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► **To cite this version:**

Didier Aurelle, Anne Haguenaue, Marc Bally, Frédéric Zuberer, Jean-Baptiste Ledoux, et al.. Symbiotic status does not preclude hybridisation in Mediterranean octocorals. 2024. hal-04568927

**HAL Id: hal-04568927**

**<https://hal.science/hal-04568927>**

Preprint submitted on 6 May 2024

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# 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 is 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

# 1 Introduction

2  
3 As corner stones of evolutionary biology, species and speciation still raise a  
4 wealth of questions fueled by the technological and conceptual  
5 advancements in genomics. Species can be defined as a part of a  
6 genealogical network, and such definition should be clearly distinguished  
7 from recognition criteria (Samadi & Barberousse, 2006). Genomic data, either  
8 from complete or partial representation of genomes, allow testing species  
9 hypotheses, understanding speciation scenarios, and go deeper in the  
10 analysis of interactions between biodiversity and environments. Species are  
11 indeed hypotheses to be tested, if possible through the integration of  
12 independent criteria, and independent molecular markers (Pante et al.,  
13 2015b). Sound species delimitation and identification is useful, among others,  
14 to better estimatespecies range and biodiversity patterns (Coelho et al.,  
15 2023; Muir et al., 2022), to avoid biases in connectivity studies (Pante et al.,  
16 2015b), and adaptive abilities (Brenner-Raffalli et al., 2022). Species  
17 delimitation can be problematic in the context of the grey zone of speciation,  
18 where different delimitation criteria may bring contradictory conclusions (De  
19 Queiroz, 2007). This is the case for cryptic species, i.e. entities assigned to  
20 the same nominal species though reproductively isolated (Cahill et al., 2023).  
21 Conversely, gene flow can be observed among morphologically differentiated  
22 entities, and this can be an important factor in adaptation and evolution  
23 (Leroy et al., 2020). The analysis of a high number of independent markers  
24 now provides the required analytical power for species delimitation in such  
25 difficult cases, for example when mitochondrial barcoding is not informative  
26 (McFadden et al., 2011). This wealth of information allows demographic  
27 inferences and testing speciation scenarios (De Jode et al., 2023; Roux et al.,  
28 2016). Incomplete speciation might still be inferred, and it's an interesting  
29 situation to study the speciation process itself (Roux et al., 2016).  
30 In the marine realm, the question of speciation was considered as particularly  
31 puzzling. From a fundamental point of view, understanding the drivers of  
32 speciation is not easy for species with high effective size, and important gene  
33 flow in a supposedly open environment (Faria et al., 2021; Mayr, 2001;  
34 Palumbi, 1992). Difficulties in sampling and rearing organisms also limits  
35 experiments to test reproductive isolation (Faria et al., 2021). Important  
36 progress has nevertheless been made in various marine organisms to better  
37 understand spatial patterns of genetic structure. One can use models of  
38 oceanographic connectivity to understand the observed genetic  
39 differentiation, and highlight potential barriers to gene flow (Reynes et al.,  
40 2021). Barriers to gene flow can also be inferred from clines in allele  
41 frequencies (Gagnaire et al., 2015). The observation of hybrid zones in  
42 marine species unveils heterogeneity in this environment, and shows the  
43 importance of the interactions between exogenous (i.e. dependent on  
44 different environments) and intrinsic (genetic incompatibilities) barriers to  
45 gene flow (Bierne et al., 2011). Some marine species also provided important  
46 models to study the role of gametes recognition in reproductive isolation  
47 (Palumbi, 1999).  
48 In this context, a poorly investigated topics remains the role of symbiotic  
49 interaction in speciation. There are various examples of the involvement of  
50 microbial species in reproductive isolation, especially in insects (Brucker &  
51 Bordenstein, 2012). For marine species, the role of microbial communities has

52 been mainly explored at the light of adaptative evolution, with the concept of  
53 hologenome which comprises the genomes of the host and associated  
54 microbiome (Rosenberg & Zilber-Rosenberg, 2018). Shallow water  
55 scleractinian corals (hexacorals) are usually associated with photosynthetic  
56 zooxanthellae (Cairns, 2007). These zooxanthellae, of the Symbiodiniaceae  
57 family, correspond to different genera and species (Lajeunesse et al., 2018).  
58 Changes in associated Symbiodiniaceae can impact the thermotolerance of  
59 the coral holobiont, and the possibility of adaptation facing climate change  
60 (Berkelmans & van Oppen, 2006; van Oppen & Medina, 2020). Inferences  
61 from the phylogeny of scleractinian corals have shown multiple acquisitions  
62 of the symbiotic state, but there is still an important diversity of non  
63 symbiotic corals (Cairns, 2007; Campoy et al., 2020). Despite the diversity of  
64 Symbiodiniaceae, the diversity in symbiotic state (presence / absence,  
65 species diversity), and the possibility of shifting at different evolutionary  
66 scales, there is no information on the potential involvement of these  
67 symbionts in reproductive isolation, or even on the possibility of gene flow  
68 among species with different symbiotic state. The symbiotic interactions  
69 between Anthozoans (hexacorals and octacorals) and Symbiodiniaceae  
70 presents important mutualistic benefits especially from a nutritional point of  
71 view (Furla et al., 2005). This interaction requires specific adaptations for the  
72 animal host, as for example protection against oxygen produced by  
73 photosynthesis (Furla et al., 2005). The association with Symbiodiniaceae can  
74 also range from mutualism to parasitism (Lesser et al., 2013; Sachs & Wilcox,  
75 2006). Therefore, one can envision that hybrids could be impaired by a  
76 modification in host - symbiont interactions. The presence or type of  
77 Symbiodiniaceae could also be involved in genetic incompatibilities with the  
78 host genome, as previously observed with bacterial species (Bordenstein,  
79 2003; Brucker & Bordenstein, 2012).

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

103

## 104 **Material and methods**

### 105 **Species distribution**

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

131

### 132 **Sampling**

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

145

### 146 **Phylogenetic relationships**

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

153

### 154 **Transcriptome sequencing and assembly**

155 Total RNA has been extracted as in Haguenaer et al. (2013). RNAs were sent  
156 to the LIGAN genomic platform for sequencing (Lille, France) on four flow cells  
157 of Illumina NextSeq 500 (2 x 75 bp). The transcriptomes have been  
158 assembled with the de novo RNA-Seq Assembly Pipeline (DRAP ; Cabau et al.,  
159 2017) with Oases (Schulz et al., 2012) and default parameters. We performed  
160 an individual assembly, and a meta-assembly to be used as reference. The  
161 statistics describing the assembled transcriptomes are given in  
162 supplementary material, Table S2.

163 We used the BLAT software (Kent, 2002) to remove potential  
164 Symbiodiniaceae sequences in the obtained transcriptomes. We used the  
165 transcriptome of the type A1 *Symbiodinium* (Genbank accession number  
166 GAKY01000000) (Baumgarten et al., 2013) to search for Symbiodiniaceae  
167 sequences separately in individual *Eunicella* transcriptomes. The output of  
168 BLAT was analysed with the `blat_parser.pl` script.

169

### 170 **Analysis of the presence of Symbiodiniaceae**

171 We analysed the presence of Symbiodiniaceae in *Eunicella* gorgonians in two  
172 ways. First, we counted the number of reads corresponding to the  
173 aforementioned Symbiodiniaceae transcriptome with Salmon (Patro et al.,  
174 2017). Second, we used the percentage of assembled sequences (i.e.  
175 contigs) in the *Eunicella* transcriptomes corresponding to Symbiodiniaceae  
176 following the BLAT analysis. We used a Kruskal-Wallis test in R to test for  
177 differences among the four groups of samples (the three *Eunicella* species  
178 and the potential hybrids) for each metric. Additionally, we performed a blast  
179 analysis with the LSU, ITS and psbA sequences of *Philozoon* (Lajeunesse et  
180 al., 2022) on individual transcriptomes to try to identify the Symbiodiniaceae  
181 genera present in the different samples.

182 As our results pointed to the potential and unexpected presence of  
183 Symbiodiniaceae in *E. cavolini* (see Results), we wanted to explore this topic  
184 further by using the preliminary data from another experiment dedicated to  
185 studying the microbiome of *E. cavolini* and *E. singularis*. This pilot study  
186 involved an analysis of microeukaryotic communities through 18S rDNA  
187 metabarcoding on two colonies of *E. cavolini*, and one *E. singularis*. This  
188 experiment and its results are presented in Supplementary File S2.

189

### 190 **SNPs calling and filtering**

191 We produced bam files by mapping the reads with `bwa` option `mem` (Li &  
192 Durbin, 2009). We used as reference the meta transcriptome filtered for  
193 Symbiodiniaceae sequences. The obtained sam files were converted in bam  
194 format with `samtools` 1.9 (Li et al., 2009), and sorted with `Picard tools` (`Picard`  
195 `Toolkit`, 2019). The SNPs calling has been performed with `reads2snp` 2.0 with  
196 default parameters (Gayral et al., 2013; Tsagkogeorga et al., 2012). The  
197 obtained dataset, including variable and non variable sites, will thereafter be  
198 referred as the “all sites” dataset. We performed separate SNP calls with  
199 `reads2snp` for pairwise comparisons among species and without the potential  
200 hybrid samples. These three datasets have been used for demographic  
201 inferences (see below), and will be referred as “all-CS” for the *cavolini* /  
202 *singularis* comparison, “all-CV” for the *cavolini* / *verrucosa* comparison, and  
203 “all-SV” for the *singularis* / *verrucosa* comparison.

