Symbiotic status does not preclude hybridisation in Mediterranean octocorals

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ABSTRACT

Understanding how species can form and remain isolated in the marine environment is still an active research area. Correctly delimiting species if also of interest for biodiversity conservation and for a wide range of biological studies. Here we study the differentiation and the possibility of hybridization among three temperate octocorals : *Eunicella cavolini*, *E. singularis* and *E. verrucosa*. These species can occur in sympatry and morphologically intermediate individuals have been observed. Among these three species, *E. singularis* is the only one known to show a mutualistic symbiosis with photosynthetic Symbiodiniaceae. As symbiotic relationships can be involved in species barriers, we tested here the possibility of hybridization between symbiotic and non-symbiotic *Eunicella* species. Through multivariate analyses and hybrid detection, we prove the existence of current gene flow between *E. singularis* and *E. cavolini*, with the observation of F1 and F2 hybrids and backcrosses. Demographic inferences indicate a scenario of secondary contact between these two species. Our data suggest an intermediate abundance of Symbiodiniaceae in the hybrids of the two species. We discuss the evolution of the Symbiodiniaceae / cnidarian symbiosis in the light of our results.

Keywords: speciation, hybridization, symbiosis, transcriptome, octocoral

Introduction

As corner stones of evolutionary biology, species and speciation still raise a wealth of questions fueled by the technological and conceptual advancements in genomics. Species can be defined as a part of a genealogical network, and such definition should be clearly distinguished from recognition criteria (Samadi & Barberousse, 2006). Genomic data, either from complete or partial representation of genomes, allow testing species hypotheses, understanding speciation scenarios, and go deeper in the analysis of interactions between biodiversity and environments. Species are indeed hypotheses to be tested, if possible through the integration of independent criteria, and independent molecular markers (Pante et al., 2015b). Sound species delimitation and identification is useful, among others, to better estimatespecies range and biodiversity patterns (Coelho et al., 2023; Muir et al., 2022), to avoid biases in connectivity studies (Pante et al., 2015b), and adaptive abilities (Brener-Raffalli et al., 2022). Species delimitation can be problematic in the context of the grey zone of speciation, where different delimitation criteria may bring contradictory conclusions (De Queiroz, 2007). This is the case for cryptic species, i.e. entities assigned to the same nominal species though reproductively isolated (Cahill et al., 2023). Conversely, gene flow can be observed among morphologically differentiated entities, and this can be an important factor in adaptation and evolution (Leroy et al., 2020). The analysis of a high number of independent markers now provides the required analytical power for species delimitation in such difficult cases, for example when mitochondrial barcoding is not informative (McFadden et al., 2011). This wealth of information allows demographic inferences and testing speciation scenarios (De Jode et al., 2023; Roux et al., 2016). Incomplete speciation might still be inferred, and it’s an interesting situation to study the speciation process itself (Roux et al., 2016).

In the marine realm, the question of speciation was considered as particularly puzzling. From a fundamental point of view, understanding the drivers of speciation is not easy for species with high effective size, and important gene flow in a supposedly open environment (Faria et al., 2021; Mayr, 2001; Palumbi, 1992). Difficulties in sampling and rearing organisms also limits experiments to test reproductive isolation (Faria et al., 2021). Important progress has nevertheless been made in various marine organisms to better understand spatial patterns of genetic structure. One can use models of oceanographic connectivity to understand the observed genetic differentiation, and highlight potential barriers to gene flow (Reynes et al., 2021). Barriers to gene flow can also be inferred from clines in allele frequencies (Gagnaire et al., 2015). The observation of hybrid zones in marine species unveils heterogeneity in this environment, and shows the importance of the interactions between exogenous (i.e. dependent on different environments) and intrinsic (genetic incompatibilities) barriers to gene flow (Bierne et al., 2011). Some marine species also provided important models to study the role of gametes recognition in reproductive isolation (Palumbi, 1999).

In this context, a poorly investigated topics remains the role of symbiotic interaction in speciation. There are various examples of the involvement of microbial species in reproductive isolation, especially in insects (Brucker & Bordenstein, 2012). For marine species, the role of microbial communities has

been mainly explored at the light of adaptative evolution, with the concept of hologenome which comprises the genomes of the host and associated microbiome (Rosenberg & Zilber-Rosenberg, 2018). Shallow water scleractinian corals (hexacorals) are usually associated with photosynthetic zooxanthellae (Cairns, 2007). These zooxanthellae, of the Symbiodiniacae family, correspond to different genera and species (LaJeunesse et al., 2018). Changes in associated Symbiodiniaceae can impact the thermotolerance of the coral holobiont, and the possibility of adaptation facing climate change (Berkelmans & van Oppen, 2006; van Oppen & Medina, 2020). Inferences from the phylogeny of scleractinian corals have shown multiple acquisitions of the symbiotic state, but there is still an important diversity of non symbiotic corals (Cairns, 2007; Campoy et al., 2020). Despite the diversity of Symbiodiniaceae, the diversity in symbiotic state (presence / absence, species diversity), and the possibility of shifting at different evolutionary scales, there is no information on the potential involvement of these symbionts in reproductive isolation, or even on the possibility of gene flow among species with different symbiotic state. The symbiotic interactions between Anthozoans (hexacorals and octocorals) and Symbiodiniaceae presents important mutualistic benefits especially from a nutrional point of view (Furla et al., 2005). This interaction requires specific adaptations for the animal host, as for example protection against oxygen produced by photosynthesis (Furla et al., 2005). The association with Symbiodiniaceae can also range from mutualism to parasitism (Lesser et al., 2013; Sachs & Wilcox, 2006). Therefore, one can envision that hybrids could be impaired by a modification in host – symbiont interactions. The presence or type of Symbiodiniaceae could also be involved in genetic incompatibilities with the host genome, as previously observed with bacterial species (Bordenstein, 2003; Brucker & Bordenstein, 2012).

Here we will study species limits between species with different symbiotic states in *Eunicella* gorgonians (octocorals). In shallow conditions (above 50 m depth), three *Eunicella* species are mainly present in the Mediterranean sea: *Eunicella cavolini*, *E. singularis*, and *E. verrucosa*. These three species have partially overlapping ranges, and they can be observed in sympatry, as is the case in the area of Marseille (France). *Eunicella singularis* hosts Symbiodiniaceae corresponding to temperate clade A (Forcioli et al., 2011; LaJeunesse et al., 2018; Porro, 2019), whereas the two other species are devoided of these symbionts (Carpine & Grasshoff, 1975). The Symbiodiniaceae contribute to the carbon metabolism o*f E. singularis*, but a non-symbiotic *aphyta* morph of this species has already been observed (Gori et al., 2012). While the lack of variability in mitochondrial DNA does not allow to distinguish these three species (Calderon et al., 2006), an initial study using two nuclear introns, and considering morphologically intermediate individuals, suggested the possibility of hybridisation between *E. singularis* and *E. verrucosa* (Aurelle et al., 2017). Demographic inferences based on the transcriptome sequences of *E. cavolini* and *E. verrucosa* indicated the possibility of current gene flow between these two species, but *E. singularis* was not analysed there (Roux et al., 2016). Here, we will go further on these questions with the following objectives: i) estimate the genomic differentiation among these three species, ii) test the possibility of hybridisation according to symbiotic state and genetic similarities, and iii) infer scenarios of speciation.

Material and methods

Species distribution

E. singularis and *E. cavolini* are only present in the Mediterranean Sea, whereas *E. verrucosa* is present both in the Eastern Atlantic Ocean and the Mediterranean Sea (Carpine & Grasshoff, 1975). In the Atlantic, *E. verrucosa* can be found from Ireland, West coasts of Britain to the north-west Africa (Readman & Hiscock, 2017). *Eunicella verrucosa* is also present in the North Western Mediterranean Sea with a patchy distribution, in Sardinia (Canessa et al., 2022), and possibly in the Adriatic and Aegean Seas (Chimienti, 2020). In the Mediterranean Sea, it can be observed from shallow conditions (20-40 m) up to 200 m depth: this presence in relatively deep conditions can lead to possible underestimation of this species range (Chimienti, 2020; Fourt & Goujard, 2012; Sartoretto & Francour, 2011). *Eunicella cavolini* is present in the Western Mediterranean, Adriatic and Aegean Seas, from 5 to 200 m depth (Carugati et al., 2022; Sini et al., 2015). As previously mentioned, *E. singularis* is the only Mediterranean octocoral known to habour Symbiodiniaceae (but see Bonacolta et al., 2024): these Symbiodiniaceae correspond to the temperate clade A (Casado-Amezúa et al., 2016; Forcioli et al., 2011), now corresponding to the *Philozoon* genus, which is sister to the *Symbiodinium* genus (LaJeunesse et al., 2018, 2022). *Eunicella singularis* can be found in the Western Mediterranean and Adriatic Seas, and less frequently in the Eastern Mediterranean (Gori et al., 2012). It is usually observed up top 40 m depth, but deeper occurrences (up to 70 m) have been mentioned, which correspond to the azooxanthellate aphyta morph (without Symbiodiniaceae; Gori et al., 2012). In the area of Marseille, these three species can be observed in sympatry, sometimes at the same depth range, up to 20 m depth (Sartoretto & Francour, 2011).

Sampling

Samples of *E. cavolini*, *E. singularis*, and *E. verrucosa* have been collected by scuba diving in the Mediterranean (for the three species), and in the Atlantic (*E. verrucosa* only; Figure 1) in 2016. In the area of Marseille, the three species have been sampled in sympatry. Four morphologically intermediate individuals (i.e. intermediate colors and branching patterns between *E. cavolini* and *E. singularis*) were collected in the area of Marseille as well: the objective was to test their hybrid status (Aurelle et al., 2017). Figure S1 presents some examples of morphology for the different species and morphologically intermediate individuals in the area of Marseille. Sampling was non destructive, with only a few centimeters of a branch collected at each time. Sampling was performed with authorizations from the local authorities.

Phylogenetic relationships

To put the three *Eunicella* species studied here in a phylogenetic context, and to test their proximity, we performed a phylogenetic analysis by using the mitochondrial MutS locus, which is widely used in octocorals (McFadden et al., 2011), on the basis of sequences available in GenBank. The methods of the phylogenetic reconstruction are detailed in the legend of supplementary Figure S2, and the list of sequences in Table S1.

Transcriptome sequencing and assembly

Total RNA has been extracted as in Haguenauer et al. (2013). RNAs were sent to the LIGAN genomic platform for sequencing (Lille, France) on four flow cells of Illumina NextSeq 500 (2 x 75 bp). The transcriptomes have been assembled with the de novo RNA-Seq Assembly Pipeline (DRAP ; Cabau et al., 2017) with Oases (Schulz et al., 2012) and default parameters. We performed an individual assembly, and a meta-assembly to be used as reference. The statistics describing the assembled transcriptomes are given in supplementary material, Table S2.
We used the BLAT software (Kent, 2002) to remove potential Symbiodiniaceae sequences in the obtained transcriptomes. We used the transcriptome of the type A1 *Symbiodinium* (Genbank accession number GAKY01000000) (Baumgarten et al., 2013) to search for Symbiodiniaceae sequences separately in individual *Eunicella* transcriptomes. The output of BLAT was analysed with the blat_parser.pl script.

Analysis of the presence of Symbiodiniaceae

We analysed the presence of Symbiodiniaceae in *Eunicella* gorgonians in two ways. First, we counted the number of reads corresponding to the aforementioned Symbiodiniaceae transcriptome with Salmon (Patro et al., 2017). Second, we used the percentage of assembled sequences (i.e. contigs) in the Eunicella transcriptomes corresponding to Symbiodiniaceae following the BLAT analysis. We used a Kruskal-Wallis test in R to test for differences among the four groups of samples (the three *Eunicella* species and the potential hybrids) for each metric. Additionally, we performed a blast analysis with the LSU, ITS and psbA sequences of *Philozoon* (LaJeunesse et al., 2022) on individual transcriptomes to try to identify the Symbiodiniaceae genera present in the different samples.
As our results pointed to the potential and unexpected presence of Symbiodiniaceae in *E. cavolini* (see Results), we wanted to explore this topic further by using the preliminary data from another experiment dedicated to studying the microbiome of *E. cavolini* and *E. singularis*. This pilot study involved an analysis of microeukaryotic communities through 18S rDNA metabarcoding on two colonies of *E. cavolini*, and one *E. singularis*. This experiment and its results are presented in Supplementary File S2.

SNPs calling and filtering

We produced bam files by mapping the reads with bwa option mem (Li & Durbin, 2009). We used as reference the meta transcriptome filtered for Symbiodiniaceae sequences. The obtained sam files were converted in bam format with samtools 1.9 (Li et al., 2009), and sorted with Picard tools (Picard Toolkit, 2019). The SNPs calling has been performed with reads2snp 2.0 with default parameters (Gayral et al., 2013; Tsagkogeorga et al., 2012). The obtained dataset, including variable and non variable sites, will thereafter be referred as the “all sites” dataset. We performed separate SNP calls with reads2snp for pairwise comparisons among species and without the potential hybrid samples. These three datasets have been used for demographic inferences (see below), and will be referred as “all-CS” for the *cavolini* / *singularis* comparison, “all-CV” for the *cavolini* / *verrucosa* comparison, and “all-SV” for the *singularis* / *verrucosa* comparison.
We filtered the “all sites” vcf file obtained with reads2snp with vcftools (Danecek et al., 2011) for an analysis of genetic diversity and differentiation. We only retained biallelic sites which have been genotyped in all individuals

(no missing data). To minimize correlation among loci, we retained SNPs separated by at least 1 kb: we will thereafter refer to this dataset as the "polymorphic sites" dataset. From this "polymorphic sites" dataset, we built a dataset focused on the differentiation between *E. cavolini* and *E. singularis*: we excluded *E. verrucosa* samples and we retained the first percent of the loci with the highest F_{ST} between *E. cavolini* and *E. singularis*. This last dataset will be referred as "1% SNPs" dataset. The characteristics of the different datasets are summarised in Table S3.

Genetic differentiation and analysis of hybrids

We analysed the genetic structure and differentiation among species with the "polymorphic sites" dataset. We used the LEA R package to estimate ancestry coefficients (Frichot et al., 2014; Frichot & François, 2015). We tested K values from 1 to 10, with 10 replicates for each K. To analyse the genetic differences among individuals, we performed a Principal Component Analysis (PCA) with the R package adegenet.The pairwise F_{ST} among species were computed with the R package Genepop (Rousset, 2008; Rousset et al., 2020), after conversion of the vcf file with PGDSpider (Lischer & Excoffier, 2012). We present here the F_{ST} estimates of Weir & Cockerham (1984). The distribution of F_{ST} among loci was obtained with vcftools.

The hybrid status (e.g. first generation hybrids) of morphologically intermediate individuals was analysed with the newhybrids software (Anderson & Thompson, 2002). We used the genepopedit R package to prepare the input file from genepop format (Stanley et al., 2017). Following the results of the LEA and PCA analyses, we focused here on the comparison between *E. cavolini*, *E. singularis* and potential hybrids. The newhybrids analysis had difficulties to converge with such a high number of loci compared to the number of markers (see discussion here: https://github.com/eriqande/newhybrids/issues/5). We therefore used the "1% SNP" dataset (with the 1% highest F_{ST} between *E. cavolini* and *E. singularis*) for the newhybrids analysis. As a prior, we used individuals with the lowest levels of admixture in LEA as potential parental individuals: this corresponded to three over five individuals for *E. cavolini*, and six over eight individuals for *E. singularis* (see results). The newhybrids analysis was repeated five times with different seeds to test the stability of the results.

