a lot of opportunities for adaptive selection for the symbiont population even 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 is still to be answered.

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Figure captions:

Figure 1 : **Sampled locations of *Eunicella singularis* colonies.**

At each location, two populations were sampled at around 10 and 30 meters deep, except for Menorca, where a single population was sampled at 10 meters. The name of each location is followed in brackets by the number of colonies sampled respectively in shallow and deep colonies.

Figure 2: **Maximum parcimony network of the zooxanthellae nuclear large ribosomal subunit and nested clade analysis (NCA) results.**

A 95% confidence parcimony network was obtained with TCS 2.1. The sequences in red were obtained from shallow colonies, the ones in blue from deep colonies. The non *Eunicella* sequences from Genebank 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 here presented, as they were more than ten 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 names of the clades within which the distribution of the shallow, deep or non *Eunicella* symbionts were significantly non random are shaded.

Figure 3: **Zooxanthellae population densities and total chlorophyll concentration for each population at each location.**

As only seven 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. Vertical bars represent the standard errors. **A)** Number of zooxanthellae per square centimeter of colony. shallow

vs. deep colonies KW = 7.393, $p = 0.0065$; **B)** Total chlorophyll concentration (µg/cm^2 of colony) . shallow vs. deep colonies KW = 7.801, $p = 0.0052$

Figure 4: **Total chlorophyll concentration per zooxanthella for each population at each location**

This was obtained by dividing the total chlorophyll content by the number of zooxanthellae per square centimetre of colony (N = 7 colonies for each population at each location). shallow vs. deep colonies KW = 0.221, $p = 0.6384$. Vertical bars represent the standard errors.

Figure 5 : **Total chlorophyll concentration in transplanted colonies**.

The total chlorophyll concentration in micrograms per square centimetre of colony was estimated for each of the control and transplanted colonies. Vertical bars denote 0,95 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, were thus analysed, on the day of the transplantation (D0), eleven (D11) and twenty days later (D20). The data were analysed following a two factor repeated measures ANOVA: Time after transplantation, $F = 1.97$, n.s., Transplantation modality, $F = 3.75$, $p < 0.05$, Time x Transplantation, $F = 0.83$, n.s. The letters indicate significantly different values for each time value (post hoc test, $p < 0.05$)

Tables and Figures

Table 1: **Distribution of the LSU/cp23S haplotypes**.

Absolute frequencies of the different haplotypes in each population (***S*** shallow, ***D*** deep) at each location. The number of sequenced LSU clones are given in brackets for each haplotype in each population. ***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			**Menorca**	**Banyuls**		**Mejean**		**Giens**		**Portofino**		*Total*
	LSU	cp23S	**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)*
Total			*1(1)*	*20(4)*	*20(6)*	*20(6)*	*20(10)*	*20(3)*	*20(3)*	*13(12)*	*17(10)*	*161(55)*