204 We filtered the “all sites” `vcf` file obtained with `reads2snp` with `vcftools`  
205 (Danecek et al., 2011) for an analysis of genetic diversity and differentiation.  
206 We only retained biallelic sites which have been genotyped in all individuals

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

215

### 216 **Genetic differentiation and analysis of hybrids**

217 We analysed the genetic structure and differentiation among species with the  
218 “polymorphic sites” dataset. We used the LEA R package to estimate  
219 ancestry coefficients (Frichot et al., 2014; Frichot & François, 2015). We  
220 tested K values from 1 to 10, with 10 replicates for each K. To analyse the  
221 genetic differences among individuals, we performed a Principal Component  
222 Analysis (PCA) with the R package adegenet. The pairwise  $F_{ST}$  among species  
223 were computed with the R package Genepop (Rousset, 2008; Rousset et al.,  
224 2020), after conversion of the vcf file with PGDSpider (Lischer & Excoffier,  
225 2012). We present here the  $F_{ST}$  estimates of Weir & Cockerham (1984). The  
226 distribution of  $F_{ST}$  among loci was obtained with vcftools.

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

242

### 243 **Scenarios of speciation**

244 We tested scenarios of speciation with the Demographic Inferences with Linked  
245 Selection (DILS) pipeline (Csilléry et al., 2012; Fraïsse et al., 2021; Pudlo et al.,  
246 2016). The DILS pipeline allows the analysis of two species scenarios only: we  
247 therefore performed separate analyses for the three two-species  
248 comparisons, with the “all-CS”, “all-CV”, and “all-SV” pairwise datasets. We  
249 first tested the priors proposed by Monnet et al. (2023) according to observed  
250 statistics of polymorphism and divergence: we then increased the range of  
251 priors to get better results on the goodness-of-fit tests. We used the same  
252 priors for all analyses, with different numbers of sequences per gene and per  
253 sample according to the dataset (Table S4). For all pairwise comparisons, we  
254 performed two DILS analyses: one with constant population sizes, and one  
255 with variable population sizes.

256

## 257 **Results**



## 258 **Phylogenetic relationships**

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

## 266 **Presence of Symbiodiniaceae**

268 The analysed samples showed low numbers of reads counts for the  
269 Symbiodiniaceae transcriptome (between 1868 and 58406 reads; Table S5).  
270 The proportion of assembled sequences corresponding to Symbiodiniaceae  
271 with BLAT was also very low (between 0.00276 and 0.03686; Table S5).  
272 Significant differences were observed among species in both cases (Kruskal-  
273 Wallis test,  $p = 0.047$  for reads counts, and  $p = 0.002$  for the proportions of  
274 assembled sequences). The pairwise Wilcoxon-Test showed significant  
275 differences only for the comparisons of proportions of assembled sequences  
276 involving *E. singularis* (Table S6; Figure S3). The frequency of assembled  
277 sequences corresponding to Symbiodiniaceae was higher in *E. singularis*  
278 compared to other species: in *E. singularis*, it varied between 0.004 and  
279 0.037 (mean = 0.022), whereas it reached 0.004 in one sample of *E. cavolini*  
280 and it was lower than 0.004 in all other non-*singularis* samples. The mean  
281 values of reads counts and assembled sequences in the hybrids were lower  
282 than in *E. cavolini* but higher than in *E. verrucosa*; the corresponding pairwise  
283 tests were not significant.

284 The blast analysis with the LSU, ITS and psbA sequences of *Philozoon* only  
285 retrieved some sequences in the transcriptomes of *E. singularis*: no hit was  
286 observed in the other samples. Regarding the pilot study of 18S rDNA  
287 metabarcoding, a diversity of 92 Operational Taxonomic Units (OTUs)  
288 corresponding to Symbiodiniaceae was observed in *E. singularis*, with a single  
289 OTU largely dominant in abundance. The same OTU was also observed in *E.*  
290 *cavolini* with a low abundance of reads, but still representing 99% of all 12 to  
291 13 Symbiodiniaceae OTUs detected in the two analysed colonies. A blast  
292 analysis of this shared OTU on Genbank indicated that it was phylogenetically  
293 related to the clade A of Symbiodiniaceae (Supplementary file S2).

## 294 **Genetic differentiation and analysis of hybrids**

295 We obtained 31369 SNPs for the “polymorphic sites” dataset. With this  
296 dataset, the highest  $F_{ST}$  values were observed for the comparisons between  
297 *E. verrucosa* and all other samples ( $F_{ST} > 0.43$ ; table S7). The  $F_{ST}$  between *E.*  
298 *cavolini* and *E. singularis* was much lower (0.21), and the lowest  $F_{ST}$  values  
299 were observed for hybrids compared to these two species ( $F_{ST}$  around 0.07 in  
300 both case). These differences corresponded to different distributions of  $F_{ST}$   
301 over SNPs for the three inter-specific comparisons (Figure S4). Both  
302 comparisons with *E. verrucosa* involved a more heterogeneous distribution of  
303  $F_{ST}$ , and more SNPs reaching the maximum estimate of 1 (1364 SNPs for  
304 *E. cavolini* vs *E. verrucosa*, and 1641 SNPs for *E. singularis* vs *E. verrucosa*),  
305 than for the comparison between *E. cavolini* and *E. singularis* ( $F_{ST} = 1$  for 41  
306 SNPs). If we consider the 1% SNPs with the highest  $F_{ST}$  estimates, 52 were  
307 shared by both comparisons involving *E. cavolini* (i.e. *E. cavolini* vs  
308 *E. singularis* and *E. cavolini* vs *E. verrucosa*), amounting to 0.17% of all SNPs,  
309

310 116 top 1% SNPs were shared by both comparisons involving *E. singularis*  
311 (0.37% of all SNPs), and 1042 top 1% SNPs were shared by both comparisons  
312 involving *E. verrucosa* (3.32% of all SNPs). All the results point to the higher  
313 differentiation of *E. verrucosa* compared to the two other species, with more  
314 outlier SNPs for the divergence of this species.

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

332 The PCA first separated *E. verrucosa* from other samples on the first axis  
333 (33.2% of variance; Figure S6). The second axis (13% of variance) separated  
334 *E. cavolini* and *E. singularis*, with the potential hybrids in intermediate  
335 position between them.

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

345

### 346 **Scenarios of speciation**

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

362 support ( $p \geq 0.99$ ) for all analyses. In the case of current migration (between  
363 *E. cavolini* and *E. singularis*), a genomic heterogeneity in migration rates was  
364 inferred ( $p \geq 0.82$ ). The inferred parameters for the different scenarios are  
365 presented in Supplementary Table S8. We will first present the results  
366 obtained for the constant population sizes models. The divergence time  
367 between *E. cavolini* and *E. singularis* (median 403 273 generations) was  
368 much lower than between *E. cavolini* and *E. verrucosa* (median 1 054 488  
369 generations), and between *E. singularis* and *E. verrucosa* (median 899 098  
370 generations). For the comparison between *E. cavolini* and *E. singularis*, the  
371 time of secondary contact was estimated at 62 039 generations (median  
372 estimate), which translates in around 85% of time in isolation since  
373 divergence. Following secondary contact, the gene flow was similar in both  
374 directions for these two species. The duration of ancestral migration roughly  
375 corresponded to 6% and 8% of the total time since divergence for the  
376 comparison between *E. cavolini* and *E. verrucosa*, and for the comparison  
377 between *E. singularis* and *E. verrucosa*, respectively. For these last two cases,  
378 the gene flow (forward in time) during ancestral migration was higher  
379 towards *E. verrucosa* than in the opposite direction. Regarding effective size,  
380 the comparisons all indicated lower values for *E. singularis* compared to both  
381 other species (around 200 000 to 300 000 for *E. singularis*, around 600 000 to  
382 750 000 for both other species). The estimated effective sizes were of similar  
383 order for *E. cavolini* and *E. verrucosa*. This aligns well with the observed  
384 estimates of nucleotide diversity around 0.007-0.009 for *E. cavolini*, 0.005-  
385 0.006 for *E. singularis*, and 0.007-0.009 for *E. verrucosa* (variations depend  
386 on the comparison which may change the retained dataset; Supplementary  
387 File S3). Similar results were obtained for the models including variations in  
388 effective size, except for the estimate of current gene flow between  
389 *E. cavolini* and *E. singularis*: with variable population size, gene flow from  
390 *E. singularis* to *E. cavolini* was higher than in the opposite direction.

## 391 **Discussion**

### 392 **Species relationships and differentiation**

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

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

420

### 421 **Speciation scenarios**

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

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

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

472

### 473 **Hybridisation and species barriers**

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

492 The alternation of parental populations with mixed populations would point to  
493 a mosaic hybrid zone (Bierne et al., 2003), where hybrids could form in  
494 different areas and from different genetic compositions of parental species.  
495 As hybridization between *E. cavolini* and *E. singularis* had not been reported  
496 before, the presence of hybrids has probably been overlooked up to now.  
497 Communicating on this subject towards scientists and diving associations  
498 might help sampling other potential hybrid zones: this would be useful to  
499 analyse the spatial and ecological distribution of hybrids. One interesting  
500 question in this context is whether changes in selection regimes induced by  
501 human activities can change the outcome of hybridization (Ålund et al.,  
502 2023). For example, Mediterranean octocorals are impacted by mortality  
503 events linked with climate change (Estaque et al., 2023; Sini et al., 2015),  
504 and it would be interesting to compare the thermotolerance of hybrids and  
505 parental individuals. In scleractinian corals, interspecific hybridisation has  
506 been reported to enhance the survival under elevated temperature conditions  
507 (Chan et al., 2018).