Scenarios of speciation

We tested scenarios of speciation with the Demographic Inferences with Linked Selection (DILS) pipeline (Csilléry et al., 2012; Fraïsse et al., 2021; Pudlo et al., 2016). The DILS pipeline allows the analysis of two species scenarios only: we therefore performed separate analyses for the three two-species comparisons, with the "all-CS", "all-CV", and "all-SV" pairwise datasets. We first tested the priors proposed by Monnet et al. (2023) according to observed statistics of polymorphism and divergence: we then increased the range of priors to get better results on the goodness-of-fit tests. We used the same priors for all analyses, with different numbers of sequences per gene and per sample according to the dataset (Table S4). For all pairwise comparisons, we performed two DILS analyses: one with constant population sizes, and one with variable population sizes.

Results

Phylogenetic relationships

The mitochondrial MutS sequences available in GenBank confirmed the proximity of the three *Eunicella* species analysed here: all sequences were identical for these three species, as well as for three other sequences of unidentified *Eunicella* samples (Figure S2). The closest species to this group was *Eunicella racemosa*. All other *Eunicella* MutS sequences (*E. tricoronata* and *E. albicans*) grouped separately with *Complexum monodi*, but with low bootstrap support.

Presence of Symbiodiniaceae

The analysed samples showed low numbers of reads counts for the Symbiodiniaceae transcriptome (between 1868 and 58406 reads; Table S5). The proportion of assembled sequences corresponding to Symbiodiniaceae with BLAT was also very low (between 0.00276 and 0.03686; Table S5). Significant differences were observed among species in both cases (Kruskal-Wallis test, $p = 0.047$ for reads counts, and $p = 0.002$ for the proportions of assembled sequences). The pairwise Wilcoxon-Test showed significant differences only for the comparisons of proportions of assembled sequences involving *E. singularis* (Table S6; Figure S3). The frequency of assembled sequences corresponding to Symbiodiniaceae was higher in *E. singularis* compared to other species: in *E. singularis*, it varied between 0.004 and 0.037 (mean = 0.022), whereas it reached 0.004 in one sample of *E. cavolini* and it was lower than 0.004 in all other non-*singularis* samples. The mean values of reads counts and assembled sequences in the hybrids were lower than in *E. cavolini* but higher than in *E. verrucosa*; the corresponding pairwise tests were not significant.

The blast analysis with the LSU, ITS and psbA sequences of *Philozoon* only retrieved some sequences in the transcriptomes of *E. singularis*: no hit was observed in the other samples. Regarding the pilot study of 18S rDNA metabarcoding, a diversity of 92 Operational Taxonomic Units (OTUs) corresponding to Symbiodiniaceae was observed in *E. singularis*, with a single OTU largely dominant in abundance. The same OTU was also observed in E. cavolini with a low abundance of reads, but still representing 99% of all 12 to 13 Symbiodiniaceae OTUs detected in the two analysed colonies. A blast analysis of this shared OTU on Genbank indicated that it was phylogenetically related to the clade A of Symbiodiniaceae (Supplementary file S2).

Genetic differentiation and analysis of hybrids

We obtained 31369 SNPs for the “polymorphic sites” dataset. With this dataset, the highest F_{ST} values were observed for the comparisons between *E. verrucosa* and all other samples ($F_{ST} > 0.43$; table S7). The F_{ST} between *E. cavolini* and *E. singularis* was much lower (0.21), and the lowest F_{ST} values were observed for hybrids compared to these two species (F_{ST} around 0.07 in both case). These differences corresponded to different distributions of F_{ST} over SNPs for the three inter-specific comparisons (Figure S4). Both comparisons with *E. verrucosa* involved a more heterogeneous distribution of F_{ST}, and more SNPs reaching the maximum estimate of 1 (1364 SNPs for *E. cavolini* vs *E. verrucosa*, and 1641 SNPs for *E. singularis* vs *E. verrucosa*), than for the comparison between *E. cavolini* and *E. singularis* ($F_{ST} = 1$ for 41 SNPs). If we consider the 1% SNPs with the highest F_{ST} estimates, 52 were shared by both comparisons involving *E. cavolini* (i.e. *E. cavolini* vs *E. singularis* and *E. cavolini* vs *E. verrucosa*), amounting to 0.17% of all SNPs,

116 top 1% SNPs were shared by both comparisons involving *E. singularis* (0.37% of all SNPs), and 1042 top 1% SNPs were shared by both comparisons involving *E. verrucosa* (3.32% of all SNPs). All the results point to the higher differentiation of *E. verrucosa* compared to the two other species, with more outlier SNPs for the divergence of this species.

The cross-entropy analysis with LEA did not give a clear signal, with the best clustering solution corresponding to K = 2 or K = 3 clusters (Figure S5). The barplots of the coancestry coefficients for K = 2 to 4 are presented in Figure 2. At K = 2, the first distinction was observed between *E. verrucosa* and all other samples. The K = 3 analysis further separated *E. cavolini* and *E. singularis*, and the morphologically intermediate individuals appeared well admixed between these two species. Conversely the individuals representative of *E. cavolini* and *E. singularis* presented low levels of admixture, apart from the *E. cavolini* of the site in Algeria (site code anb), and, at a small level, two *E. singularis* individuals from Banyuls (ban). At K = 4, the two *E. cavolini* individuals from Algeria separated from their conspecifics from the northern part of the Mediterranean, without admixture. The low signal of introgression in two *E. singularis* individuals from Banyuls appeared to correspond to *E. cavolini* from the northern and the southern part, or only the southern part of the Mediterranean depending on the individuals. A very low signal of *E. verrucosa* ancestry was observed in most *E. singularis* individuals, but not in *E. cavolini*.

The PCA first separated *E. verrucosa* from other samples on the first axis (33.2% of variance; Figure S6). The second axis (13% of variance) separated *E. cavolini* and *E. singularis*, with the potential hybrids in intermediate position between them.

The newhybrids analysis indicated that the morphologically intermediate individuals, which displayed intermediate ancestry with LEA, were indeed hybrids with a probability of one in all five iterations of the analysis. One individual was a first-generation hybrid, another one was a second-generation hybrid, and the two other ones corresponded to backcrossing with *E. singularis* (Figure 2). In the same analysis, the *E. cavolini* and *E. singularis* individuals not included as priors for parental species (see Figure 2 for the individuals used as priors), were indeed inferred as parental with a probability of one.

Scenarios of speciation

The Supplementary File S3 gives the complete results of the DILS analysis including estimated parameters distribution, comparisons between observed and expected joint Site Frequency Spectrum (jSFS), and the PCA comparing the observed dataset with the prior and posterior distributions. The average pairwise net divergence estimated from DILS was 0.0018 between *E. cavolini* and *E. singularis,* and around 0.007 for the two comparisons with *E. verrucosa* (Table S7). The DILS analysis indicated the existence of current gene flow between *E. cavolini* and *E. singularis* with high probability, both with constant and variable population sizes ($p = 0.87$ and 0.88 respectively; Table 1). This possibility of gene flow corresponded to a scenario of secondary contact. Conversely, a model of current isolation was inferred for the comparisons between *E. verrucosa* and each of the two other species, with a probability $p \geq 0.87$: in these two cases, the inferred scenario included a period of ancestral migration, though with moderate support (p between 0.61 and 0.69). A genomic heterogeneity in effective size was inferred with strong

support ($p \geq 0.99$) for all analyses. In the case of current migration (between *E. cavolini* and *E. singularis*), a genomic heterogeneity in migration rates was inferred ($p \geq 0.82$). The inferred parameters for the different scenarios are presented in Supplementary Table S8. We will first present the results obtained for the constant population sizes models. The divergence time between *E. cavolini* and *E. singularis* (median 403 273 generations) was much lower than between *E. cavolini* and *E. verrucosa* (median 1 054 488 generations), and between *E. singularis* and *E. verrucosa* (median 899 098 generations). For the comparison between *E. cavolini* and *E. singularis,* the time of secondary contact was estimated at 62 039 generations (median estimate), which translates in around 85% of time in isolation since divergence. Following secondary contact, the gene flow was similar in both directions for these two species. The duration of ancestral migration roughly corresponded to 6% and 8% of the total time since divergence for the comparison between *E. cavolini* and *E. verrucosa*, and for the comparison between *E. singularis* and *E. verrucosa*, respectively. For these last two cases, the gene flow (forward in time) during ancestral migration was higher towards *E. verrucosa* than in the opposite direction. Regarding effective size, the comparisons all indicated lower values for *E. singularis* compared to both other species (around 200 000 to 300 000 for *E. singularis*, around 600 000 to 750 000 for both other species). The estimated effective sizes were of similar order for *E. cavolini* and *E. verrucosa*. This aligns well with the observed estimates of nucleotide diversity around 0.007-0.009 for *E. cavolini*, 0.005-0.006 for *E. singularis*, and 0.007-0.009 for *E. verrucosa* (variations depend on the comparison which may change the retained dataset; Supplementary File S3). Similar results were obtained for the models including variations in effective size, except for the estimate of current gene flow between *E. cavolini* and *E. singularis*: with variable population size, gene flow from *E. singularis* to *E. cavolini* was higher than in the opposite direction.

Discussion

Species relationships and differentiation

As already observed with COI (Aurelle et al., 2017), the mitochondrial MutS marker did not allow to discriminate the three species. This is the consequence of the usually slow evolution of mitochondrial DNA in octocorals (Muthye et al., 2022). The use of transcriptome sequences first confirmed that *E. cavolini* and *E. singularis* are sister species, with *E. verrucosa* being more distantly related. The F_{ST} estimate between *E. verrucosa* and the two other species was more than twice higher than the estimate between *E. cavolini* and *E. singularis*. This had been previously suggested with two intron sequences, but incomplete lineage sorting for these markers did not allow a formal conclusion (Aurelle et al., 2017). The Mediterranean *Eunicella* then add a new example of the lack of power of mitochondrial DNA to discriminate genetically differentiated octocoral species, as shown in other genera by the use of RAD sequencing or ultra conserved elements and exons (Erickson et al., 2021; Pante et al., 2015a). The slow rate of evolution of mitochondrial DNA in octocorals has been linked to the presence of the mitochondrial locus MutS, an homolog of a bacterial gene involved in DNA repair. One can note that counter examples exist which show that the presence of this locus is not the only factor explaining the slow evolution of mitochondrial DNA in octocorals (Muthye et al., 2022).

The divergence between *E. singularis* and *E. cavolini* would then have occurred after the divergence from *E. verrucosa.* One can note the highest divergence of *E. singularis* from *E. verrucosa* compared to *E. cavolini* (pairwise F_{ST} 0.529 and 0.432 respectively): this could point to a more complex evolutionary history than a single divergence, for example involving different demographic histories, or gene flow from unsampled taxa (Tricou et al., 2022).

Speciation scenarios

The speciation scenarios inferred from DILS are in agreement with the observed pattern of differentiation. For the results obtained here with pairwise analyses, a much higher divergence time was observed for both comparisons involving *E. verrucosa* than for those involving other species. A scenario of constant isolation was strongly supported in both cases, with the possibility of ancestral migration. The current isolation of *E. verrucosa* from *E. cavolini* is at odds with previous results which showed the possibility of current gene flow between these two species despite an important divergence (Roux et al., 2016). It will be interesting to explore the reasons for the discrepancy between this study and the present one, which are both based on transcriptome datasets but obtained from different samples and sequencing platforms.

Eunicella verrucosa is currently more widely distributed in the North Eastern Atlantic Ocean, and less frequent in the Mediterranean Sea, whereas both other species analyzed here are only present in the Mediterranean Sea. The Atlantic / Mediterranean Sea transition does not seem to act as a phylogeographic barrier for *E. verrucosa* (Macleod et al., 2024). One could then envision a scenario where the split between *E. verrucosa* and both other species occurred in allopatry between the Atlantic Ocean and the Mediterranean Sea, followed by the colonization of the Mediterranean Sea by *E. verrucosa*. The generation time remains unknown for the *Eunicella* species studied here, and previous studies have shown important variation in the age at first reproduction in gorgonians, from 2 to 13 years (see references in Munro, 2004). If we suppose a lower hypothesis of generation time of two years for *Eunicella* species, with a median estimate of divergence time around 900 000 generations for *E. verrucosa* / *E. singularis* and 1 000 000 for *E. verrucosa* / *E. cavolini*, and based on a mutation rate set at 3.10^{-9}, this would indicate a divergence at least around 2 000 000 years (2 Ma). As a comparison, this is of the same order as estimates of divergence time for sister scleractinian species which can go from around 1 Ma to 4-5 Ma (Aurelle et al., 2024; Johnston et al., 2017). Note that higher generation time would point to divergence time older than 5 Ma, where the Messinian crisis (between 5.5 and 6 Ma; Rouchy & Caruso, 2006) could have played a role in initiating divergence. Obviously, more data on generation time and mutation rate are needed to tentatively link speciation times with fluctuations in paleo-environments. With a median estimate around 400 000 generations, the divergence time between *E. cavolini* and *E. singularis* would be 2.5 times more recent, around 800 000 years. The median time of secondary contact between these two species would be around 60 000 generations, corresponding to 15% of the time spent since divergence. It is difficult to infer past distributions of *E. singularis* and *E. cavolini*, but one can note that even if they are currently found in sympatry in different areas (such as near Marseille), their range do not completely overlap. For example *E. cavolini* is

nearly absent at the West of the Rhone on French coasts, whereas *E. singularis* is present there. The ecological range of *E. singularis* and *E. cavolini* is also not completely overlapping, as *E. cavolini* can be observed deeper than *E. singularis* (Carugati et al., 2022; Gori et al., 2012). Therefore one can envision an historical separation of these two species either geographically or ecologically, followed by a secondary contact where gene flow took place.

Hybridisation and species barriers

The analysis of genetic ancestry with LEA and the hybrid inferences both confirmed that morphologically intermediate individuals are indeed hybrids between *E. singularis* and *E. cavolini*, with the identification of one F1, one F2 and one backcross with *E. singularis*: the last two categories indicate that first generation hybrids can be fertile and can participate in reproduction. The possibility of current gene flow is confirmed with the DILS analysis, with a probability of 0.87 and 0.88 for models with constant and variable population sizes respectively. Importantly the DILS analysis did not include hybrid individuals, which indicates that gene flow between these two species indeed goes further than the aforementioned hybrid levels. Accordingly, the LEA analysis also indicated a small level of *E. cavolini* ancestry in some *E. singularis* individuals, but the reverse was not observed: this would point to asymmetric gene flow, more frequent towards *E. singularis*, but this does not agree with the results of DILS. Quantifying hybrid frequency in situ may be difficult, as we don't know the range of morphological variations in hybrids. Nevertheless, the ease to find such hybrids in the area studied here, as well as similar observations in other sites near Marseille (S. Sartoretto, pers. com.) indicates that hybridization is not rare at an evolutionary scale.