Figure 1

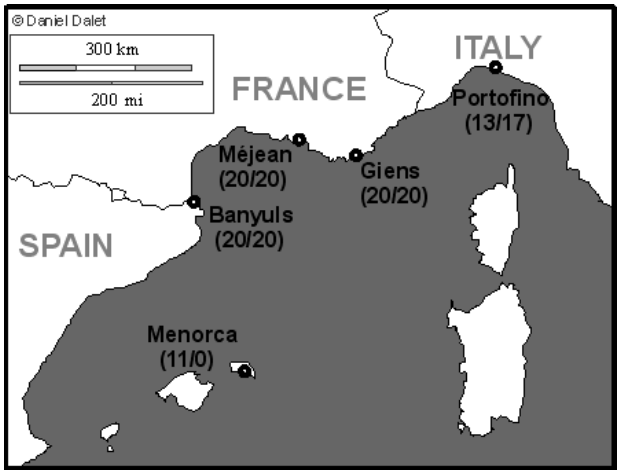
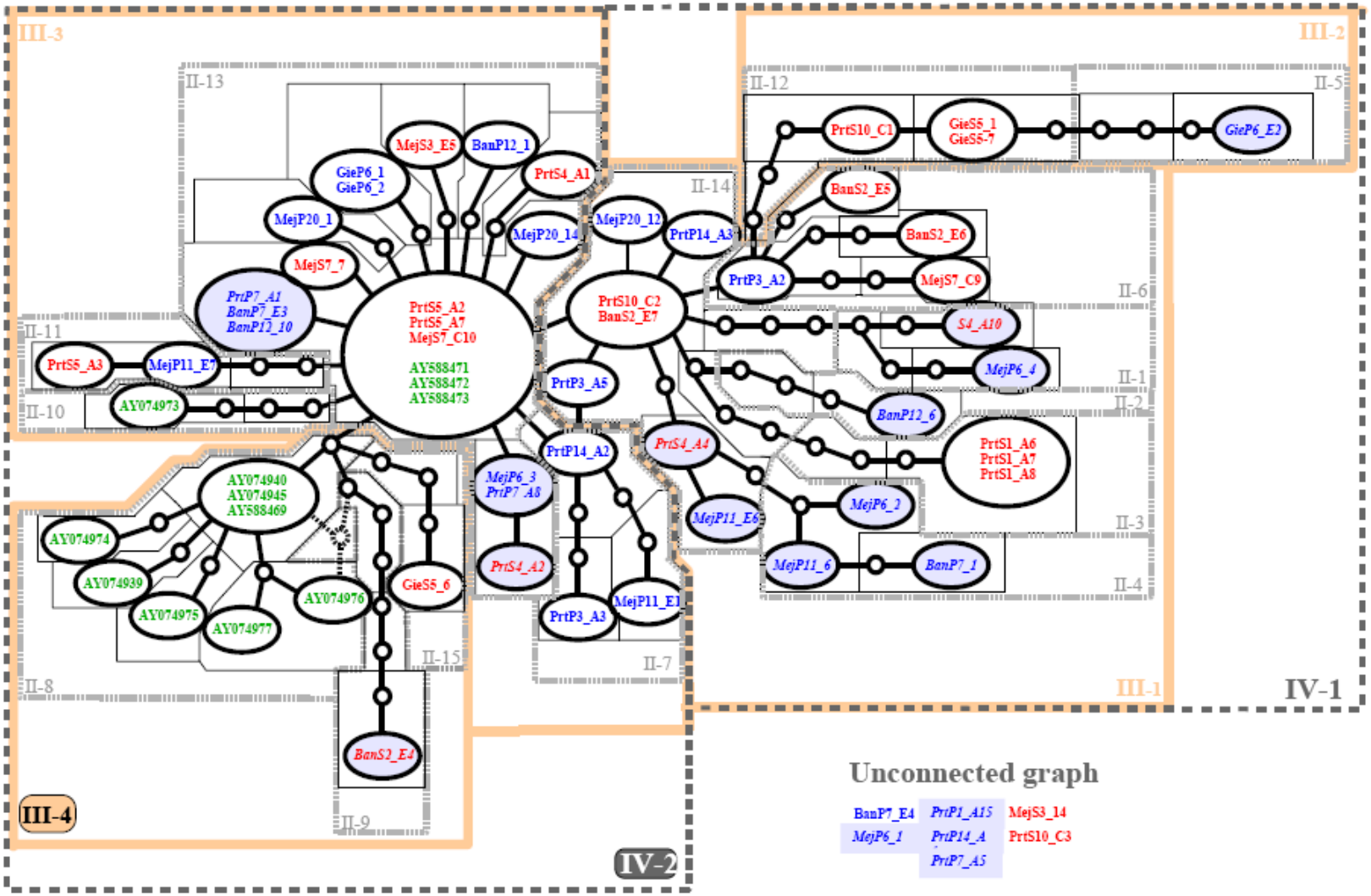

© Daniel Dalet
300 km
200 mi
FRANCE
ITALY
Portofino
(13/17)
Méjean
(20/20)
Giens
(20/20)
Banyuls
(20/20)
SPAIN
Menorca
(11/0)