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

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

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

533

### 534 **Evolution of symbiosis**

535 As previously discussed, we clearly demonstrated here the possibility of gene  
536 flow between a symbiotic (i.e. hosting Symbiodiniaceae) and a non-symbiotic  
537 octocoral species: the hybrid status then does not lead to complete isolation,  
538 even if Symbiodiniaceae could be involved in genetic incompatibilities. The  
539 methods used here did not aim at a precise quantification of  
540 Symbiodiniaceae, and one can note the general low levels of sequences  
541 corresponding to these symbionts, even in *E. singularis*: this may be due to  
542 difficulties in extracting the RNA of the symbionts with this protocol or to loss  
543 of symbionts before extractions. This is not a general result: for example, a  
544 whole transcriptome sequencing of the octocorals *Heliopora coerulea* and  
545 *Briareum asbestinum* recovered 29% and 17.2% of Symbiodiniaceae  
546 sequences respectively (Guzman et al., 2018; Rivera-Garc a et al., 2019).  
547 Despite these limits we observed a clear signal of higher Symbiodiniaceae  
548 concentration in *E. singularis* than in *E. cavolini* and *E. verrucosa*.  
549 Interestingly, the hybrids showed a lower frequency of Symbiodiniaceae than  
550 *E. singularis*, and possibly than *E. cavolini*, though this last result remains to  
551 be tested with more samples. These results indicate a breakdown of  
552 symbiosis following hybridization with potential consequences on the fitness  
553 of hybrids. In *E. singularis*, the transmission of Symbiodiniaceae seems to  
554 occur both vertically, through ovules, and horizontally, from the environment  
555 (Forcioli et al., 2011). Vertical transmission may change the fate of hybrids  
556 depending on the species of the mother, as it can change the initial load in  
557 Symbiodiniaceae: this hypothesis is nevertheless difficult to test as these  
558 species can not be reproduced in aquarium. More generally, the question of  
559 the link between symbiosis and the fitness of hybrids would require a  
560 dedicated study involving more precise estimates of Symbiodiniaceae  
561 abundance (e.g. with quantitative PCR), and of physiological parameters such  
562 as photosynthetic and respiration rates in controlled conditions (Ezzat et al.,  
563 2013).

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

569 reverse direction (Campoy et al., 2020). This could indicate that investing in  
570 such mutualistic interactions for the cnidarian would lead to increasingly  
571 relying on autotrophy for energetic supply, making reversal to heterotrophy  
572 difficult. In octocorals, an evolutionary versatility in symbiotic state seems  
573 possible, as in various families and genera, both symbiotic and non-symbiotic  
574 species are present (Van Oppen et al., 2005). In the Mediterranean Sea, all  
575 octocoral species are non-symbiotic, except for *E. singularis*. The most  
576 parsimonious scenario here would be an acquisition of symbiosis in  
577 *E. singularis* during or following its divergence from *E. cavolini*. The symbiotic  
578 status of *E. singularis* nevertheless could be facultative as non-symbiotic  
579 colonies of *E. singularis* have been observed between 40 and 60 m depths  
580 (Gori et al., 2012). Additionally, experimental physiological studies have  
581 demonstrated the nutritional plasticity of *E. singularis* which is able to use  
582 either heterotrophy or autotrophy for its metabolism (Ezzat et al., 2013).  
583 Nevertheless, in natural conditions, autotrophy seems to provide an  
584 important contribution to the metabolism of *E. singularis*, and the collapse of  
585 photosynthetic capacities in too warm conditions could contribute to  
586 mortality events in this species (Coma et al., 2015). The question of  
587 symbiosis could be reversed as well: why don't we observe any symbiosis  
588 with Symbiodiniaceae in *E. cavolini*? This species can be observed in shallow  
589 conditions (less than 10 m depth) where there is enough light for  
590 photosynthesis, and in syntopy with *E. singularis*. The availability of preys or  
591 particulate organic matter may provide enough energy to *E. cavolini* in its  
592 habitat, but this species may have never engaged in mutualistic interaction  
593 with Symbiodiniaceae. Interestingly we observed a low rate of sequences  
594 related to Symbiodiniaceae in the transcriptomes of *E. cavolini* (and even  
595 lower, but not null in *E. verrucosa*). This could either correspond to a signal  
596 from free living Symbiodiniaceae, or to background, transient, associations  
597 with the cnidarian. In addition, a Symbiodiniaceae OTU that is common to  
598 *E. singularis* and *E. cavolini* was identified among the microeukaryotes  
599 associated with the two species, which probably corresponds to the  
600 Symbiodiniaceae species symbiotic with *E. singularis*., and is related to  
601 strains observed in symbiosis with other cnidarians. Rare Symbiodiniaceae  
602 strains can also be observed in symbiotic hexacorals, probably with low  
603 impact on the hosts physiology (Lee et al., 2016). Molecular markers also  
604 allowed to evidence the presence of Symbiodiniaceae in species previously  
605 supposed to be asymbiotic, as in the Mediterranean octocoral *Paramuricea*  
606 *clavata*, and in several Hawaiian antipatharian species (Bonacolta et al.,  
607 2024; Wagner et al., 2011). These results, and our observations in *Eunicella*  
608 species, obviously underline the dynamic nature of interactions between  
609 Symbiodiniaceae and cnidarians: the establishment of these symbiotic  
610 interactions may be preceded by various types of interactions, more or less  
611 stable, and more or less mutualistic. The development of effective symbiosis,  
612 with stable relationships, and higher abundance of symbiont, would require  
613 specific adaptation from both partners. We can see here that even if on a  
614 macro-evolutionary scale, the acquisition of symbiosis is much more frequent  
615 than its loss, on a micro-evolutionary scale the gene flow between the  
616 *Eunicella* species analysed here did not lead to the full development of  
617 symbiosis in *E. cavolini*.

618  
619

## Conclusions and perspectives

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

635

### 636 **Acknowledgements:**

637 We thank the ECCOREV Research Federation (FR 3098) for the financial  
638 support of part of this study (<https://www.eccorev.fr/>). The project leading to  
639 this publication has received funding from European FEDER Fund under  
640 project 1166-39417. The project leading to this publication has received  
641 funding from Excellence Initiative of Aix-Marseille University - A\*MIDEX, a  
642 French "Investissements d'Avenir" programme. The authors thank the UMR  
643 8199 LIGAN-PM Genomics platform (Lille, France, especially Véronique  
644 Dhennin) which belongs to the 'Federation de Recherche' 3508 Labex EGID  
645 (European Genomics Institute for Diabetes; ANR-10-LABX-46) and was  
646 supported by the ANR Equipex 2010 session (ANR-10-EQPX-07-01; 'LIGAN-  
647 PM'). The LIGAN-PM Genomics platform (Lille, France) is also supported by the  
648 FEDER and the Region Nord-Pas-de-Calais-Picardie. JBL was supported by the  
649 strategic funding UIDB/04423/2020, UIDP/04423/2020 and  
650 2021.00855.CEECIND through national funds provided by FCT -Fundação para  
651 a Ciência e a Tecnologia. Camille Roux, Jonathan Romiguiier and Christelle  
652 Fraïsse were of a great help for the analysis of speciation scenarios. We thank  
653 the SCUBA diver team of INSU/OSU Pytheas (Dorian Guillemain) for their help  
654 during the fieldwork. We acknowledge the staff of the "Cluster de calcul  
655 intensif HPC" Platform of the OSU Institut Pythéas (Aix-Marseille Université,  
656 INSU-CNRS) for providing the computing facilities. We are grateful to the  
657 Genotoul bioinformatics platform Toulouse Occitanie (Bioinfo Genotoul,  
658 <https://doi.org/10.15454/1.5572369328961167E12>) for providing help,  
659 computing and storage resources. We thank Christophe Klopp for his help. We  
660 acknowledge the use of the computing cluster of MNHN (Plateforme de Calcul  
661 Intensif et Algorithmique PCIA, Muséum National d'Histoire Naturelle, Centre  
662 national de la recherche scientifique, UAR 2700 2AD, CP 26, 57 rue Cuvier, F-  
663 75231 Paris Cedex 05, France). Part of the bioinformatics analyses have been  
664 performed on the Core Cluster of the Institut Français de Bioinformatique  
665 (IFB) (ANR-11-INBS-0013). Part of this work has been performed during a  
666 CNRS detachment position of D. Aurelle at the ISYEB laboratory.

667

668

### 669 **Data availability**



670 The raw sequences are available in Genbank under BioProject ID  
671 PRJNA1037721 and SRA accession number SRA PRJNA1037721.  
672 The scripts used in this study are available at  
673 <https://doi.org/10.5281/zenodo.10966625>  
674

### 675 **Conflict of interest disclosure**

676 The authors declare that they comply with the PCI rule of having no financial  
677 conflicts of interest in relation to the content of the article  
678

### 679 **References**

- 680  
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682
- Ålund, M., Cenzer, M., Bierne, N., Boughman, J. W., Cerca, J., Comerford, M. S., Culicchi, A., Langerhans, B., McFarlane, S. E., & Möst, M. H. (2023). Anthropogenic Change and the Process of Speciation. *Cold Spring Harbor Perspectives in Biology*, 15(12), a041455.
- Anderson, E., & Thompson, E. A. (2002). A model-based method for identifying species hybrids using multilocus genetic data. *Genetics*, 160(3), 1217–1229.
- Arnold, M. L., Bulger, M. R., Burke, J. M., Hempel, A. L., & Williams, J. H. (1999). Natural hybridization: How low can you go and still be important? *Ecology*, 80(2), 371–381.
- Aurelle, D., Haguenaer, A., Blaise, C., Reynes, L., Arnaud-Haond, S., Boavida, J., Cabau, C., Klopp, C., Lundalv, T., Noûs, C., Sartoretto, S., Wienberg, C., Jiménez, C. E., & Orejas, C. (2024). On the specific status of eastern Mediterranean Dendrophyllia corals (Cnidaria, Anthozoa): Genetic characterization and speciation scenarios. *Zoologica Scripta*, 53(2), 235–247. <https://doi.org/10.1111/zsc.12643>
- Aurelle, D., Pivotto, I. D., Malfant, M., Topcu, N. E., Masmoudi, M. B., Chaoui, L., Kara, H. M., Coelho, M. A., Castilho, R., & Haguenaer, A. (2017). Fuzzy species limits in Mediterranean gorgonians (Cnidaria, Octocorallia): Inferences on speciation processes. *Zoologica Scripta*, 46(6), 767–778.
- Baumgarten, S., Bayer, T., Aranda, M., Liew, Y. J., Carr, A., Micklem, G., & Voolstra, C. R. (2013). Integrating microRNA and mRNA expression profiling in *Symbiodinium microadriaticum*, a dinoflagellate symbiont of reef-building corals. *BMC Genomics*, 14(1), 704. <https://doi.org/10.1186/1471-2164-14-704>
- Berkelmans, R., & van Oppen, M. J. H. (2006). The role of zooxanthellae in the thermal tolerance of corals: A “nugget of hope” for coral reefs in an era of climate change. *Proceedings of the Royal Society B: Biological Sciences*, 273(1599), 2305–2312.
- Bierne, N., Borsa, P., Daguin, C., Jollivet, D., Viard, F., Bonhomme, F., & David, P. (2003). Introgression patterns in the mosaic hybrid zone between *Mytilus edulis* and *M. galloprovincialis*. *Molecular Ecology*, 12, 447–462.
- Bierne, N., Welch, J., Loire, E., Bonhomme, F., & David, P. (2011). The coupling hypothesis: Why genome scans may fail to map local adaptation genes. *Molecular Ecology*, 20(10), 2044–2072.
- Bonacolta, A. M., Miravall, J., Gómez-Gras, D., Ledoux, J., López-Sendino, P., Garrabou, J., Massana, R., & Del Campo, J. (2024). Differential apicomplexan presence predicts thermal stress mortality in the Mediterranean coral *Paramuricea clavata*. *Environmental Microbiology*, 26(1), e16548.