The alternation of parental populations with mixed populations would point to a mosaic hybrid zone (Bierne et al., 2003), where hybrids could form in different areas and from different genetic compositions of parental species. As hybridization between *E. cavolini* and *E. singularis* had not been reported before, the presence of hybrids has probably been overlooked up to now. Communicating on this subject towards scientists and diving associations might help sampling other potential hybrid zones: this would be useful to analyse the spatial and ecological distribution of hybrids. One interesting question in this context is whether changes in selection regimes induced by human activities can change the outcome of hybridization (Ålund et al., 2023). For example, Mediterranean octocorals are impacted by mortality events linked with climate change (Estaque et al., 2023; Sini et al., 2015), and it would be interesting to compare the thermotolerance of hybrids and parental individuals. In scleractinian corals, interspecific hybridisation has been reported to enhance the survival under elevated temperature conditions (Chan et al., 2018).

Regarding the comparison between *E. cavolini* and *E. singularis*, the DILS analysis pointed to an homogeneity of gene flow. The net divergence between *E. cavolini* and *E. singularis* (0.0018) puts these two species below the grey zone of speciation in the analyses of Roux et al. (2016).They may correspond to an early stage of speciation where genomic islands of differentiation did not expand in the genome of these species (see Peñalba et al., 2024, and references therein). One can note that in the meta-analysis evolutionary inferences of De Jode et al. (2023), an heterogeneity of gene flow was more frequently inferred than homogeneity for scenarios of

secondary contact, which points to a diversity of stages of divergence in the tested species.

The distribution of F_{ST} between *E. cavolini* and *E. singularis*, indicated a slight heterogeneity, with few loci with F_{ST} higher than 0.5 for example. Conversely, both comparisons with *E. verrucosa* showed much more loci with high F_{ST}, as expected with higher divergence time. Among the list of the most highly differentiated loci, more overlap was also observed for the two comparisons involving *E. verrucosa* than for the other pairwise comparisons: this may indicate that few areas of potential incompatibilities with *E. verrucosa* are involved in the divergence between *E. cavolini* and *E. singularis*. Such incompatibilities could explain the persistence of interspecific differentiation despite a quite long time since secondary contact. These conclusions should be investigated more thoroughly with genome wide analysis of differentiation, and by taking into account the alternative hypotheses explaining genomic islands of divergence such as background selection or local adaptation (Quilodrán et al., 2020).

Evolution of symbiosis

As previously discussed, we clearly demonstrated here the possibility of gene flow between a symbiotic (i.e. hosting Symbiodiniaceae) and a non-symbiotic octocoral species: the hybrid status then does not lead to complete isolation, even if Symbiodiniaceae could be involved in genetic incompatibilities. The methods used here did not aim at a precise quantification of Symbiodiniaceae, and one can note the general low levels of sequences corresponding to these symbionts, even in *E. singularis*: this may be due to difficulties in extracting the RNA of the symbionts with this protocol or to loss of symbionts before extractions. This is not a general result: for example, a whole transcriptome sequencing of the octocorals *Heliopora coerulea* and *Briareum asbestinum* recovered 29% and 17.2% of Symbiodiniaceae sequences respectively (Guzman et al., 2018; Rivera-García et al., 2019). Despite these limits we observed a clear signal of higher Symbiodiniaceae concentration in *E. singularis* than in *E. cavolini* and *E. verrucosa.* Interestingly, the hybrids showed a lower frequency of Symbiodiniaceae than *E. singularis*, and possibly than *E. cavolini*, though this last result remains to be tested with more samples. These results indicate a breakdown of symbiosis following hybridization with potential consequences on the fitness of hybrids. In *E. singularis*, the transmission of Symbiodiniaceae seems to occur both vertically, through ovules, and horizontally, from the environment (Forcioli et al., 2011). Vertical transmission may change the fate of hybrids depending on the species of the mother, as it can change the initial load in Symbiodiniaceae: this hypothesis is nevertheless difficult to test as these species can not be reproduced in aquarium. More generally, the question of the link between symbiosis and the fitness of hybrids would require a dedicated study involving more precise estimates of Symbiodiniaceae abundance (e.g. with quantitative PCR), and of physiological parameters such as photosynthetic and respiration rates in controlled conditions (Ezzat et al., 2013).

Our results also question the evolution and significance of octocoral / Symbiodiniaceae symbiosis. A phylogenetic study of scleractinians has shown that in these hexacorals, the transition between symbiotic and non-symbiotic states happened repeatedly, but mostly in the direction of the acquisition of symbiosis with Symbiodiniaceae, with very low rates of transition in the

reverse direction (Campoy et al., 2020). This could indicate that investing in such mutualistic interactions for the cnidarian would lead to increasingly relying on autotrophy for energetic supply, making reversal to heterotrophy difficult. In octocorals, an evolutionary versatility in symbiotic state seems possible, as in various families and genera, both symbiotic and non-symbiotic species are present (Van Oppen et al., 2005). In the Mediterranean Sea, all octocoral species are non-symbiotic, except for *E. singularis*. The most parsimonious scenario here would be an acquisition of symbiosis in *E. singularis* during or following its divergence from *E. cavolini*. The symbiotic status of *E. singularis* nevertheless could be facultative as non-symbiotic colonies of *E. singularis* have been observed between 40 and 60 m depths (Gori et al., 2012). Additionally, experimental physiological studies have demonstrated the nutritional plasticity of *E. singularis* which is able to use either heterotrophy or autotrophy for its metabolism (Ezzat et al., 2013). Nevertheless, in natural conditions, autotrophy seems to provide an important contribution to the metabolism of *E. singularis*, and the collapse of photosynthetic capacities in too warm conditions could contribute to mortality events in this species (Coma et al., 2015). The question of symbiosis could be reversed as well: why don't we observe any symbiosis with Symbiodiniaceae in *E. cavolini*? This species can be observed in shallow conditions (less than 10 m depth) where there is enough light for photosynthesis, and in syntopy with *E. singularis*. The availability of preys or particulate organic matter may provide enough energy to *E. cavolini* in its habitat, but this species may have never engaged in mutalistic interaction with Symbiodiniaceae. Interestingly we observed a low rate of sequences related to Symbiodiniaceae in the transcriptomes of *E. cavolini* (and even lower, but not null in *E. verrucosa*). This could either correspond to a signal from free living Symbiodiniaceae, or to background, transient, associations with the cnidarian. In addition, a Symbiodiniaceae OTU that is common to *E. singularis* and *E. cavolini* was identified among the microeukaryotes associated with the two species, which probably corresponds to the Symbiodiniaceae species symbiotic with *E. singularis*., and is related to strains observed in symbiosis with other cnidarians. Rare Symbiodiniaceae strains can also be observed in symbiotic hexacorals, probably with low impact on the hosts physiology (Lee et al., 2016). Molecular markers also allowed to evidence the presence of Symbiodiniaceae in species previously supposed to be asymbiotic, as in the Mediterranean octocoral *Paramuricea clavata*, and in several Hawaiian antipatharian species (Bonacolta et al., 2024; Wagner et al., 2011). These results, and our observations in *Eunicella* species, obviously underline the dynamic nature of interactions between Symbiodiniaceae and cnidarians: the establishment of these symbiotic interactions may be preceded by various types of interactions, more or less stable, and more or less mutualistic. The development of effective symbiosis, with stable relationships, and higher abundance of symbiont, would require specific adaptation from both partners. We can see here that even if on a macro-evolutionary scale, the acquisition of symbiosis is much more frequent than its loss, on a micro-evolutionary scale the gene flow between the *Eunicella* species analysed here did not lead to the full development of symbiosis in *E. cavolini*.

Conclusions and perspectives

We here demonstrated the semi-permeable nature of species barriers between two octocoral species with and without mutualistic interaction with Symbiodiniaceae. This opens the way to further researches. On these model species it will be useful to estimate the frequency and spatial extent of hybrid zone: does it correlate with particular environments with a coupling between endogenous and exogenous barriers to gene flow (Bierne et al., 2011)? Characterizing the genomic landscape of introgression would help to search for islands of divergence, and to look for the impact on introgression on adaptation or symbiosis for example. Indeed, even low levels of interspecific gene flow can have important consequences on the evolution of species (Arnold et al., 1999). Finally, various cases of hybridization have been demonstrated between species of symbiotic scleractinian corals (e.g. Combosch & Vollmer, 2015): it would then be interesting to study the dynamics of symbiosis in these cases when different Symbiodiniaceae species are involved.

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Data availability

The raw sequences are available in Genbank under BioProject ID PRJNA1037721 and SRA accession number SRA PRJNA1037721.
The scripts used in this study are available at https://doi.org/10.5281/zenodo.10966625

Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article

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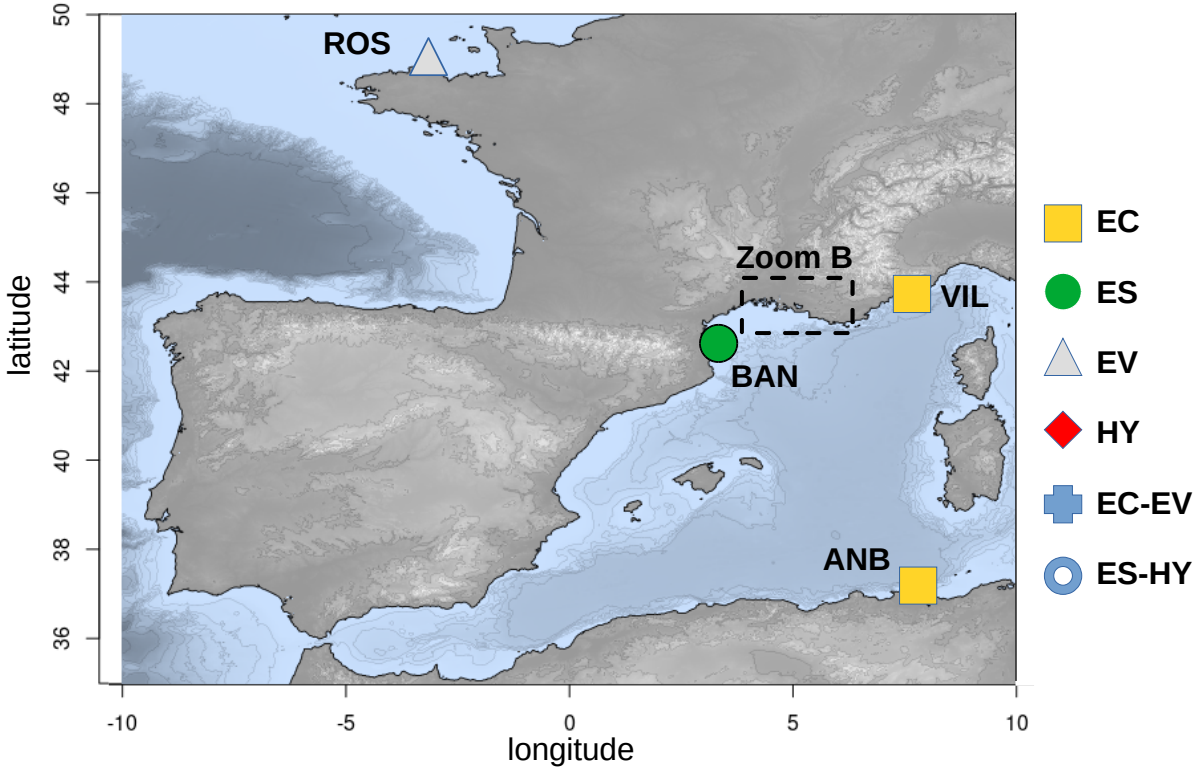
Figure 1: map of sampling sites; A) general view, B) zoom on the area of Marseille. The symbols present the different samples: EC *E. cavolini*, ES *E. singularis*, EV *E. verrucosa*, HY hybrids, EC-EV *E. cavolini* and *E. verrucosa* in sympatry, ES-HY *E. singularis* and hybrids in sympatry. The three letters correspond to the codes of the sampling. The maps have been produced with the marmap R package (Pante & Simon-Bouhet, 2013) and following the tutorial of Krueger-Hadfield (2015).

A)



B)

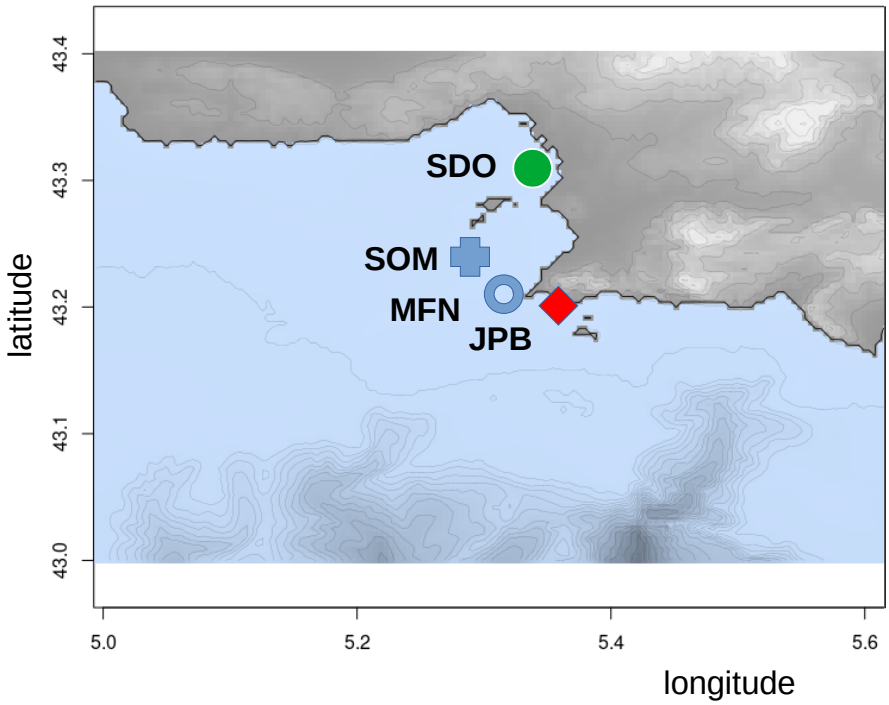

SDO
SOM
MFN
JPB
latitude
longitude
43.4
43.3
43.2
43.1
43.0
5.0
5.2
5.4
5.6

Figure 2: barplots of coancestry coefficients inferred with the LEA R package. The analysis is based on the “polymorphic sites” dataset, with 31369 SNPs. The red asterisks indicate the individuals used as prior for parental status in the newhybrids analysis. The results of the newhybrids analysis are indicated below the hybrid individuals: F1, 1st generation; F2, 2nd generation; Sbx, backcross with *E. singularis*. Note that the coancestry analysis is based on 31 369 SNPs, whereas the newhybrids analysis is based on 326 SNPs showing high differentiation between *E. cavolini* and *E. singularis*.