Figure 2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III-2
II-13
II-12
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II-6
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II-15
II-7
II-8
II-9
III-1
IV-1
III-4
IV-2
PrtS5_A2
PrtS6_A7
MejS7_C10
AY588471
AY588472
AY588473
PrtS10_C2
BanS2_E7
PrtS10_C1
GieS5_1
GieS5-7
GieP6_E2
BanS2_E5
BanS2_E6
MejS7_C9
PrtP3_A2
PrtP14_A3
MejP20_12
MejP20_14
MejP20_1
GieP6_1
GieP6_2
MejS3_E5
BanP12_1
PrtS4_A1
MejS7_7
PrtP7_A1
BanP7_E3
BanP12_10
PrtS5_A3
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S4_A10
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PrtS1_A8
MejP6_2
MejP11_E6
MejP11_6
BanP7_1
MejP6_3
PrtP7_A8
PrtS4_A2
PrtP3_A3
MejP11_E1
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AY074945
AY588469
AY074974
AY074939
AY074975
AY074977
AY074976
GieS5_6
BanS2_E4
Unconnected graph
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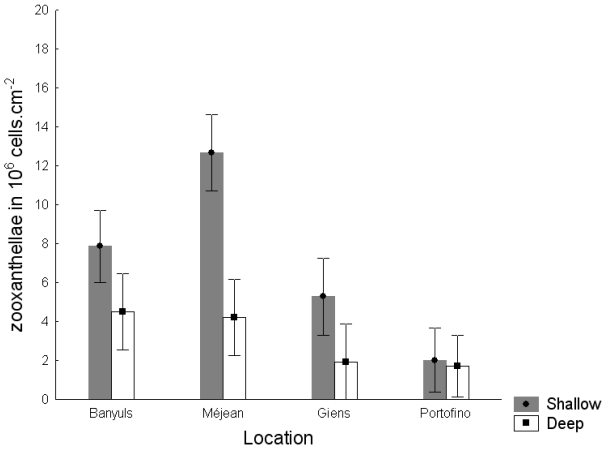
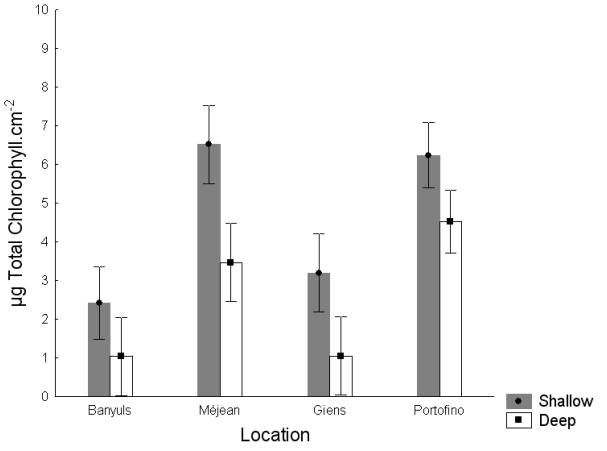
MejS3_14
PrtS10_C3



1

2 Figure 3a

3



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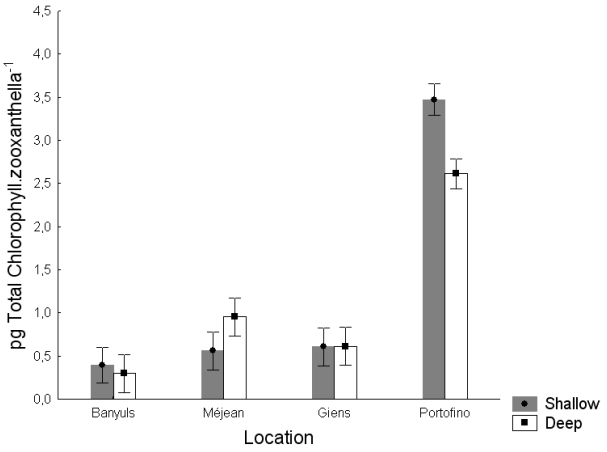
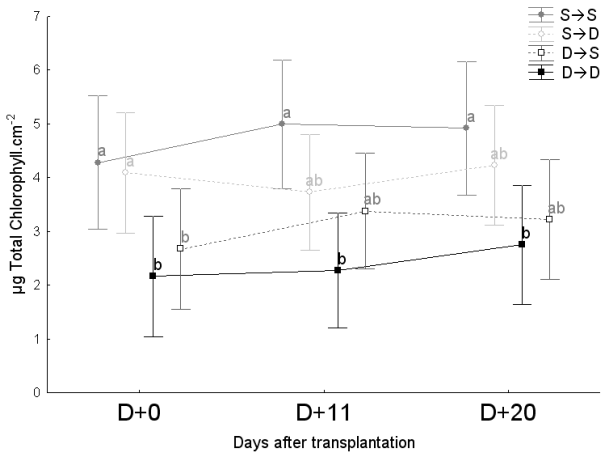
5 Figure 3b

6



7

8 Figure 4


S→S
S→D
D→S
D→D
µg Total Chlorophyll.cm-2
0
1
2
3
4
5
6
7
a
a
a
ab
a
ab
b
ab
b
b
b
ab
D+0
D+11
D+20
Days after transplantation

1

2 Figure 5

3