- Bordenstein, S. (2003). Symbiosis And The Origin Of Species. In *Insect Symbiosis* (pp. 283–303). CRC Press.
- Brener-Raffalli, K., Vidal-Dupirol, J., Adjeroud, M., Rey, O., Romans, P., Bonhomme, F., Pratlong, M., Haguenaue, A., Pillot, R., Feuillassier, L., Claereboudt, M., Magalon, H., Gélín, P., Pontarotti, P., Aurelle, D., Mitta, G., & Toulza, E. (2022). Gene expression plasticity and frontloading promote thermotolerance in *Pocillopora* corals. *Peer Community Journal*, 2. <https://doi.org/10.24072/pcjournal.79>
- Brucker, R. M., & Bordenstein, S. R. (2012). Review: Speciation by symbiosis. *Trends in Ecology & Evolution*, 27(8), 443. ScienceDirect. <https://doi.org/10.1016/j.tree.2012.03.011>
- Cabau, C., Escudié, F., Djari, A., Guiguen, Y., Bobe, J., & Klopp, C. (2017). Compacting and correcting Trinity and Oases RNA-Seq de novo assemblies. *PeerJ*, 5, e2988.
- Cahill, A. E., Megléc, E., & Chenuil, A. (2023). Scientific history, biogeography, and biological traits predict presence of cryptic or overlooked species. *Biological Reviews*, 99, 546-561. <https://doi.org/10.1111/brv.13034>
- Cairns, S. D. (2007). Deep-water corals: An overview with special reference to diversity and distribution of deep-water scleractinian corals. *Bulletin of Marine Science*, 81(3), 311–322.
- Calderon, I., Garrabou, J., & Aurelle, D. (2006). Evaluation of the utility of COI and ITS markers as tools for population genetic studies of temperate gorgonians. *Journal of Experimental Marine Biology and Ecology*, 336, 184–197.
- Campoy, A. N., Addamo, A. M., Machordom, A., Meade, A., Rivadeneira, M. M., Hernández, C. E., & Venditti, C. (2020). The Origin and Correlated Evolution of Symbiosis and Coloniality in Scleractinian Corals. *Frontiers in Marine Science*, 7. <https://www.frontiersin.org/articles/10.3389/fmars.2020.00461>
- Canessa, M., Bavestrello, G., Bo, M., Enrichetti, F., & Trainito, E. (2022). Filling a gap: A population of *Eunicella verrucosa* (pallas, 1766)(Anthozoa, Alcyonacea) in the Tavolara-punta Coda Cavallo marine protected area (Ne Sardinia, Italy). *Diversity*, 14(5), 405.
- Carpine, C., & Grasshoff, M. (1975). Les gorgonaires de la Méditerranée. *Bulletin de l'Institut Océanographique de Monaco*, 71(140).
- Carugati, L., Moccia, D., Bramanti, L., Cannas, R., Follesa, M. C., Salvadori, S., & Cau, A. (2022). Deep-Dwelling Populations of Mediterranean *Corallium rubrum* and *Eunicella cavolini*: Distribution, Demography, and Co-Occurrence. *Biology*, 11(2). <https://doi.org/10.3390/biology11020333>
- Casado-Amezúa, P., Terrón-Sigler, A., Pinzón, J. H., Furla, P., Forcioli, D., Allemand, D., Ribes, M., & Coma, R. (2016). General ecological aspects of Anthozoan-Symbiodinium interactions in the Mediterranean Sea. *The Cnidaria, Past, Present and Future: The World of Medusa and Her Sisters*, 375–386.
- Chan, W. Y., Peplow, L. M., Menéndez, P., Hoffmann, A. A., & Van Oppen, M. J. (2018). Interspecific hybridization may provide novel opportunities for coral reef restoration. *Frontiers in Marine Science*, 5, 160.
- Chimienti, G. (2020). Vulnerable forests of the pink sea fan *Eunicella verrucosa* in the Mediterranean Sea. *Diversity*, 12(5), 176.
- Coelho, M., Pearson, G., Boavida, J., Paulo, D., Aurelle, D., Arnaud-haond, S., Gómez-gras, D., Bensoussan, N., López-sendino, P., Cerrano, C., Kipson, S., Bakran-petricioli, T., Ferretti, E., Linares, C., Garrabou, J., Serrão, E., & Ledoux, J. (2023). Not out of the Mediterranean: Atlantic populations of the gorgonian *Paramuricea clavata* are a separate sister species under further

- lineage diversification. *Ecology and Evolution*, 13, e9740.  
<https://onlinelibrary.wiley.com/doi/full/10.1002/ece3.9740>#
- Coma, R., Llorente-Llurba, E., Serrano, E., Gili, J.-M., & Ribes, M. (2015). Natural heterotrophic feeding by a temperate octocoral with symbiotic zooxanthellae: A contribution to understanding the mechanisms of die-off events. *Coral Reefs*, 34, 549–560.
- Combosch, D. J., & Vollmer, S. V. (2015). Trans-Pacific RAD-Seq population genomics confirms introgressive hybridization in Eastern Pacific Pocillopora corals. *Molecular Phylogenetics and Evolution*, 88, 154–162.
- Csilléry, K., François, O., & Blum, M. G. B. (2012). abc: An R package for approximate Bayesian computation (ABC). *Methods in Ecology and Evolution*, 3(3), 475–479. <https://doi.org/10.1111/j.2041-210X.2011.00179.x>
- Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., Handsaker, R. E., Lunter, G., Marth, G. T., & Sherry, S. T. (2011). The variant call format and VCFtools. *Bioinformatics*, 27(15), 2156–2158.
- De Jode, A., Le Moan, A., Johannesson, K., Faria, R., Stankowski, S., Westram, A. M., Butlin, R. K., Rafajlović, M., & Fraïsse, C. (2023). Ten years of demographic modelling of divergence and speciation in the sea. *Evolutionary Applications*, 16(2), 542–559.
- De Queiroz, K. (2007). Species Concepts and Species Delimitation. *Systematic Biology*, 56(6), 879–886. <https://doi.org/10.1080/10635150701701083>
- Erickson, K. L., Pentico, A., Quattrini, A. M., & McFadden, C. S. (2021). New approaches to species delimitation and population structure of anthozoans: Two case studies of octocorals using ultraconserved elements and exons. *Molecular Ecology Resources*, 21(1), 78–92.
- Estaque, T., Richaume, J., Bianchimani, O., Schull, Q., Mérigot, B., Bensoussan, N., Bonhomme, P., Vouriot, P., Sartoretto, S., & Monfort, T. (2023). Marine heatwaves on the rise: One of the strongest ever observed mass mortality event in temperate gorgonians. *Global Change Biology*, 29, 6159–6162. <https://doi.org/10.1111/gcb.16931>
- Ezzat, L., Merle, P.-L., Furla, P., Buttler, A., & Ferrier-Pagès, C. (2013). The Response of the Mediterranean Gorgonian Eunicella singularis to Thermal Stress Is Independent of Its Nutritional Regime. *PLoS ONE*, 8(5), e64370. <https://doi.org/10.1371/journal.pone.0064370>
- Faria, R., Johannesson, K., & Stankowski, S. (2021). Speciation in marine environments: Diving under the surface. *Journal of Evolutionary Biology*, 34(1), 4–15.
- Forcioli, D., Merle, P. L., Caligara, C., Ciosi, M., Muti, C., Francour, P., Cerrano, C., & Allemand, D. (2011). Symbiont diversity is not involved in depth acclimation in the Mediterranean sea whip Eunicella singularis. *Marine Ecology Progress Series*, 439, 57–71.
- Fourt, M., & Goujard, A. (2012). Rapport final de la campagne MEDSEACAN (Têtes des canyons méditerranéens continentaux) novembre 2008–avril 2010. *Partenariat Agence Des Aires Marines Protégées-GIS Posidonie*, 1–218.
- Fraïsse, C., Popovic, I., Mazoyer, C., Spataro, B., Delmotte, S., Romiguier, J., Loire, E., Simon, A., Galtier, N., & Duret, L. (2021). DILS: Demographic inferences with linked selection by using ABC. *Molecular Ecology Resources*, 21(8), 2629–2644.
- François Monnet, Zoé Postel, Pascal Touzet, Christelle Fraïsse, Yves Van de Peer, Xavier Vekemans, & Camille Roux. (2023). Rapid establishment of species barriers in plants compared to animals. *bioRxiv*, 2023.10.16.562535. <https://doi.org/10.1101/2023.10.16.562535>