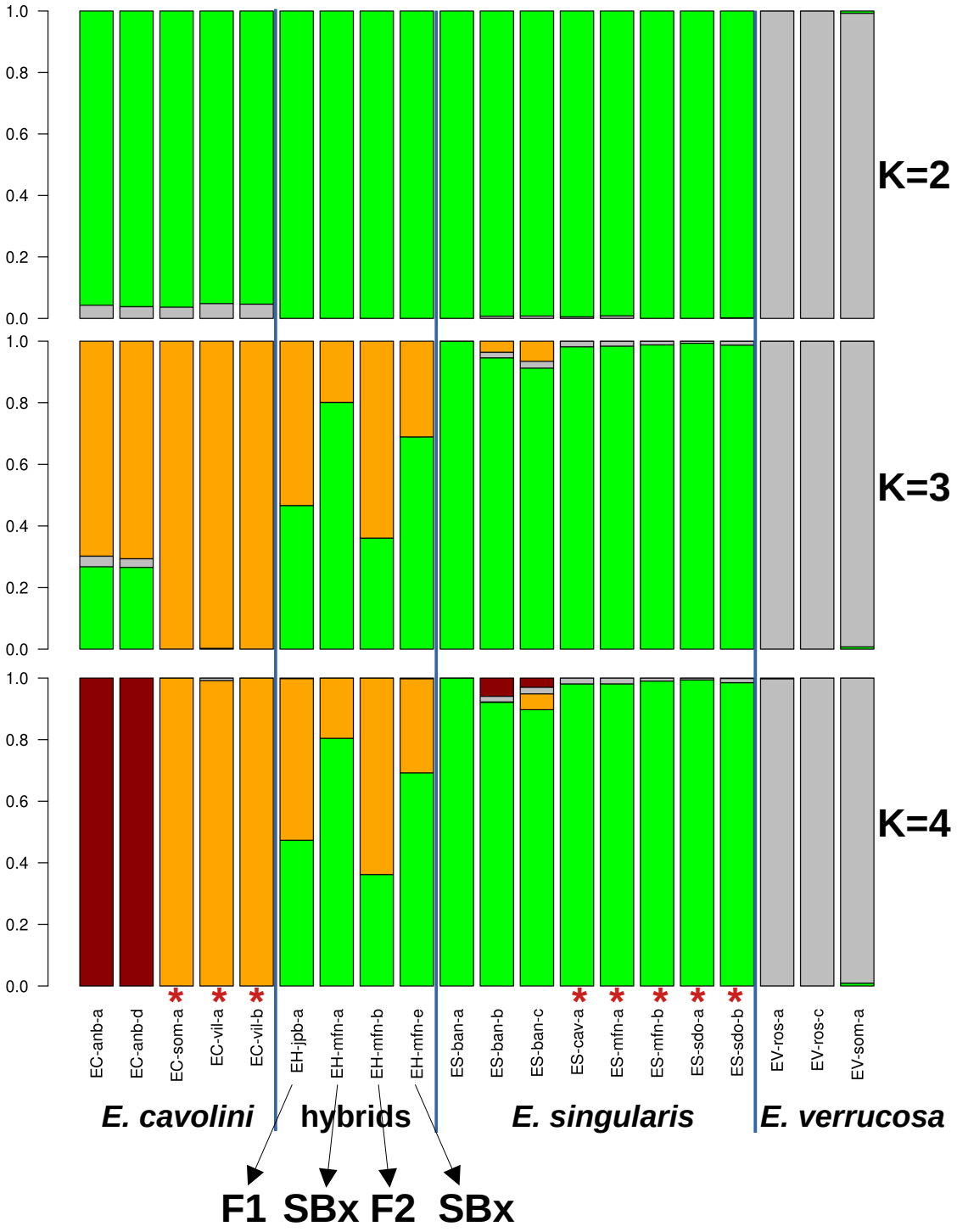

1.0
0.8
0.6
0.4
0.2
0.0
K=2
K=3
K=4
EC-anb-a
EC-anb-d
EC-som-a
EC-vil-a
EC-vil-b
EH-jpb-a
EH-mfn-a
EH-mfn-b
EH-mfn-e
ES-ban-a
ES-ban-b
ES-ban-c
ES-cav-a
ES-mfn-a
ES-mfn-b
ES-sdo-a
ES-sdo-b
EV-ros-a
EV-ros-c
EV-som-a
E. cavolini
hybrids
E. singularis
E. verrucosa
F1
SBx
F2
SBx

Table 1: results of demographic inferences with DILS. The columns indicate the species comparison, the model choice for population size (constant vs. variable), and the results of inferences: current gene flow (migration vs isolation); if current migration, isolation / migration (IM) vs ancestral migration (AM); if no current migration, strict isolation (SI) vs ancestral migration (AM); homogeneity (N-homo) vs heterogeneity in effective size (N-hetero) among loci; homogeneity (M-homo) vs heterogeneity (M-hetero) in gene flow among loci. The probability of each scenario is given in the same case.

Comparison	Population size	Current gene flow	IM / SC	SI / AM	Heterogeneity effective size	Heterogeneity gene flow
cavolini / singularis	constant	Migration; 0.87	SC; 0.79	-	N-hetero; 0.99	M-homo; 0.82
cavolini / singularis	variable	Migration; 0.88	SC; 0.77	-	N-hetero; 1	M-homo; 0.87
cavolini / verrucosa	constant	Isolation; 0.90	-	AM; 0.65	N-hetero; 1	-
cavolini / verrucosa	variable	Isolation; 0.89	-	AM; 0.69	N-hetero; 1	-
singularis / verrucosa	constant	Isolation; 0.87	-	AM; 0.61	N-hetero; 1	-
singularis / verrucosa	variable	Isolation; 0.87	-	AM; 0.61	N-hetero; 1	-

Symbiotic status does not preclude hybridisation in Mediterranean octocorals

Supplementary Material S1

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Table S1: list of mitochondrial MutS sequences used for the phylogenetic reconstruction with the corresponding Genbank accession numbers.

Accession number	**Genus**	**species**
KP036906	*Complexum*	*monodi*
NC_035666	*Eunicella*	*albicans*
JQ397290	*Eunicella*	*cavolini*
JQ397291	*Eunicella*	*cavolini*
JQ397292	*Eunicella*	*cavolini*
NC_035667	*Eunicella*	*cavolinii*
KX051577	*Eunicella*	*racemosa*
JQ397293	*Eunicella*	*singularis*
JQ397294	*Eunicella*	*singularis*
KX051571	*Eunicella*	*singularis*
KX051572	*Eunicella*	*singularis*
JQ397307	*Eunicella*	*sp.*
JQ397308	*Eunicella*	*sp.*
JQ397311	*Eunicella*	*sp.*
JX203795	*Eunicella*	*tricoronata*
NC_062012	*Eunicella*	*tricoronata*
JQ397300	*Eunicella*	*verrucosa*
JQ397302	*Eunicella*	*verrucosa*
JQ397305	*Eunicella*	*verrucosa*
JQ397306	*Eunicella*	*verrucosa*
NC_073494	*Eunicella*	*verrucosa*
KX904973	*Swiftia*	*pacifica*
KX905018	*Swiftia*	*simplex*

Table S2 : statistics of assembled transcriptomes for individual samples and meta-transcriptomes. The assembly is based on paired-ends sequencing (2 x 75 bp) and the number of raw sequences corresponds to the number of pairs. Contigs indicates the number of contigs for each assembly, with the corresponding N50 and L50. The Lg columns corresponds to the contigs length in bp, with the sum, minimum, mean, median and maximum of Lg. The last two lanes refer to the meta-transcriptome obtained from all individual transcriptomes with or without potential Symbiodiniaceae sequences. See main text for details.

Genus	**Species**	**Sample**	**raw sequences**	**contigs**	**N50**	**L50**	**Lg sum**	**Lg min**	**Lg mean**	**Lg median**	**Lg max**
Eunicella	*cavolini*	e-cavol-anb-a	21432997	33627	1978	7240	46041698	201	1369.19	1023	13533
Eunicella	*cavolini*	e-cavol-anb-d	20070761	33624	2002	7025	46288676	201	1376.66	1004	24422
Eunicella	*cavolini*	e-cavol-som-a	22986734	43541	1757	8987	52113269	201	1196.88	840	24228
Eunicella	*cavolini*	e-cavol-vil-a	31846763	36908	2056	7687	51709228	201	1401.03	1018	18381
Eunicella	*cavolini*	e-cavol-vil-b	28751407	34961	2044	7470	48971519	201	1400.75	1040	19504
Eunicella	hybrid	e-hybri-jpb-a	34392918	39407	2031	8098	54290945	201	1377.7	998	25557
Eunicella	hybrid	e-hybri-mfn-a	44256795	40762	2081	8451	57794280	201	1417.85	1039	25573
Eunicella	hybrid	e-hybri-mfn-b	34705411	39672	2046	8040	54738734	201	1379.78	981.5	16650
Eunicella	hybrid	e-hybri-mfn-e	36536647	39532	2038	8090	54685655	201	1383.33	995.5	25578
Eunicella	*singularis*	e-singu-ban-a	44325669	45364	1919	9379	58576839	201	1291.26	928	28882
Eunicella	*singularis*	e-singu-ban-b	33184944	38095	1930	8114	50868966	201	1335.32	987	20211
Eunicella	*singularis*	e-singu-ban-c	46271612	43821	2023	9132	60512898	201	1380.91	1007	21714
Eunicella	*singularis*	e-singu-cav-a	48947180	51120	1967	10031	65261049	201	1276.62	868	22527
Eunicella	*singularis*	e-singu-mfn-a	52588076	70114	1761	13336	79649263	201	1136	739	16808
Eunicella	*singularis*	e-singu-mfn-b	43713977	55035	1894	10583	67120524	201	1219.6	808	21143
Eunicella	*singularis*	e-singu-sdo-a	37444166	55928	1741	10464	62326140	201	1114.4	715	16387
Eunicella	*singularis*	e-singu-sdo-b	39266148	72419	1652	13837	78950323	201	1090.19	715	24245
Eunicella	*verrucosa*	e-verru-ros-a	19398629	31195	1936	6630	41727111	201	1337.62	981	16974
Eunicella	*verrucosa*	e-verru-ros-c	20495748	31526	1968	6729	42779660	201	1356.96	1005	16663
Eunicella	*verrucosa*	e-verru-som-a	23332185	33133	2005	6944	45531674	201	1374.21	1005	25577
Meta transcriptome			**number of contigs**	**retained contigs**	**N50**	**L50**	**Lg sum**	**Lg min**	**Lg mean**	**Lg median**	**Lg max**
Eunicella	meta		891354	68386	2144	14309	102621319	201	1500,62	1098	28882
Eunicella	meta no Symb		300085	59697	1975	12316	80903965	201	1355,24	967	25577

Table S3 : summary of the different datasets; the first four datasets include variable and non variable sites (all sites), while the “polymorphic sites” and the “1% SNPs” datasets only consider SNPs, i.e. variable sites. For the “all” datasets we indicate the number of contigs and the number of sites retained from reads2snp. See main text for details

dataset	samples	sites	number of contigs / SNPs	analyses
all sites	all	all from reads2snp	61500 contigs / 101516577 sites	build SNPs datasets
all-CS	*cavolini* / *singularis*	all from reads2snp	61947 contigs / 101515803 sites	speciation scenarios with DILS
all-CV	*cavolini* / *verrucosa*	all from reads2snp	59702 contigs / 100704015 sites	speciation scenarios with DILS
all-SV	*singularis* / *verrucosa*	all from reads2snp	61373 contigs / 101444729 sites	speciation scenarios with DILS
polymorphic sites	all	polymorphic sites ; no missing data	31369 SNPs	F_{ST}, LEA, PCA
1 % SNPs	without *verrucosa*	polymorphic sites ; no missing data ; 1 % highest F_{ST} *cavolini* / *singularis*	326 SNPs	newhybrids

Table S4: parameters used in the DILS analyses: Max_NA : maximum proportion of missing data ; Lmin : minimum sequence length per gene ; nMin : minimum number of sequences per gene and per species ; jSFS : use of joint Site Frequency Spectrum as an additional set of summary statistics ; constant / variable : consider constant or variable population size ; minimum and maximum values for the following priors : Tsplit : time of split, Ne : population size, M : migration rate. All other priors were kept at default values. For all analyses we used the option for coding regions, we didn’t use any outgroup, we used the bimodal model for barriers, and the "normal" computation mode. The last column indicates the code used to describe the corresponding analysis in the text. The ranges of prior were chosen after preliminary analyses where we analysed the goodness of fit of the data to the models and priors. We used a mutation rate of 3.10^{-9}.

dataset	**max_NA**	**Lmin**	**nMin**	**jSFS**	**Tsplit**	**Ne**	**M**
all-CS	0.1	30	10	yes	100 – 2 000 000	100 – 2 000 000	0-30
all-CV	0.1	30	6	yes	100 – 2 000 000	100 – 2 000 000	0-30
all-SV	0.1	30	6	yes	100 – 2 000 000	100 – 2 000 000	0-30

Table S5: frequency of Symbiodiniaceae sequences in the individual transcriptomes on the basis i) of the proportion of raw reads mapped on the Symbiodiniaceae transcriptome, and ii) on the proportion of sequences in individual transcriptomes following the BLAT analysis. “meta” indicate the meta-transcriptome assembly based on all samples. See Table S2 for the codes of samples.

Sample	Species	Raw reads	Transcriptome
e-cavol-anb-a	*E. cavolini*	0.0171	0.00305
e-cavol-anb-d	*E. cavolini*	0.0087	0.00268
e-cavol-som-a	*E. cavolini*	0.0087	0.00426
e-cavol-vil-a	*E. cavolini*	0.0184	0.00350
e-cavol-vil-b	*E. cavolini*	0.0255	0.00333
e-hybri-jpb-a	hybrid	0.0123	0.00262
e-hybri-mfn-a	hybrid	0.0076	0.00321
e-hybri-mfn-b	hybrid	0.0079	0.00270
e-hybri-mfn-e	hybrid	0.0162	0.00302
e-singu-ban-a	*E. singularis*	0.0192	0.00675
e-singu-ban-b	*E. singularis*	0.0140	0.00410
e-singu-ban-c	*E. singularis*	0.0080	0.00647
e-singu-cav-a	*E. singularis*	0.0261	0.02263
e-singu-mfn-a	*E. singularis*	0.0233	0.03419
e-singu-mfn-b	*E. singularis*	0.0129	0.02745
e-singu-sdo-a	*E. singularis*	0.0158	0.03644
e-singu-sdo-b	*E. singularis*	0.0207	0.03686
e-verru-ros-a	*E. verrucosa*	0.0075	0.00276
e-verru-ros-c	*E. verrucosa*	0.0082	0.00282
e-verru-som-a	*E. verrucosa*	0.0098	0.00279
meta			0.01393

Table S6: p-values of the Pairwise-Wilcoxon test on the frequency of Symbiodiniaceae. A) on the basis of read counts with Salmon; B) on the proportion of assembled sequences with the BLAT analysis

A)

	E. cavolini	hybrids	*E. singularis*
hybrids	0.69		
E. singularis	0.69	0.36	
E. verrucosa	0.57	0.69	0.15

B)

	E. cavolini	hybrids	*E. singularis*
hybrids	0.571		
E. singularis	0.019	0.020	
E. verrucosa	0.571	1	0.048

Table S7: above diagonal: average net divergence estimated from DILS for the “all” pairwise datasets (the hybrids were not included in the DILS analysis); below diagonal pairwise F_{ST} estimated from variable sites only (“polymorphic SNPs” dataset; see main text and Table S3 for details)

	E. cavolini	hybrids	*E. singularis*	*E. verrucosa*
E. cavolini	-	-	0.0018	0.0067
hybrids	0.069	-	-	-
E. singularis	0.207	0.073	-	0.0070
E. verrucosa	0.432	0.456	0.529	-

Table S8: estimated parameters for the different evolutionary scenarios for the three pairwise comparisons. We present here the results of estimations for the optimized posterior with the random forests approach implemented in DILS. For each parameter we present the highest posterior density, with the median, and the lower and higher 2.5 % limits. Models : SC : secondary contact ; AM : ancestral migration. Parameters : N : effective size ; $founders_X$: number of founder individuals in species X ; T_{split} : time of split at which the ancestral population subdivides in two populations ; T_{SC} : time of secondary contact ; T_{AM} : time of the end of gene flow for ancestral migration ; T_{dem_X} : time of demographic event for species X ; M_{XY} : introgression rate from Y to X . For all parameters, the subscripts indicate the species : A for ancestral, C for *E. cavolini*, S for *E. singularis,* and V for *E. verrucosa*. Times are given in generations, migration in numbers of migrants per generation.

A) comparison *E. cavolini* / *E. singularis*

	HPD 0.025	HPD median	HPD 0.0975
constant size, SC			
N_C	545985	633894	733842
N_S	168290	192199	225073
N_A	537403	581831	632310
T_{split}	336413	403273	476196
T_{SC}	51536	62039	71760
M_{CS}	12	15	17
M_{SC}	12	15	18
variable size, SC			
N_C	531986	665965	875780
N_S	185826	222258	276889
N_A	515018	578861	640504
$founders_C$	0	1	1
$founders_S$	0	1	1
T_{dem_C}	250520	339056	418963
$T_{demS\_}$	245400	350132	454320
T_{split}	330907	434060	542765
T_{SC}	40560	57552	75405
M_{CS}	14	19	24
M_{SC}	8	12	16

B) comparison *E. cavolini* / *E. verrucosa*

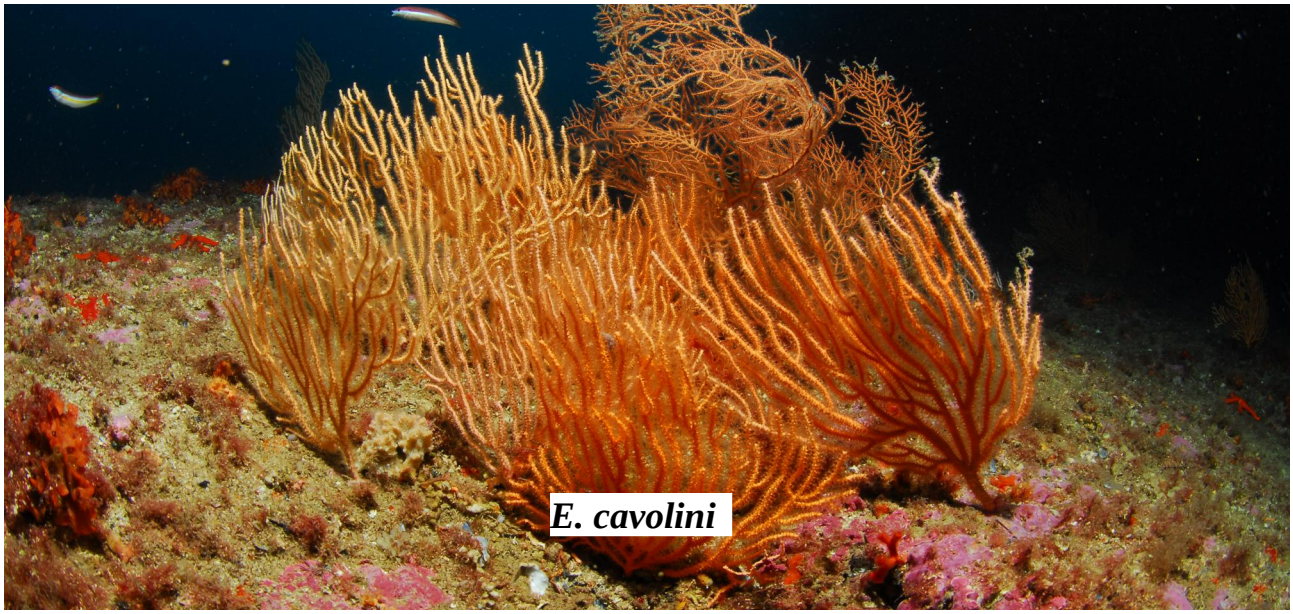
	HPD 0.025	HPD median	HPD 0.0975
constant size, AM			
N_C	630969	744556	875220
N_V	648850	755298	920095
N_A	698501	784512	879664
T_{split}	909392	1054488	1225792
T_{AM}	840920	991118	1147073
M_{CV}	4	6	7
M_{VC}	9	12	14
variable size, AM			
N_C	777526	1099410	1694348
N_V	871210	1230360	1803231
N_A	692366	793880	930000
$founders_C$	0	1	1
$founders_V$	0	0	1
T_{dem_C}	237960	369260	496633
T_{dem_V}	335620	509096	679348
T_{split}	819714	1051517	1367074
T_{AM}	782210	930590	1104120
M_{CV}	7	12	16
M_{VC}	11	22	31

C) comparison *E. singularis* / *E. verrucosa*

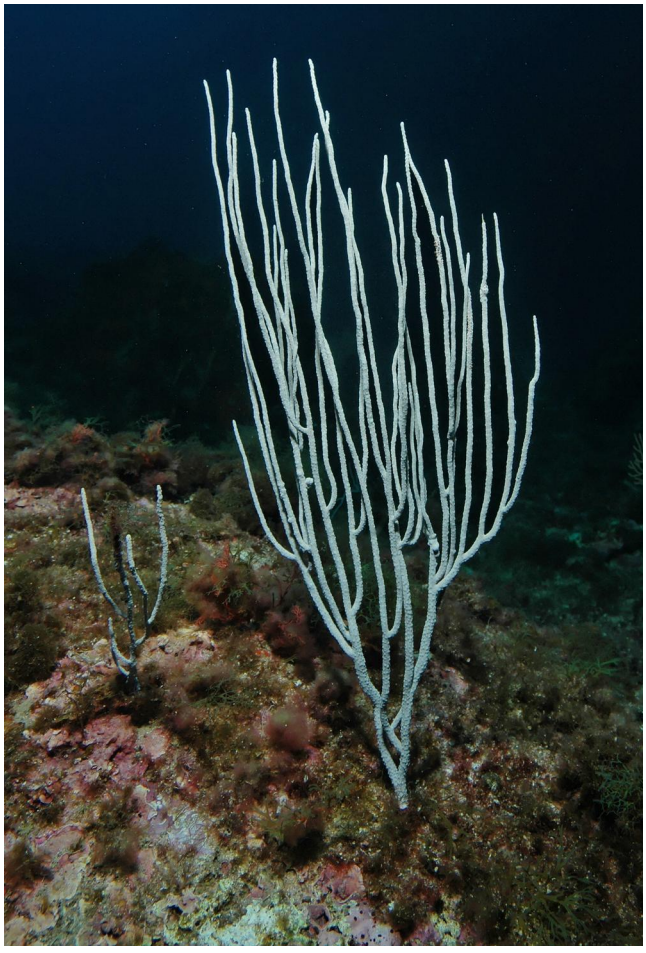
	HPD 0.025	HPD median	HPD 0.0975
constant size, AM			
N_S	263390	298162	336536
N_V	490519	592796	715930
N_A	632004	708517	790246
T_{split}	741840	899098	1091610
T_{AM}	698891	811827	934655
M_{SV}	10	14	17
M_{VS}	21	27	33
variable size, AM			
N_S	281023	386388	494606
N_V	856542	1165039	1566087
N_A	592428	697054	797828
$founders_C$	0	0	0
$founders_V$	0	0	1
T_{dem_S}	166517	273546	374076
T_{dem_V}	226988	360174	493360
T_{split}	713634	926756	1207281
T_{AM}	454059	659458	858558
M_{SV}	3	4	6
M_{VS}	1	1	2

Figure S1: examples of morphological diversity in *Eunicella* species in the area of Marseille.

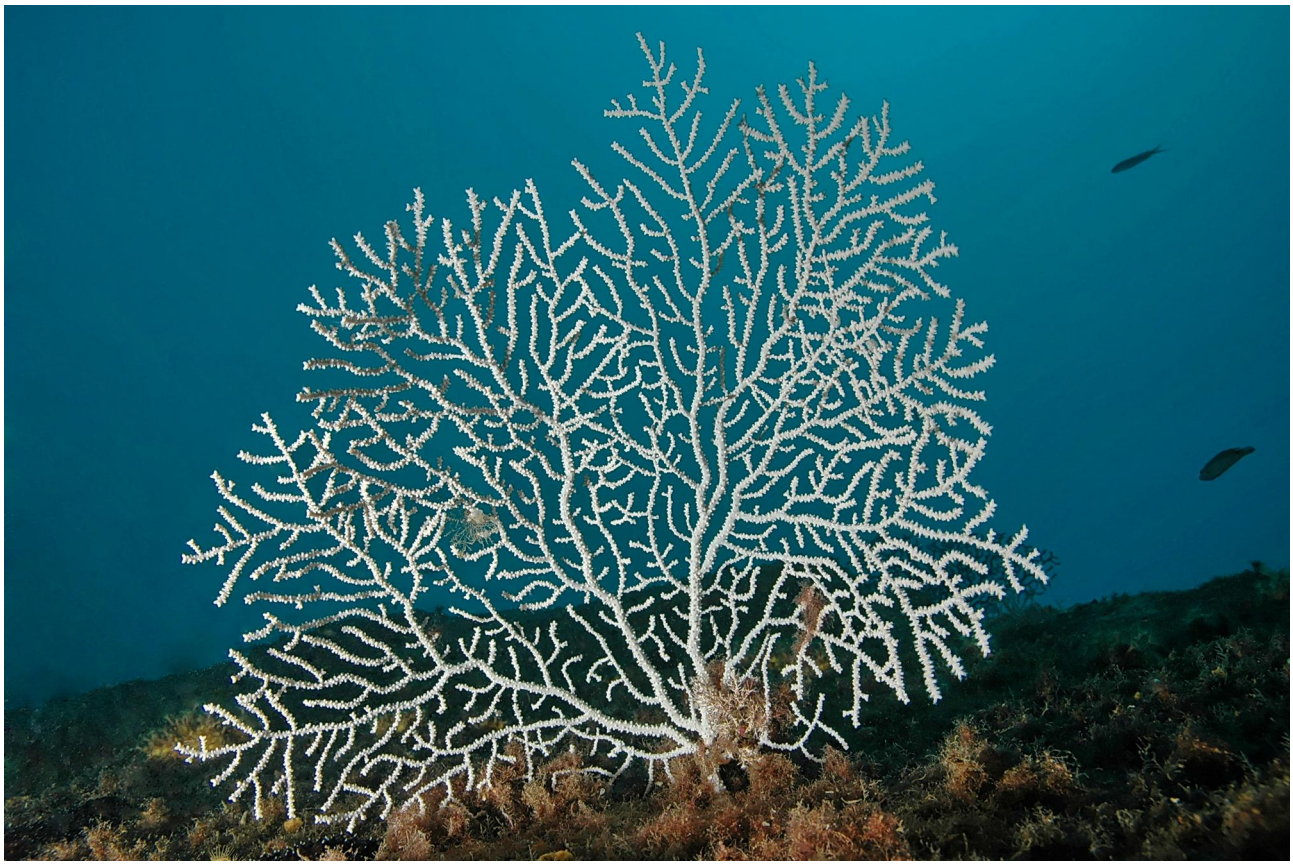
A) example of typical *E. cavolini* colonies (in the foreground)