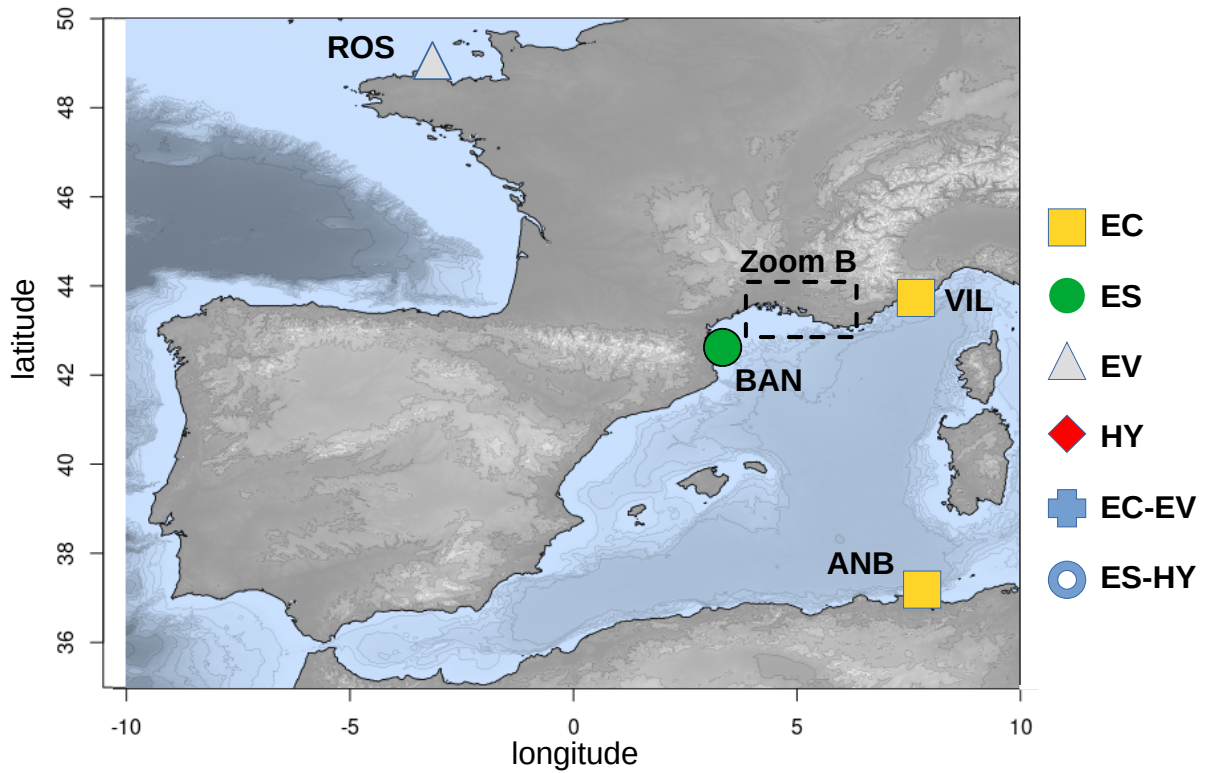
- Frichot, E., & François, O. (2015). LEA: an R package for landscape and ecological association studies. *Methods in Ecology and Evolution*, 6(8), 925–929.
- Frichot, E., Mathieu, F., Trouillon, T., Bouchard, G., & François, O. (2014). Fast and efficient estimation of individual ancestry coefficients. *Genetics*, 196(4), 973–983.
- Furla, P., Allemand, D., Shick, J. M., Ferrier-Pagès, C., Richier, S., Plantivaux, A., Merle, P.-L., & Tambutté, S. (2005). The symbiotic anthozoan: A physiological chimera between alga and animal. *Integrative and Comparative Biology*, 45(4), 595–604.
- Gagnaire, P., Broquet, T., Aurelle, D., Viard, F., Souissi, A., Bonhomme, F., Arnaud-Haond, S., & Bierne, N. (2015). Using neutral, selected, and hitchhiker loci to assess connectivity of marine populations in the genomic era. *Evolutionary Applications*, 8(8), 769–786.
- Gayral, P., Melo-Ferreira, J., Glemin, S., Bierne, N., Carneiro, M., Nabholz, B., Lourenco, J. M., Alves, P. C., Ballenghien, M., & Faivre, N. (2013). Reference-free population genomics from next-generation transcriptome data and the vertebrate–invertebrate gap. *PLoS Genetics*, 9(4), e1003457.
- Gori, A., Bramanti, L., López-González, P., Thoma, J. N., Gili, J.-M., Grinyó, J., Uceira, V., & Rossi, S. (2012). Characterization of the zooxanthellate and azooxanthellate morphotypes of the Mediterranean gorgonian *Eunicella singularis*. *Marine Biology*, 159(7), 1485–1496.
- Guzman, C., Shinzato, C., Lu, T.-M., & Conaco, C. (2018). Transcriptome analysis of the reef-building octocoral, *Heliopora coerulea*. *Scientific Reports*, 8(1), 8397. <https://doi.org/10.1038/s41598-018-26718-5>
- Haguenaer, A., Zuberer, F., Ledoux, J.-B., & Aurelle, D. (2013). Adaptive abilities of the Mediterranean red coral *Corallium rubrum* in a heterogeneous and changing environment: From population to functional genetics. *Journal of Experimental Marine Biology and Ecology*, 449(0), 349–357. <https://doi.org/10.1016/j.jembe.2013.10.010>
- Johnston, E. C., Forsman, Z. H., Flot, J.-F., Schmidt-Roach, S., Pinzón, J. H., Knapp, I. S., & Toonen, R. J. (2017). A genomic glance through the fog of plasticity and diversification in Pocillopora. *Scientific Reports*, 7(1), 5991.
- Kent, W. J. (2002). BLAT—the BLAST-like alignment tool. *Genome Research*, 12(4), 656–664.
- Krueger-Hadfield, S. (2015). *Marmap*. <https://www.molecularecologist.com/2015/07/03/marmap/>
- Lajeunesse, T. C., Parkinson, J. E., Gabrielson, P. W., Jeong, H. J., Reimer, J. D., Voolstra, C. R., & Santos, S. R. (2018). Systematic revision of Symbiodiniaceae highlights the antiquity and diversity of coral endosymbionts. *Current Biology*, 28(16), 2570–2580.
- Lajeunesse, T. C., Wiedenmann, J., Casado-Amezúa, P., D’ambra, I., Turnham, K. E., Nitschke, M. R., Oakley, C. A., Goffredo, S., Spano, C. A., & Cubillos, V. M. (2022). Revival of Philozoon Geddes for host-specialized dinoflagellates, ‘zooxanthellae’, in animals from coastal temperate zones of northern and southern hemispheres. *European Journal of Phycology*, 57(2), 166–180.
- Lee, M. J., Jeong, H. J., Jang, S. H., Lee, S. Y., Kang, N. S., Lee, K. H., Kim, H. S., Wham, D. C., & Lajeunesse, T. C. (2016). Most low-abundance “background” Symbiodinium spp. are transitory and have minimal functional significance for symbiotic corals. *Microbial Ecology*, 71, 771–783.
- Leroy, T., Louvet, J.-M., Lalanne, C., Le Provost, G., Labadie, K., Aury, J.-M., Delzon, S., Plomion, C., & Kremer, A. (2020). Adaptive introgression as a

- driver of local adaptation to climate in European white oaks. *New Phytologist*, 226(4), 1171–1182. <https://doi.org/10.1111/nph.16095>
- Lesser, M. P., Stat, M., & Gates, R. D. (2013). The endosymbiotic dinoflagellates (*Symbiodinium* sp.) of corals are parasites and mutualists. *Coral Reefs*, 32(3), 603–611. <https://doi.org/10.1007/s00338-013-1051-z>
- Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, 25(14), 1754–1760.
- Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., & Durbin, R. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics*, 25(16), 2078–2079.
- Lischer, H. E., & Excoffier, L. (2012). PGDSpider: An automated data conversion tool for connecting population genetics and genomics programs. *Bioinformatics*, 28(2), 298–299.
- Macleod, K. L., Jenkins, T. L., Witt, M. J., & Stevens, J. R. (2024). Rare, long-distance dispersal underpins genetic connectivity in the pink sea fan, *Eunicella verrucosa*. *Evolutionary Applications*, 17(3), e13649. <https://doi.org/10.1111/eva.13649>
- Mayr, E. (2001). Wu's genic view of speciation. *Journal of Evolutionary Biology*, 14(6), 866–867. <https://doi.org/10.1046/j.1420-9101.2001.00336.x>
- McFadden, C., Benayahu, Y., Pante, E., Thoma, J. N., Nevarez, P. A., & France, S. C. (2011). Limitations of mitochondrial gene barcoding in Octocorallia. *Molecular Ecology Resources*, 11(1), 19–31. <https://doi.org/10.1111/j.1755-0998.2010.02875.x>
- Muir, P. R., Obura, D. O., Hoeksema, B. W., Sheppard, C., Pichon, M., & Richards, Z. T. (2022). Conclusions of low extinction risk for most species of reef-building corals are premature. *Nature Ecology & Evolution*, 6(4), 357–358.
- Munro, L. (2004). Determining the reproductive cycle of *Eunicella verrucosa*. *Reef Research: ETR*, 11. [https://www.marine-bio-images.com/RR\\_Eunicella\\_PDFS/Report\\_RR12Jul2004reproductive%20cycle%20pdf.pdf](https://www.marine-bio-images.com/RR_Eunicella_PDFS/Report_RR12Jul2004reproductive%20cycle%20pdf.pdf)
- Muthye, V., Mackereth, C. D., Stewart, J. B., & Lavrov, D. V. (2022). Large dataset of octocoral mitochondrial genomes provides new insights into mt-mutS evolution and function. *DNA Repair*, 110, 103273.
- Palumbi, S. R. (1992). Marine speciation on a small planet. *Trends in Ecology & Evolution*, 7(4), 114–118.
- Palumbi, S. R. (1999). All males are not created equal: Fertility differences depend on gamete recognition polymorphisms in sea urchins. *Proceedings of the National Academy of Sciences*, 96(22), 12632–12637.
- Pante, E., Abdelkrim, J., Viricel, A., Gey, D., France, S., Boisselier, M.-C., & Samadi, S. (2015a). Use of RAD sequencing for delimiting species. *Heredity*, 114(5), 450–459.
- Pante, E., Puillandre, N., Viricel, A., Arnaud-Haond, S., Aurelle, D., Castelin, M., Chenuil, A., Destombe, C., Forcioli, D., & Valero, M. (2015b). Species are hypotheses: Avoid connectivity assessments based on pillars of sand. *Molecular Ecology*, 24(3), 525–544.
- Pante, E., & Simon-Bouhet, B. (2013). marmap: A package for importing, plotting and analyzing bathymetric and topographic data in R. *PLoS One*, 8(9), e73051. <https://doi.org/10.1371/journal.pone.0073051>
- Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., & Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods*, 14(4), 417–419.
- Peñalba, J. V., Runemark, A., Meier, J. I., Singh, P., Wogan, G. O., Sánchez-Guillén, R., Mallet, J., Rometsch, S. J., Menon, M., & Seehausen, O. (2024). The Role