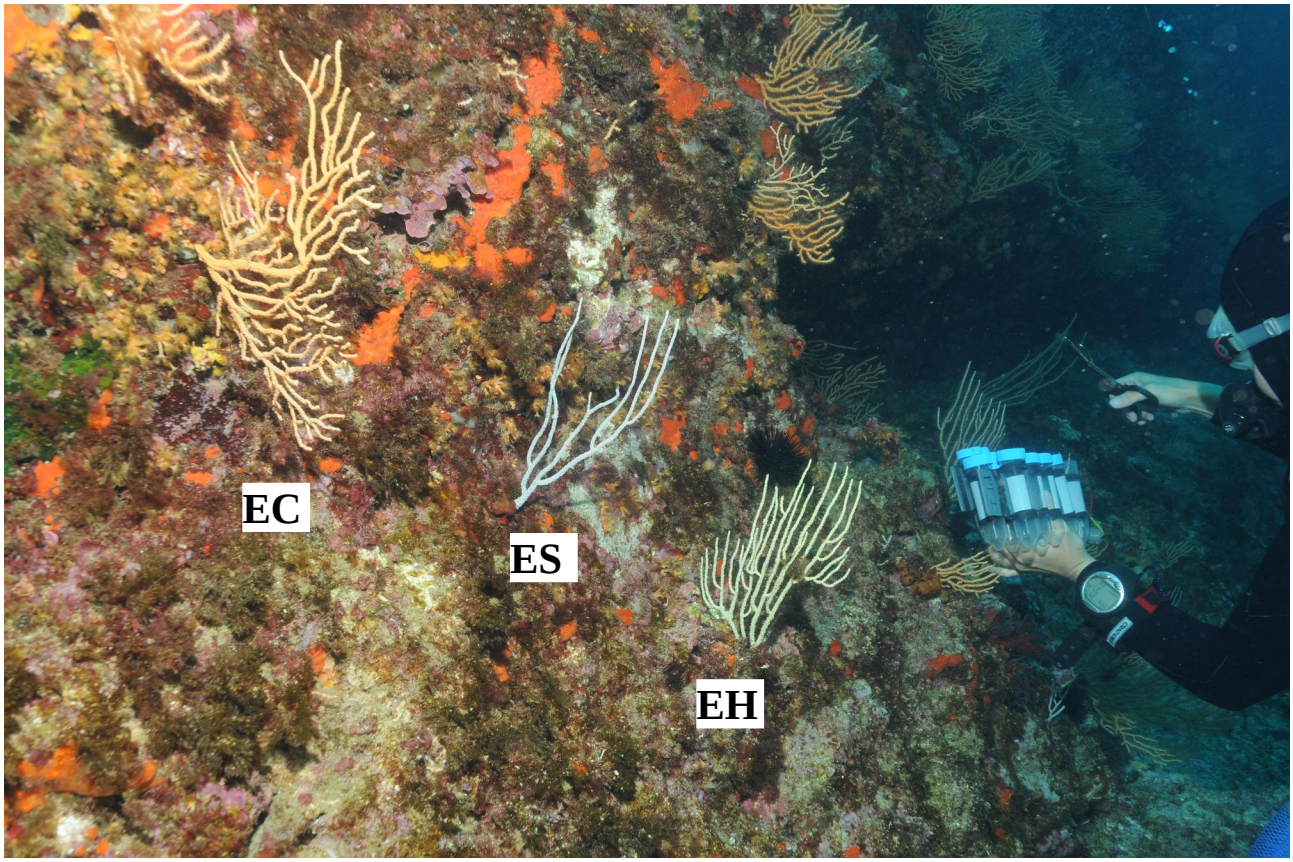
B) example of a typical *E. singularis*



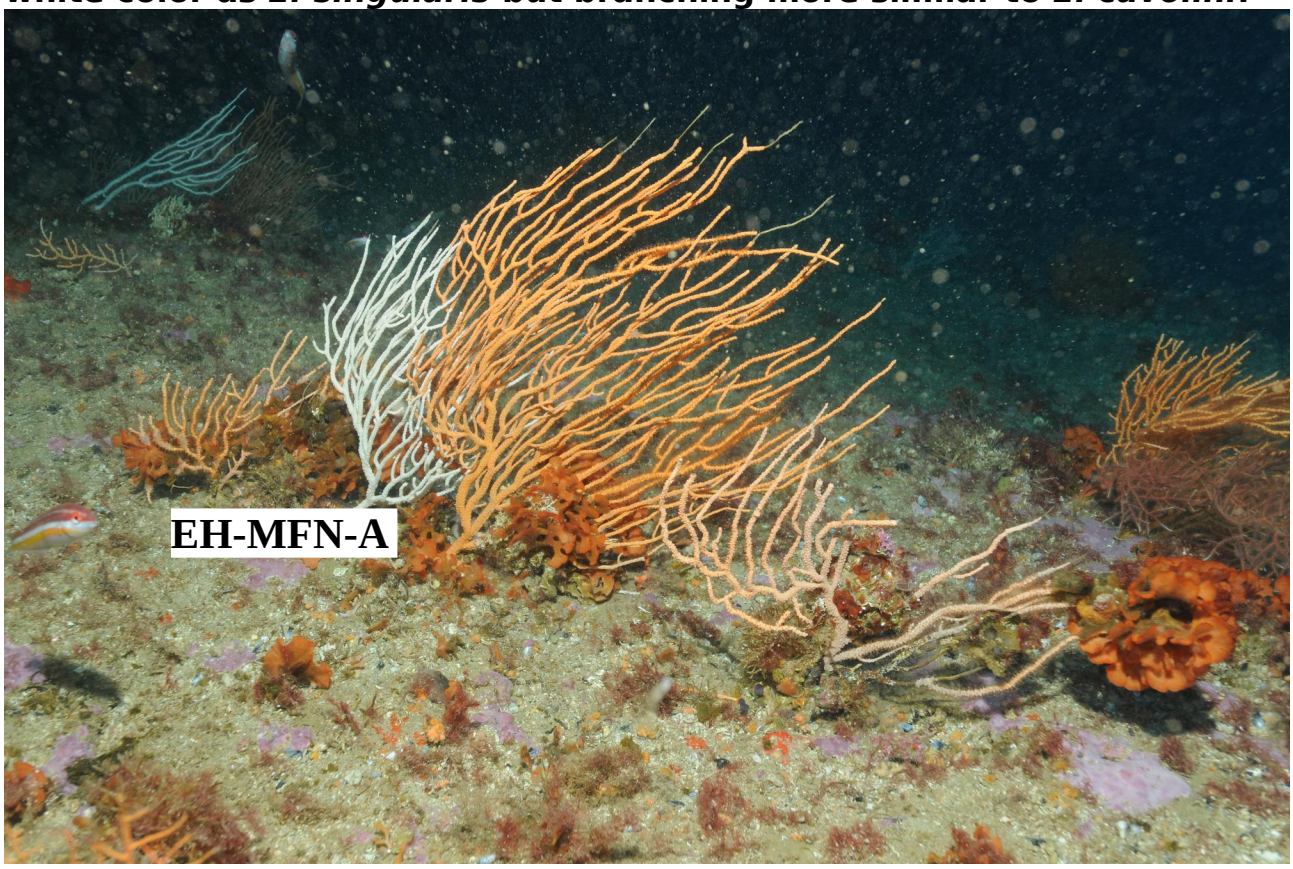
C) example of a typical *E. verrucosa*:



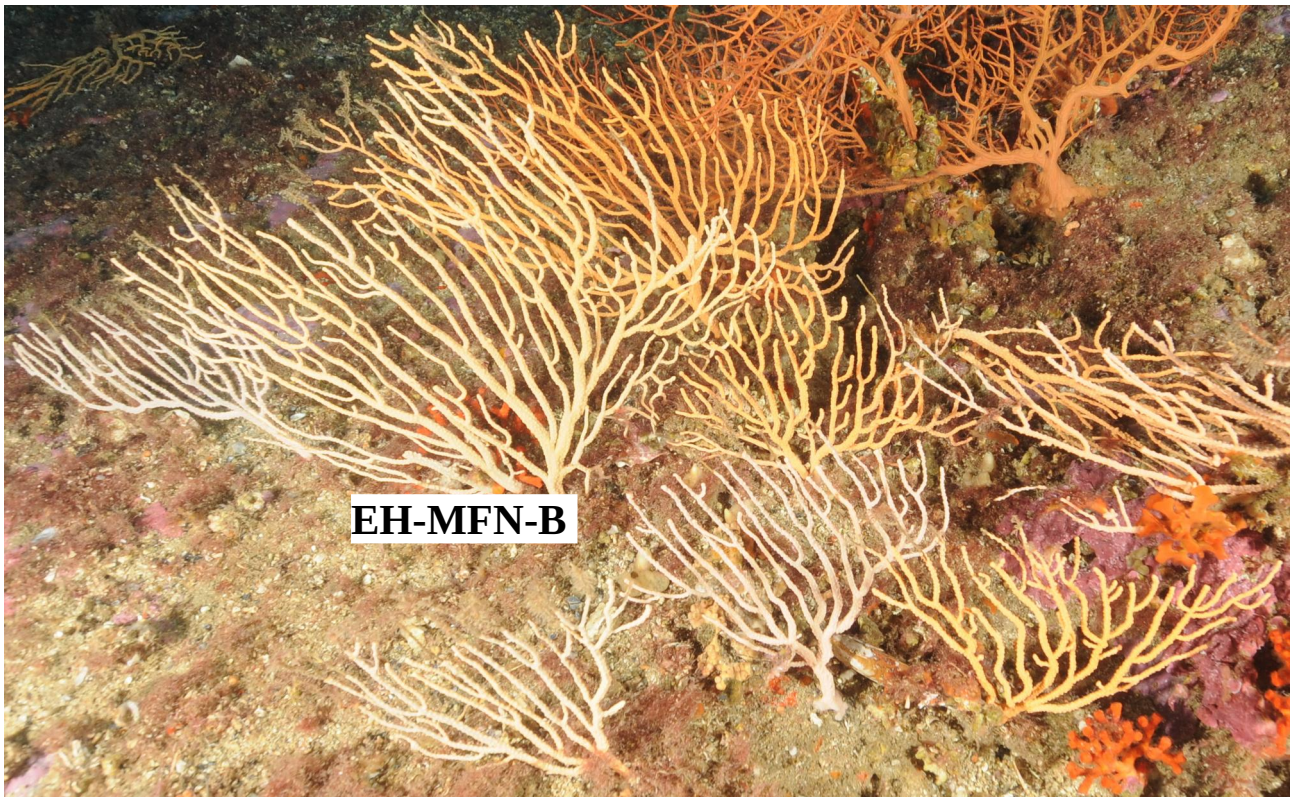
D) sampling with the presence of *E. cavolini* (EC), *E. singularis* (ES) and a potential hybrid (EH)



E) morphology of the colony EH-MFN-A (white, in the background) with white color as *E. singularis* but branching more similar to *E. cavolini*.



F) morphology of the colony EH-MFN-B with intermediate branching and color between *E. cavolini* and *E. singularis*



F) morphology of the colony EH-MFN-E with intermediate branching and color between *E. cavolini* and *E. singularis*

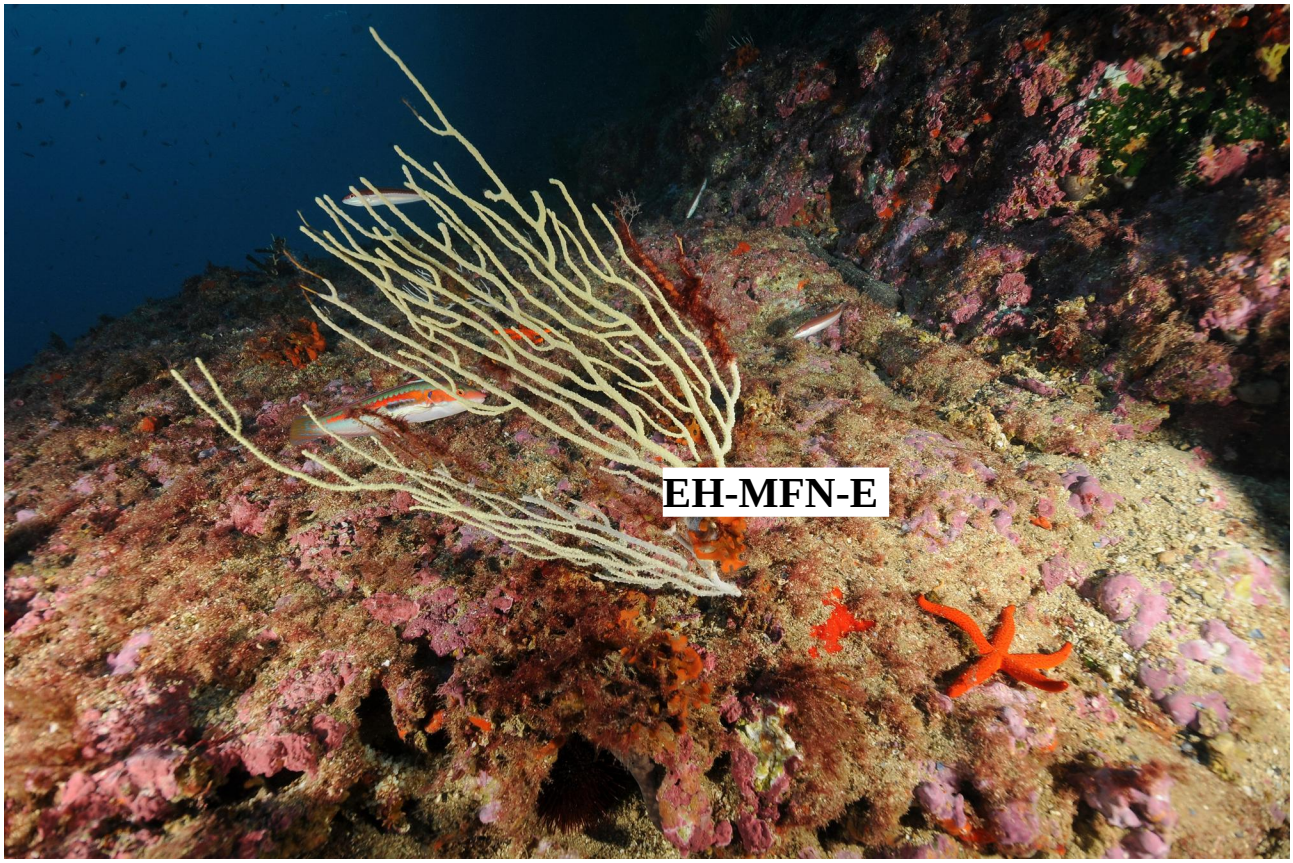


Figure S2: phylogenetic relationships among *Eunicella* species. The phylogenetic reconstruction has been performed with mitochondrial MutS sequences obtained from Genbank, with a search focused on *Eunicella* species. Sequences from the *Complexum* and *Swiftia* genera have been used as outgroups on the basis of a Blast search with the MutS sequence of *E. cavolini*, and according to the current systematics of octocorals (McFadden et al., 2022). The sequences have been edited with ugene (Okonechnikov et al., 2012). The phylogenetic reconstructions have been performed with the Maximum-Likelihood (ML) approach of IQ-TREE 2.1.1 (Nguyen et al., 2015). We used the ModelFinder option (Kalyaanamoorthy et al., 2017), and robustness was evaluated with 1000 ultrafast bootstraps (Hoang et al., 2018). The tree has been visualized with FigTree 1.4.4 (Rambaut, 2006) and was rooted with *Swiftia simplex* as outgroup. The numbers to the left of the nodes indicate the percentages of bootsraps. The Genbank accession numbers are listed in table S1.

References:

Hoang, D. T., Chernomor, O., Von Haeseler, A., Minh, B. Q., & Vinh, L. S. (2018). UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution*, *35*(2), 518–522.

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Okonechnikov, K., Golosova, O., Fursov, M., & Ugene Team. (2012). Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics*, *28*(8), 1166–1167.

Rambaut, A. (2006). *FigTREE v1.4*. University of Edinburgh. http://tree.bio.ed.ac.uk/software/figtree/

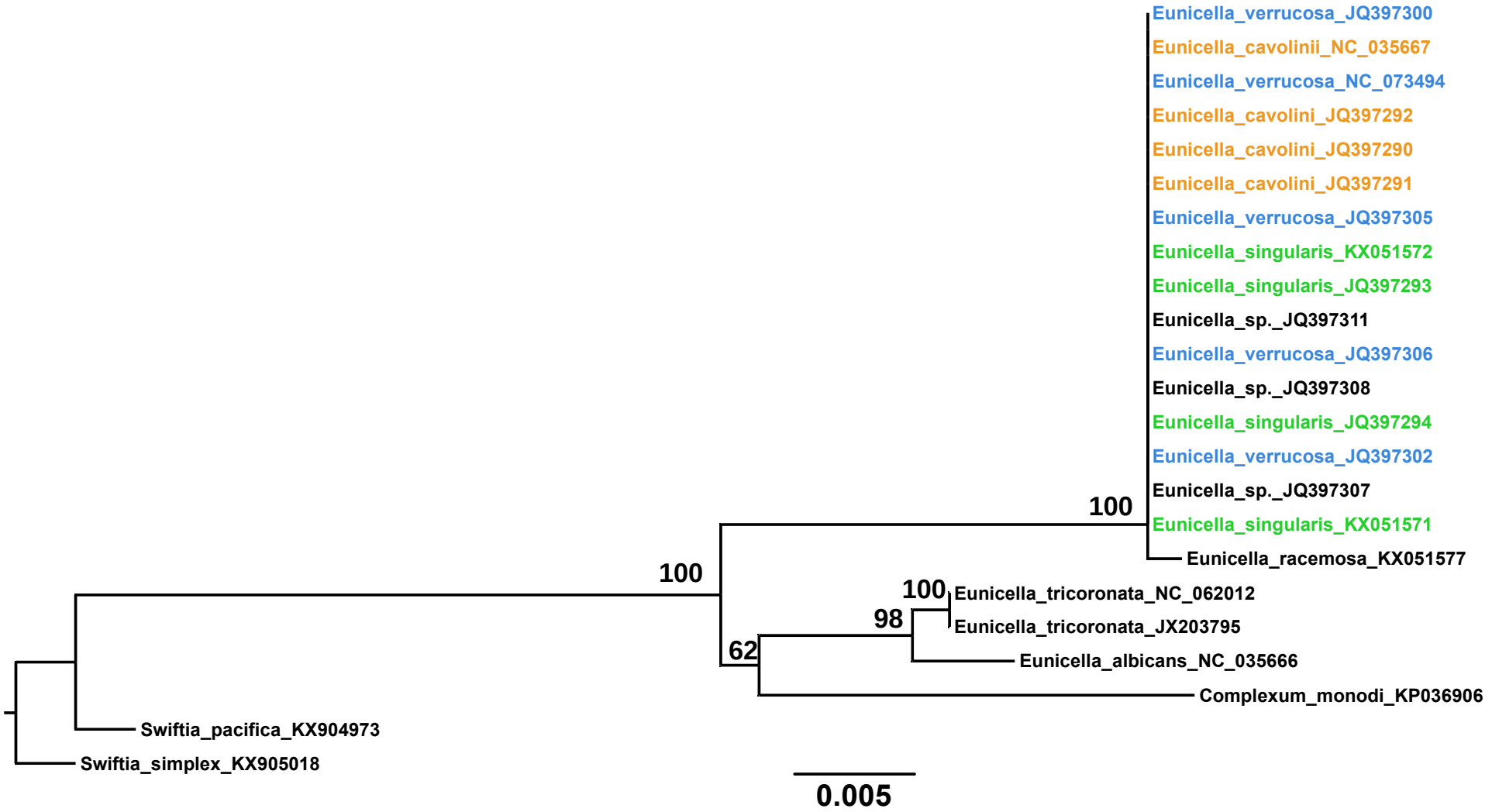
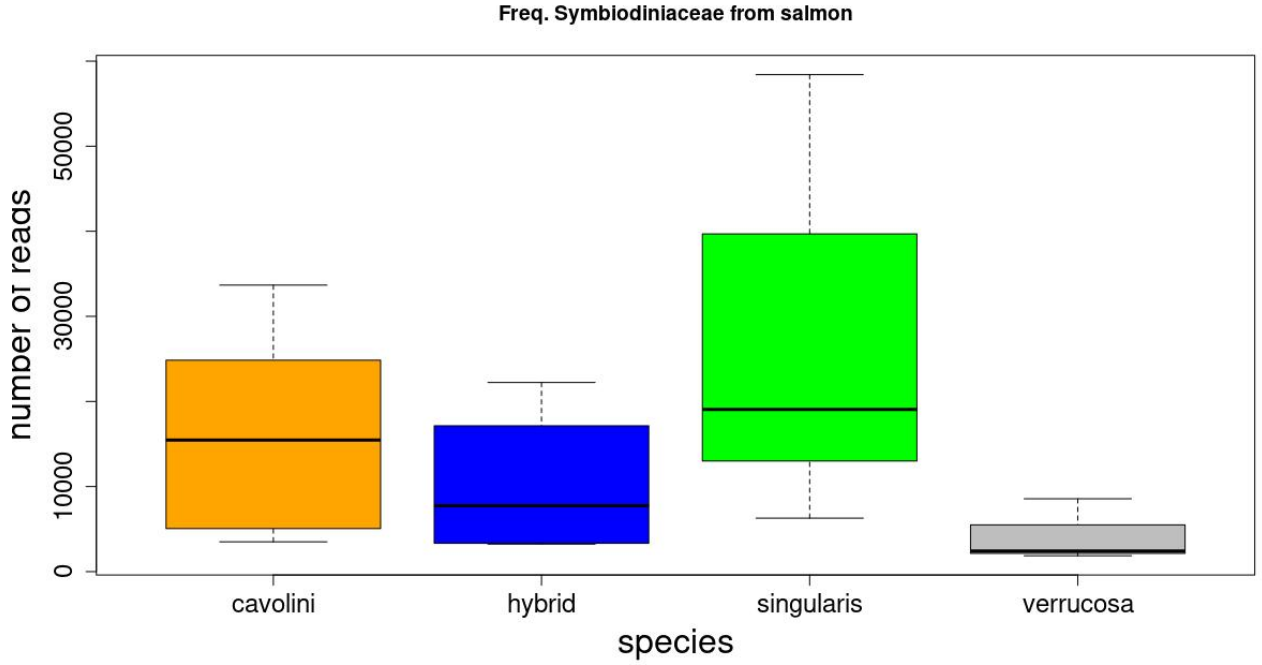