- of Hybridization in Species Formation and Persistence. *Cold Spring Harbor Perspectives in Biology*, a041445.
- Picard Toolkit. (2019). Broad Institute. GitHub Repository.  
<https://broadinstitute.github.io/picard/>
- Porro, B. (2019). *Diversités génétiques chez l'holobionte Anemonia viridis: Des morphotypes de l'hôte à la différenciation symbiotique*. (Doctoral dissertation, COMUE Université Côte d'Azur (2015-2019)).
- Pudlo, P., Marin, J.-M., Estoup, A., Cornuet, J.-M., Gautier, M., & Robert, C. P. (2016). Reliable ABC model choice via random forests. *Bioinformatics*, 32(6), 859–866.
- Quilodrán, C. S., Rugg, K., Sendell-Price, A. T., Anderson, E. C., Coulson, T., & Clegg, S. M. (2020). The multiple population genetic and demographic routes to islands of genomic divergence. *Methods in Ecology and Evolution*, 11(1), 6–21. <https://doi.org/10.1111/2041-210X.13324>
- Readman, J., & Hiscock, K. (2017). *Eunicella verrucosa*. Pink sea fan.  
<https://www.marlin.ac.uk/species/detail/1121>
- Reynes, L., Aurelle, D., Chevalier, C., Pinazo, C., Valero, M., Mauger, S., Sartoretto, S., Blafuné, A., Ruitton, S., Boudouresque, C.-F., Verlaque, M., & Thibaut, T. (2021). Population genomics and Lagrangian modeling shed light on dispersal events in the Mediterranean endemic *Ericaria zosteroides* (= *Cystoseira zosteroides*) (Fucales). *Frontiers in Marine Science*, 8, 683528.
- Rivera-García, L., Rivera-Vicéns, R. E., Veglia, A. J., & Schizas, N. V. (2019). De novo transcriptome assembly of the digitate morphotype of *Briareum asbestinum* (Octocorallia: Alcyonacea) from the southwest shelf of Puerto Rico. *Marine Genomics*, 47, 100676.  
<https://doi.org/10.1016/j.margen.2019.04.001>
- Rosenberg, E., & Zilber-Rosenberg, I. (2018). The hologenome concept of evolution after 10 years. *Microbiome*, 6(1), 78.
- Rouchy, J. M., & Caruso, A. (2006). The Messinian salinity crisis in the Mediterranean basin: A reassessment of the data and an integrated scenario. *Sedimentary Geology*, 188, 35–67.
- Rousset, F. (2008). genepop'007: A complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources*, 8(1), 103–106. <https://doi.org/10.1111/j.1471-8286.2007.01931.x>
- Rousset, F., Lopez, J., & Belkhir, K. (2020). Package 'genepop.' *R Package Version*, 1(7).
- Roux, C., Fraïsse, C., Romiguier, J., Anciaux, Y., Galtier, N., & Bierne, N. (2016). Shedding Light on the Grey Zone of Speciation along a Continuum of Genomic Divergence. *PLOS Biology*, 14(12), e2000234.  
<https://doi.org/10.1371/journal.pbio.2000234>
- Sachs, J. L., & Wilcox, T. P. (2006). A shift to parasitism in the jellyfish symbiont *Symbiodinium microadriaticum*. *Proceedings of the Royal Society B: Biological Sciences*, 273(1585), 425–429.
- Samadi, S., & Barberousse, A. (2006). The tree, the network, and the species. *Biological Journal of the Linnean Society*, 89(3), 509–521.
- Sartoretto, S., & Francour, P. (2011). Bathymetric distribution and growth rates of *Eunicella verrucosa* (Cnidaria: Gorgoniidae) populations along the Marseilles coast (France). *Scientia Marina*, 76(2), 349–355.
- Schulz, M. H., Zerbino, D. R., Vingron, M., & Birney, E. (2012). Oases: Robust de novo RNA-seq assembly across the dynamic range of expression levels. *Bioinformatics*, 28(8), 1086–1092.
- Sini, M., Kipson, S., Linares, C., Koutsoubas, D., & Garrabou, J. (2015). The Yellow Gorgonian *Eunicella cavolini*: Demography and Disturbance Levels across

- the Mediterranean Sea. *PLoS ONE*, *10*(5), e0126253.  
<https://doi.org/10.1371/journal.pone.0126253>
- Stanley, R. R. E., Jeffery, N. W., Wringe, B. F., DiBacco, C., & Bradbury, I. R. (2017). genepopedit: A simple and flexible tool for manipulating multilocus molecular data in R. *Molecular Ecology Resources*, *17*(1), 12–18.  
<https://doi.org/10.1111/1755-0998.12569>
- Tricou, T., Tannier, E., & de Vienne, D. M. (2022). Ghost lineages can invalidate or even reverse findings regarding gene flow. *PLoS Biology*, *20*(9), e3001776.
- Tsagkogeorga, G., Cahais, V., & Galtier, N. (2012). The population genomics of a fast evolver: High levels of diversity, functional constraint, and molecular adaptation in the tunicate *Ciona intestinalis*. *Genome Biology and Evolution*, *4*(8), 852–861.
- van Oppen, M. J. H., & Medina, M. (2020). Coral evolutionary responses to microbial symbioses. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1808), 20190591.  
<https://doi.org/10.1098/rstb.2019.0591>
- Van Oppen, M., Mieog, J. C., Sanchez, C., & Fabricius, K. (2005). Diversity of algal endosymbionts (zooxanthellae) in octocorals: The roles of geography and host relationships. *Molecular Ecology*, *14*(8), 2403–2417.
- Wagner, D., Pochon, X., Irwin, L., Toonen, R. J., & Gates, R. D. (2011). Azooxanthellate? Most Hawaiian black corals contain Symbiodinium. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1710), 1323–1328.
- Weir, B. S., & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution*, *38*, 1358–1370.

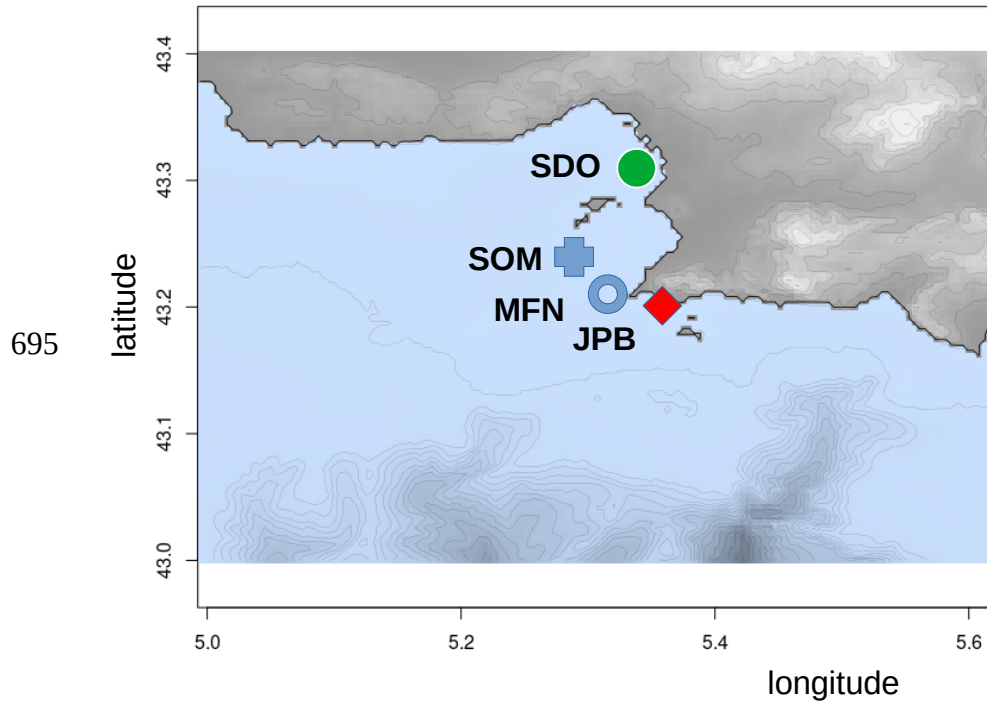
684 **Figure 1:** map of sampling sites; A) general view, B) zoom on the area of  
685 Marseille. The symbols present the different samples: EC *E. cavolini*, ES  
686 *E. singularis*, EV *E. verrucosa*, HY hybrids, EC-EV *E. cavolini* and *E. verrucosa* in  
687 sympatry, ES-HY *E. singularis* and hybrids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