Eunicella_verrucosa_JQ397300
Eunicella_cavolinii_NC_035667
Eunicella_verrucosa_NC_073494
Eunicella_cavolini_JQ397292
Eunicella_cavolini_JQ397290
Eunicella_cavolini_JQ397291
Eunicella_verrucosa_JQ397305
Eunicella_singularis_KX051572
Eunicella_singularis_JQ397293
Eunicella_sp._JQ397311
Eunicella_verrucosa_JQ397306
Eunicella_sp._JQ397308
Eunicella_singularis_JQ397294
Eunicella_verrucosa_JQ397302
Eunicella_sp._JQ397307
Eunicella_singularis_KX051571
Eunicella_racemosa_KX051577
Eunicella_tricoronata_NC_062012
Eunicella_tricoronata_JX203795
Eunicella_albicans_NC_035666
Complexum_monodi_KP036906
Swiftia_pacifica_KX904973
Swiftia_simplex_KX905018
100
100
100
98
62
0.005

Figure S3: distribution of the frequency of Symbiodiniaceae sequences in the individual transcriptomes according to the species based A) on the number of reads estimated with Salmon, and B) on the proportion of assembled sequences with the BLAT analyses.

A) Read counts with Salmon: Mean values per group: *E. cavolini*: 16508; hybrids: 10238; *E. singularis*: 26023; *E. verrucosa*: 4285. Kruskal-Wallis test of the differences among groups: chi-squared = 7.9467, df = 3, p-value = 0.047.



B) Assembled sequences with BLAT: Mean values per group: *E. cavolini*: 0.0034; hybrids: 0.0029; *E. singularis*: 0.0219; *E. verrucosa*: 0.0028. Kruskal-Wallis test of the differences among groups: chi-squared = 14.352, df = 3, p-value = 0.002.

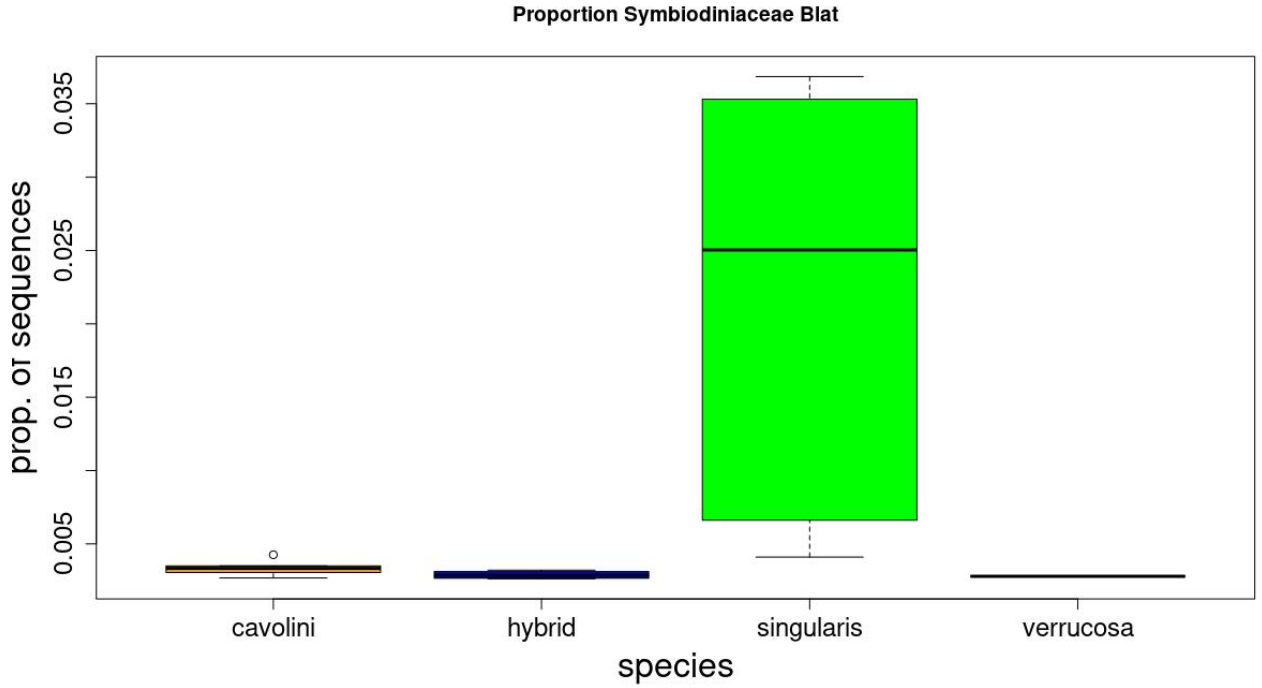
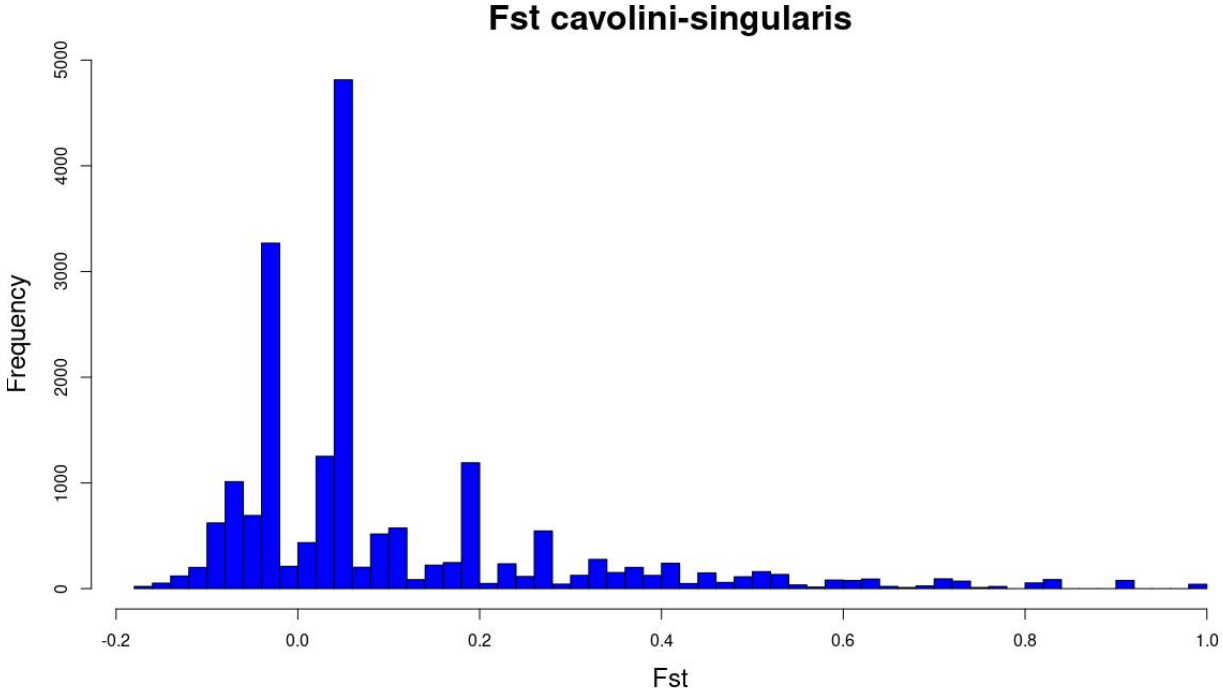
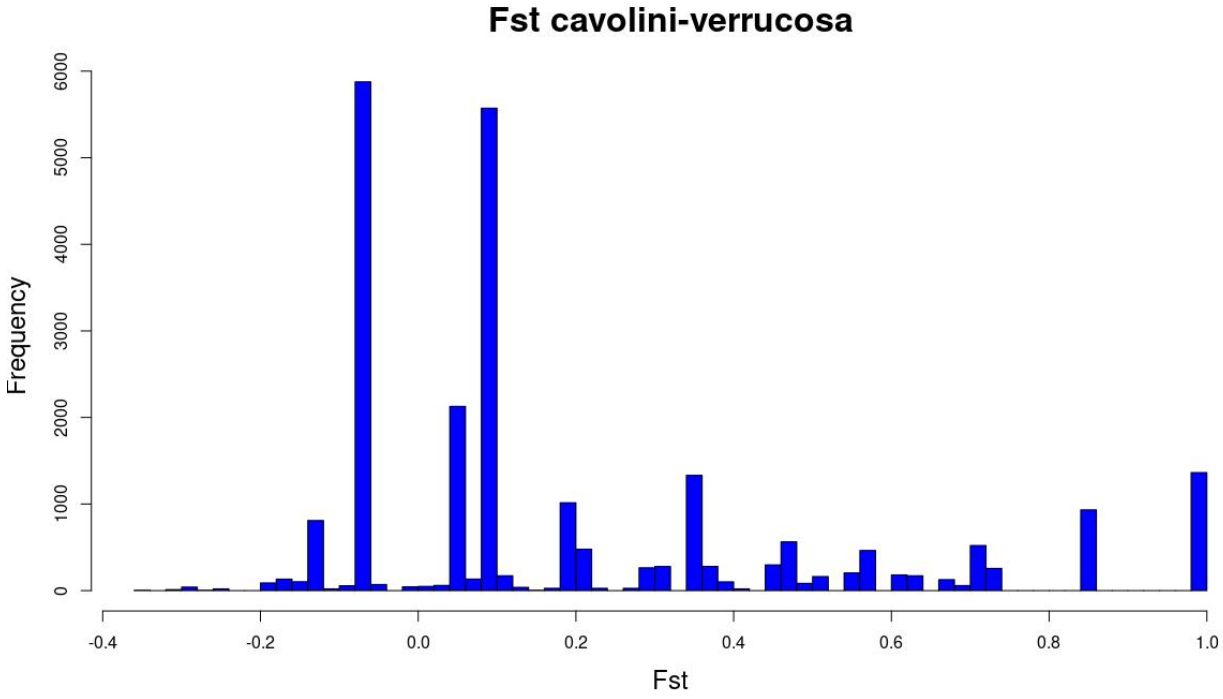


Figure S6: distribution of F_{ST} estimates over loci, for the pairwise comparisons among the three species, with the exclusion of potential hybrids.