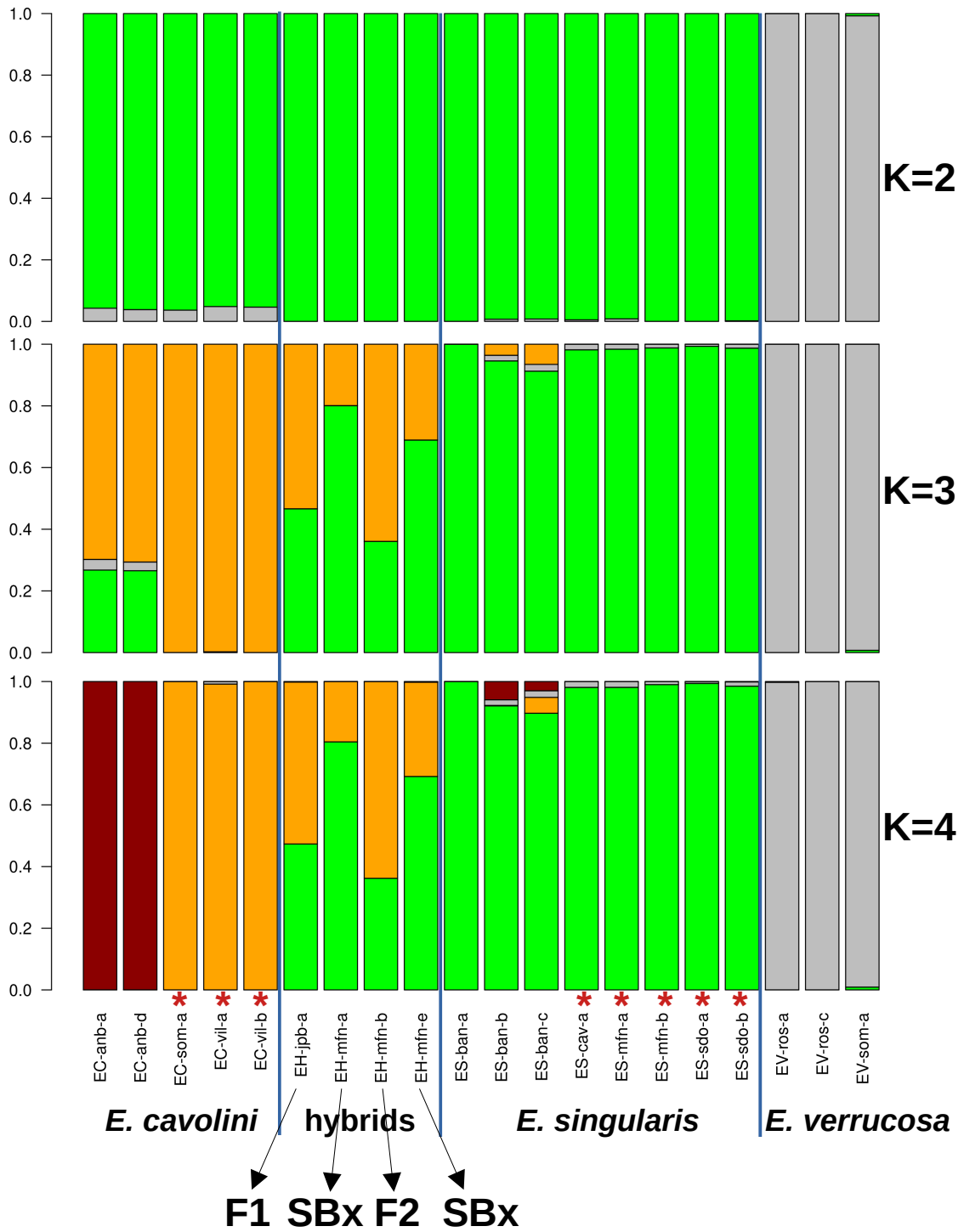
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**B)**



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



706 **Table 1:** results of demographic inferences with DILS. The columns indicate the species comparison, the model choice for  
 707 population size (constant vs. variable), and the results of inferences: current gene flow (migration vs isolation); if current migration,  
 708 isolation / migration (IM) vs ancestral migration (AM); if no current migration, strict isolation (SI) vs ancestral migration (AM);  
 709 homogeneity (N-homo) vs heterogeneity in effective size (N-hetero) among loci; homogeneity (M-homo) vs heterogeneity (M-  
 710 hetero) in gene flow among loci. The probability of each scenario is given in the same case.  
 711

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

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# 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.

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

**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		
									Lg mean	median	Lg max
<i>Eunicella</i>	<i>cavolini</i>	e-cavol-anb-a	21432997	33627	1978	7240	46041698	201	1369.19	1023	13533
<i>Eunicella</i>	<i>cavolini</i>	e-cavol-anb-d	20070761	33624	2002	7025	46288676	201	1376.66	1004	24422
<i>Eunicella</i>	<i>cavolini</i>	e-cavol-som-a	22986734	43541	1757	8987	52113269	201	1196.88	840	24228
<i>Eunicella</i>	<i>cavolini</i>	e-cavol-vil-a	31846763	36908	2056	7687	51709228	201	1401.03	1018	18381
<i>Eunicella</i>	<i>cavolini</i>	e-cavol-vil-b	28751407	34961	2044	7470	48971519	201	1400.75	1040	19504
<i>Eunicella</i>	hybrid	e-hybri-jpb-a	34392918	39407	2031	8098	54290945	201	1377.7	998	25557
<i>Eunicella</i>	hybrid	e-hybri-mfn-a	44256795	40762	2081	8451	57794280	201	1417.85	1039	25573
<i>Eunicella</i>	hybrid	e-hybri-mfn-b	34705411	39672	2046	8040	54738734	201	1379.78	981.5	16650
<i>Eunicella</i>	hybrid	e-hybri-mfn-e	36536647	39532	2038	8090	54685655	201	1383.33	995.5	25578
<i>Eunicella</i>	<i>singularis</i>	e-singu-ban-a	44325669	45364	1919	9379	58576839	201	1291.26	928	28882
<i>Eunicella</i>	<i>singularis</i>	e-singu-ban-b	33184944	38095	1930	8114	50868966	201	1335.32	987	20211
<i>Eunicella</i>	<i>singularis</i>	e-singu-ban-c	46271612	43821	2023	9132	60512898	201	1380.91	1007	21714
<i>Eunicella</i>	<i>singularis</i>	e-singu-cav-a	48947180	51120	1967	10031	65261049	201	1276.62	868	22527
<i>Eunicella</i>	<i>singularis</i>	e-singu-mfn-a	52588076	70114	1761	13336	79649263	201	1136	739	16808
<i>Eunicella</i>	<i>singularis</i>	e-singu-mfn-b	43713977	55035	1894	10583	67120524	201	1219.6	808	21143
<i>Eunicella</i>	<i>singularis</i>	e-singu-sdo-a	37444166	55928	1741	10464	62326140	201	1114.4	715	16387
<i>Eunicella</i>	<i>singularis</i>	e-singu-sdo-b	39266148	72419	1652	13837	78950323	201	1090.19	715	24245
<i>Eunicella</i>	<i>verrucosa</i>	e-verru-ros-a	19398629	31195	1936	6630	41727111	201	1337.62	981	16974
<i>Eunicella</i>	<i>verrucosa</i>	e-verru-ros-c	20495748	31526	1968	6729	42779660	201	1356.96	1005	16663
<i>Eunicella</i>	<i>verrucosa</i>	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	median	Lg max
<i>Eunicella</i>	meta		891354	68386	2144	14309	10262131	201	1500.62	1098	28882
<i>Eunicella</i>	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	<i>cavolini</i> <i>singularis</i>	/ all from reads2snp	61947 contigs / 101515803 sites	speciation scenarios with DILS
all-CV	<i>cavolini</i> <i>verrucosa</i>	/ all from reads2snp	59702 contigs / 100704015 sites	speciation scenarios with DILS
all-SV	<i>singularis</i> <i>verrucosa</i>	/ all from reads2snp	61373 contigs / 101444729 sites	speciation scenarios with DILS
polymorphic sites	all	polymorphic sites ; no missing data	31369 SNPs	F <sub>ST</sub> , LEA, PCA
1 % SNPs	without <i>verrucosa</i>	polymorphic sites ; no missing data ; 1 % highest F <sub>ST</sub> <i>cavolini</i> / <i>singularis</i>	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 : Tsplitt : 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  $3.10^{-9}$ .

<b>dataset</b>	<b>max_NA</b>	<b>Lmin</b>	<b>nMin</b>	<b>jSFS</b>	<b>Tsplitt</b>	<b>Ne</b>	<b>M</b>
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.