A) comparison between *E. cavolini* and *E. singularis*



B) comparison between *E. cavolini* and *E. verrucosa*



C) comparison between *E.singularis* and *E. verrucosa*

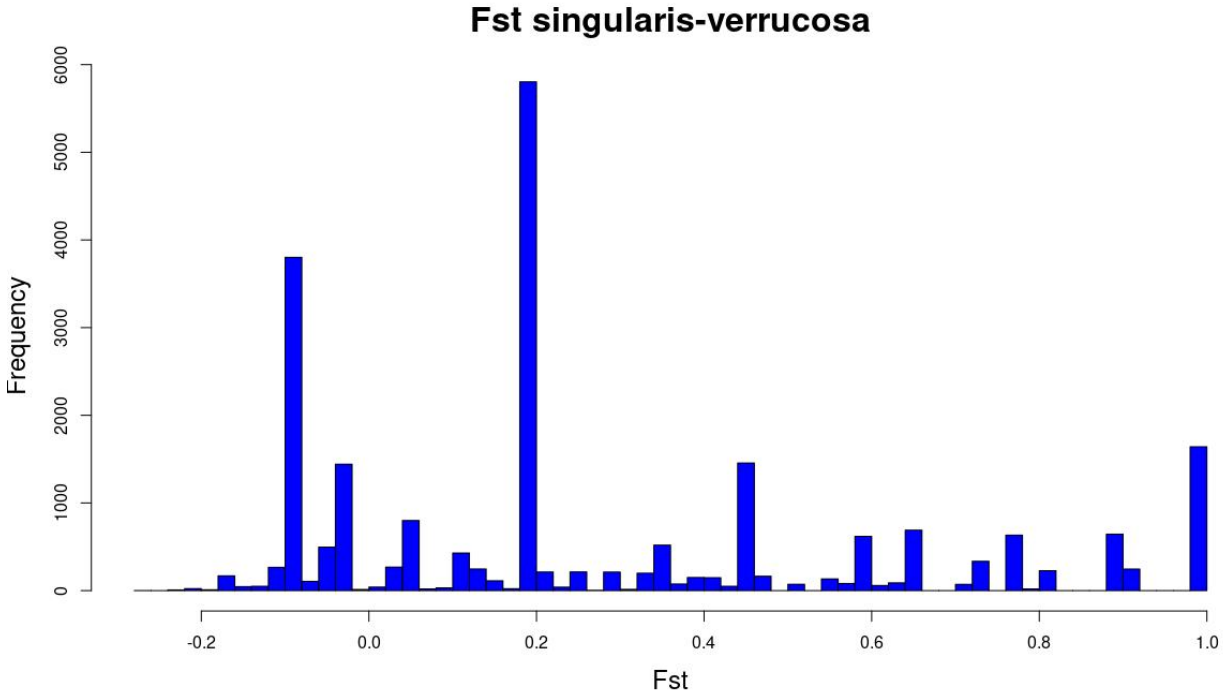


Figure S5: result of the cross-entropy analysis with the LEA R package

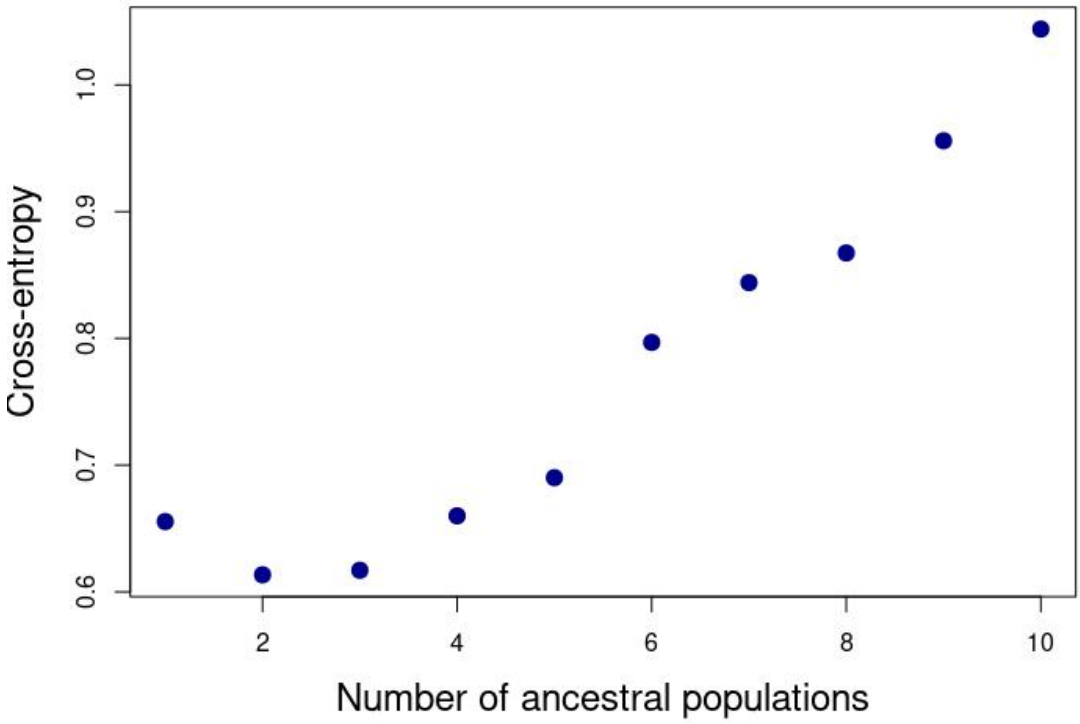
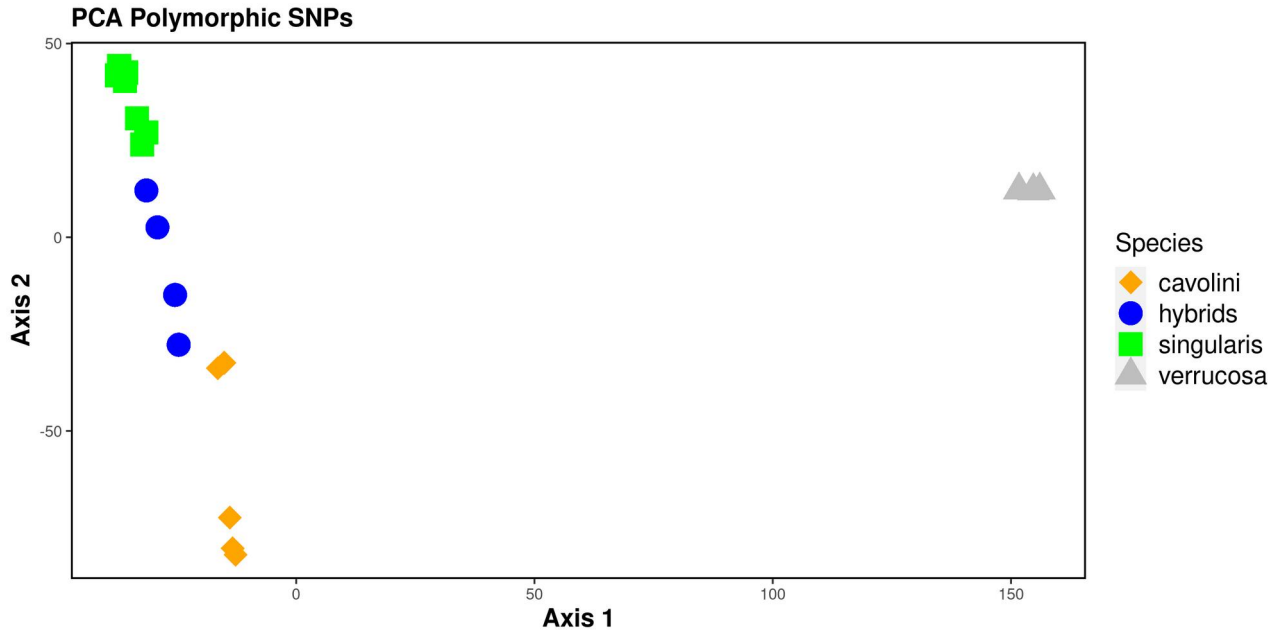


Figure S6: Principal Component Analysis based on the “polymorphic SNPs” dataset. The axis 1 corresponded to 33.2% of the variance, and axis 2 to 13% of the variance.



Symbiotic status does not preclude hybridisation in Mediterranean octocorals

Supplementary Material S2: 18S rDNA metabarcoding

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Objectives of the study

This section describes a preliminary sequencing test carried out to analyse the microeukaryotic community associated with gorgonians of the genus *Eunicella*.

Given that gorgonian host DNA accounts for the vast majority of DNA extracted from colonies, the detection of microeukaryotic diversity is challenging as their less abundant sequences are severely disadvantaged by PCR, which favours amplification of dominant matrices, here the ribosomal DNA (rDNA) of the host. To circumvent this problem, we tested a strategy that relies on the use of a blocking primer complementary to the gorgonian rDNA sequence to reduce the proportion of host amplicons. This approach has been reported, for example, in previous studies on coral-associated protists (Clerissi et al., 2018) and krill stomach contents (Vestheim & Jarman, 2008).

Methods

PCR amplification and metabarcoding of 18S rDNA

Using the 18S rDNA gorgonian sequences available in GenBank and one *Eunicella cavolini* sequence determined in the laboratory, we confirmed that the 18SV4 blocking primer of Clerissi et al. (2018) (5'-TCTTGATTAATGAAAACATTCTTGGC-3' modified with a C3 spacer at the 3' end) initially designed for scleractinian corals was also complementary to the octocorallia sequences.

We therefore tested the efficiency of amplification of microeukaryotes on DNA samples obtained from one colony of *E. singularis* and two colonies of *E. cavolini* sampled in Marseille Bay, using the blocking primer 18SV4 in combination with the primer pair 18SV4-F (5'-CCAGCASCYGCGGTAATTCC-3') and 18SV4-R (5'-ACTTTCGTTCTTGATYRA-3') (Stoek et al., 2010) targeting a fragment of approximately 420 base pairs in the V4 variable region of the 18S rRNA gene.

Gorgonian DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) and PCR reactions were performed according to the conditions of Clerissi et al. (2018), except that different concentration ratios between the blocking primer and the 18SV4 primers (1.5:1, 3:1, 5:1 and 10:1) were tested to optimise the proportion of microeukaryotic amplicons.

Sequencing of the final library of pooled amplicons was performed at the Génome Québec Centre of Expertise and Services (Montréal, Canada) on Illumina MiSeq platform using 2 x 250 bp v2 chemistry and following the manufacturer's guidelines.

Analysis of sequencing data

The FROGS pipeline v4.0 (Escudié et al., 2018) implemented in a Galaxy instance at GenoToul bioinformatics facility (Toulouse, France; https://bioinfo.genotoul.fr/) was used to align reads, remove chimera sequences, define Operational Taxonomic Units (OTUs), and to assign taxonomy based on the Silva 138.1 18S reference database (Quast et al., 2012).

For the phylogenetic analysis, the sequences were edited with ugene (Okonechnikov et al., 2012). The phylogenetic reconstructions were performed on a 376 bp alignment with the Maximum-Likelihood (ML) approach of IQ-TREE 2.1.1 (Nguyen et al., 2015). We used the ModelFinder option (Kalyaanamoorthy et al., 2017), and robustness was evaluated with 1000 ultrafast bootstraps (Hoang et al., 2018). The tree has been visualized with FigTree 1.4.4 (Rambaut, 2006) and rooted at mid-point.

Results

Idenfication of Symbiodiniaceae sequences in *E. singularis* and *E. cavolini*

Depending on the primer ratio used, the proportion of non-cnidarian 18S sequences reached up to 37.6% and 23.2% of the total sequences for *E. singularis* and *E. cavolini*, respectively. For both species, inhibition of host 18S rDNA gene amplification was most effective with the highest concentration of blocking primer.
In *E. singularis*, we identified 92 OTUs belonging to the family Symbiodiniaceae, in good agreement with the intracolonial diversity of zooxanthellae genotypes previously reported in this host species (Forcioli et al., 2011). Among these OTUs, a single OTU (OTU_7) was highly dominant and contributed up to 45.6% of the total Symbiodiniaceae abundance in the studied colony.
A small number of Symbiodiniaceae OTUs were detected in *E. cavolini* colonies (12 to 13 OTUs depending on the colony). Between the two colonies analysed, the proportion of Symbiodiniaceae sequences varied considerably, accounting for 0.21% to 2,3% of the non-cnidarian sequences when the blocking primer concentration was the highest. However, in both cases OTU_7 was the most abundant, representing up to 99% of all Symbiodiniaceae sequences. The sequence of this OTU_7 (381 bp in length) has been submitted to GenBank under reference SUB14400021.

Phylogenetic analysis

The 18S rDNA OTU_7 shared between *E. cavolini* and *E. singularis* was used for a Blast search in GenBank. In the list of Blast hits, we retained a subset of sequences corresponding to different levels of identity and to different clades of Symbiodiniaceae for phylogenetic reconstruction. The phylogenetic inference on these data produced a tree (Fig. S2.1) that allowed sequences belonging to clades A, B, C and D to be distinguished (most previously defined clades were recovered with more than 90% bootstrap support). According to the tree topology, the putative Symbiodiniaceae species associated with OTU_7 is sister to a clade containing *Symbiodinium microadriaticum*, *S.*

pilosum and other symbiotic *Symbiodinium* species belonging to Clade A (now corresponding to the *Philozoon* genus; Lajeunesse et al., 2022). The closest sequence to OTU_7 is from a dinoflagellate isolated from a South China Sea reef (accession MZ621018; to be released upon publication).

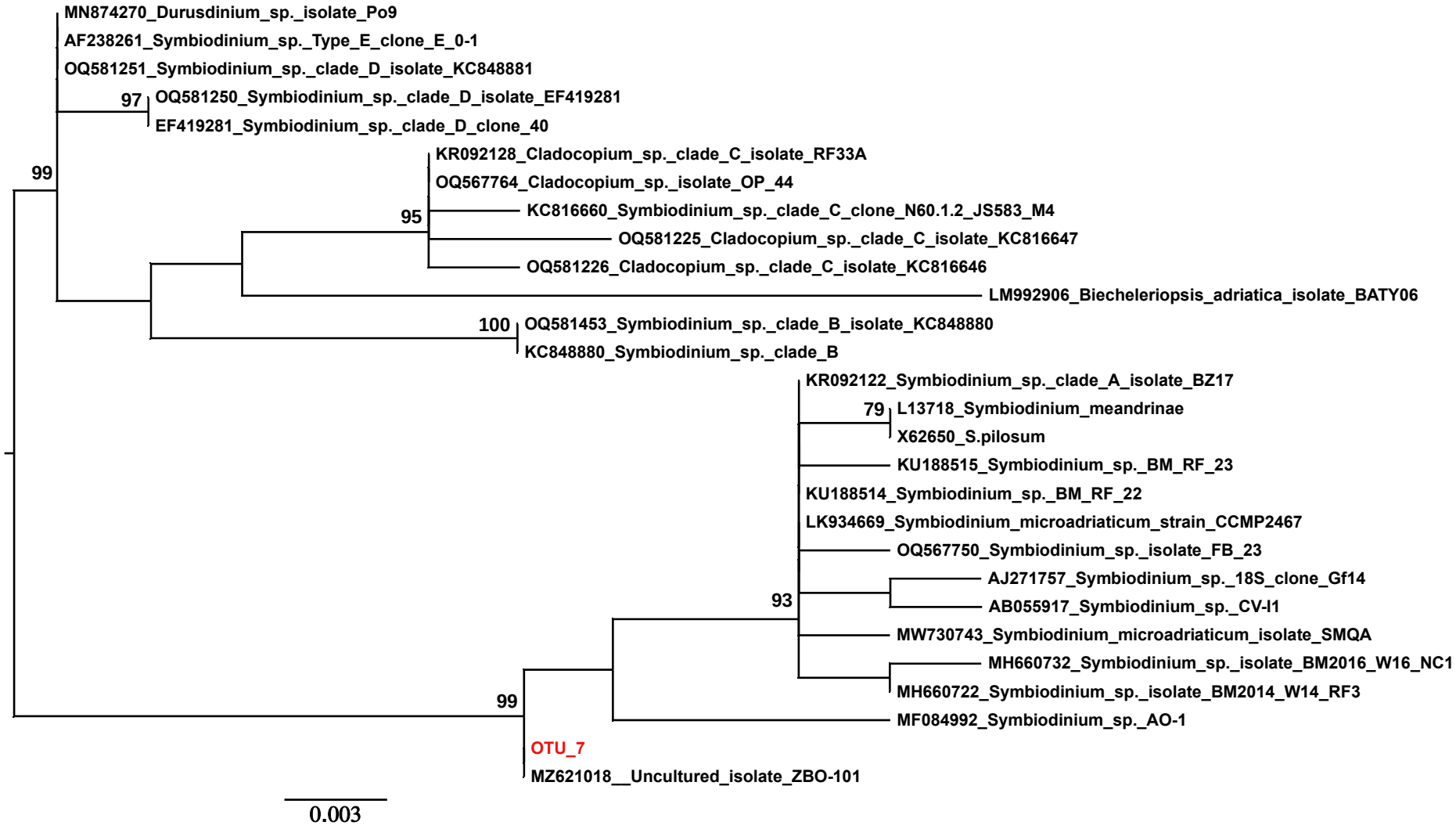

Figure S2.1. Maximum-likelihood phylogeny of Symbiodiniaceae based on the variable V4 region of the 18S rRNA gene, illustrating the relationship of OTU_7 sequence (in red) with Symbiodiniaceae spp. belonging to Clade A. The numbers to the left of the nodes indicate the percentages of bootstraps, for values superior to 75%. The first part of each sequence name corresponds to the accession number in GenBank.

References

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