<b>Sample</b>	<b>Species</b>	<b>Raw reads</b>	<b>Transcriptome</b>
e-cavol-anb-a	<i>E. cavolini</i>	0.0171	0.00305
e-cavol-anb-d	<i>E. cavolini</i>	0.0087	0.00268
e-cavol-som-a	<i>E. cavolini</i>	0.0087	0.00426
e-cavol-vil-a	<i>E. cavolini</i>	0.0184	0.00350
e-cavol-vil-b	<i>E. cavolini</i>	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	<i>E. singularis</i>	0.0192	0.00675
e-singu-ban-b	<i>E. singularis</i>	0.0140	0.00410
e-singu-ban-c	<i>E. singularis</i>	0.0080	0.00647
e-singu-cav-a	<i>E. singularis</i>	0.0261	0.02263
e-singu-mfn-a	<i>E. singularis</i>	0.0233	0.03419
e-singu-mfn-b	<i>E. singularis</i>	0.0129	0.02745
e-singu-sdo-a	<i>E. singularis</i>	0.0158	0.03644
e-singu-sdo-b	<i>E. singularis</i>	0.0207	0.03686
e-verru-ros-a	<i>E. verrucosa</i>	0.0075	0.00276
e-verru-ros-c	<i>E. verrucosa</i>	0.0082	0.00282
e-verru-som-a	<i>E. verrucosa</i>	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)**

	<i>E. cavolini</i>	hybrids	<i>E. singularis</i>
hybrids	0.69		
<i>E. singularis</i>	0.69	0.36	
<i>E. verrucosa</i>	0.57	0.69	0.15

**B)**

	<i>E. cavolini</i>	hybrids	<i>E. singularis</i>
hybrids	0.571		
<i>E. singularis</i>	0.019	0.020	
<i>E. verrucosa</i>	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)

	<i>E. cavolini</i>	hybrids	<i>E. singularis</i>	<i>E. verrucosa</i>
<i>E. cavolini</i>	-	-	0.0018	0.0067
hybrids	0.069	-	-	-
<i>E. singularis</i>	0.207	0.073	-	0.0070
<i>E. verrucosa</i>	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***

	<b>HPD 0.025</b>	<b>HPD median</b>	<b>HPD 0.0975</b>
<b>constant size, SC</b>			
$N_C$	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
<b>variable size, SC</b>			
$N_C$	531986	665965	875780
$N_S$	185826	222258	276889
$N_A$	515018	578861	640504
founders <sub>C</sub>	0	1	1
founders <sub>S</sub>	0	1	1
$T_{dem\_C}$	250520	339056	418963
$T_{dem\_S}$	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***

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

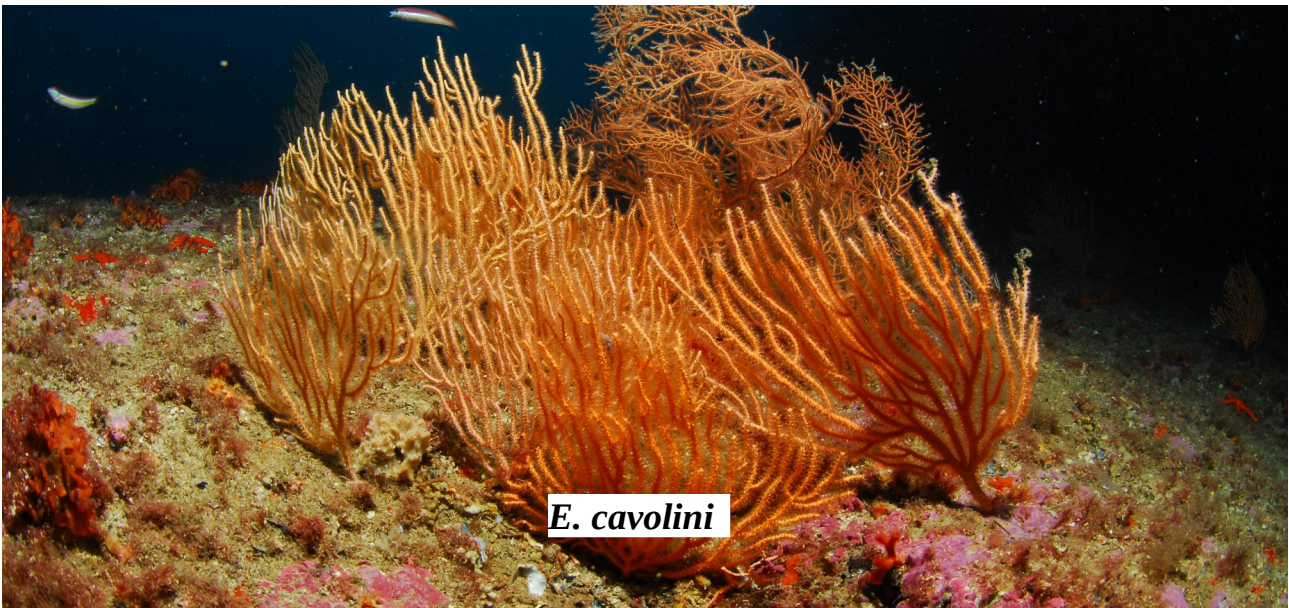
**C) comparison *E. singularis* / *E. verrucosa***

	<b>HPD 0.025</b>	<b>HPD median</b>	<b>HPD 0.0975</b>
<b>constant size, AM</b>			
$N_S$	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
<b>variable size, AM</b>			
$N_S$	281023	386388	494606
$N_V$	856542	1165039	1566087
$N_A$	592428	697054	797828
founders <sub>c</sub>	0	0	0
founders <sub>v</sub>	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

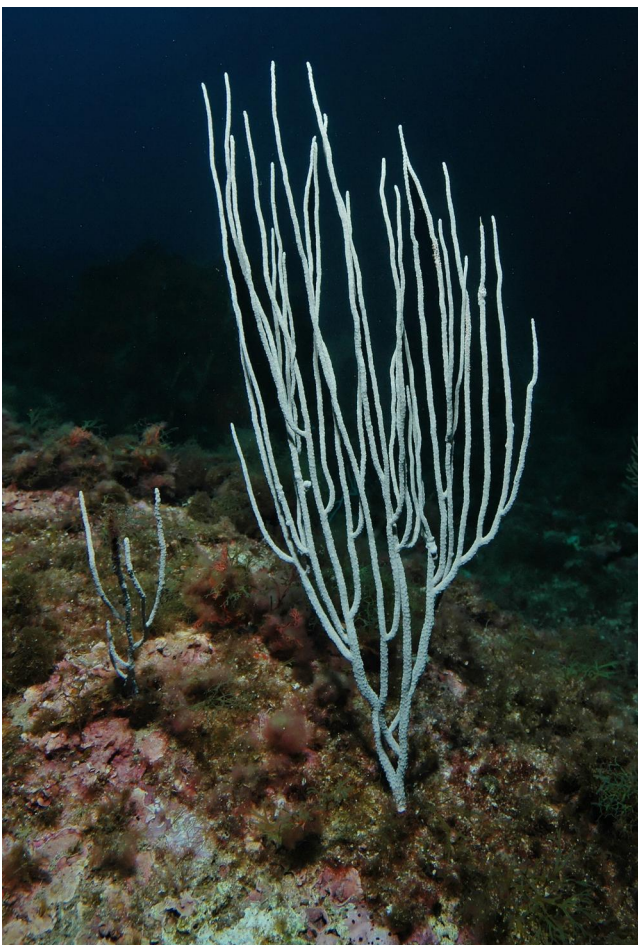


**Figure S1:** examples of morphological diversity in *Eunicella* species in the area of Marseille.

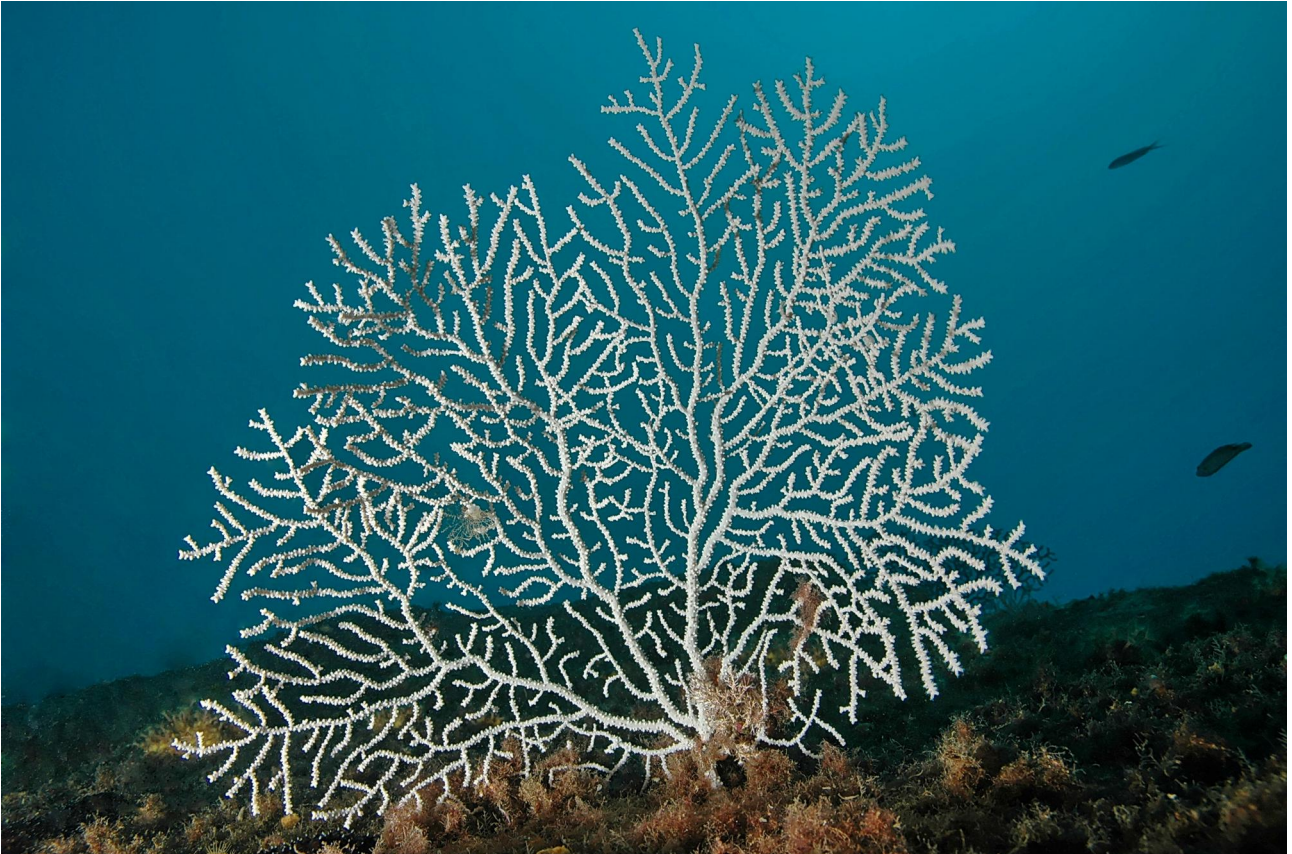
**A) example of typical *E. cavolini* colonies (in the foreground)**



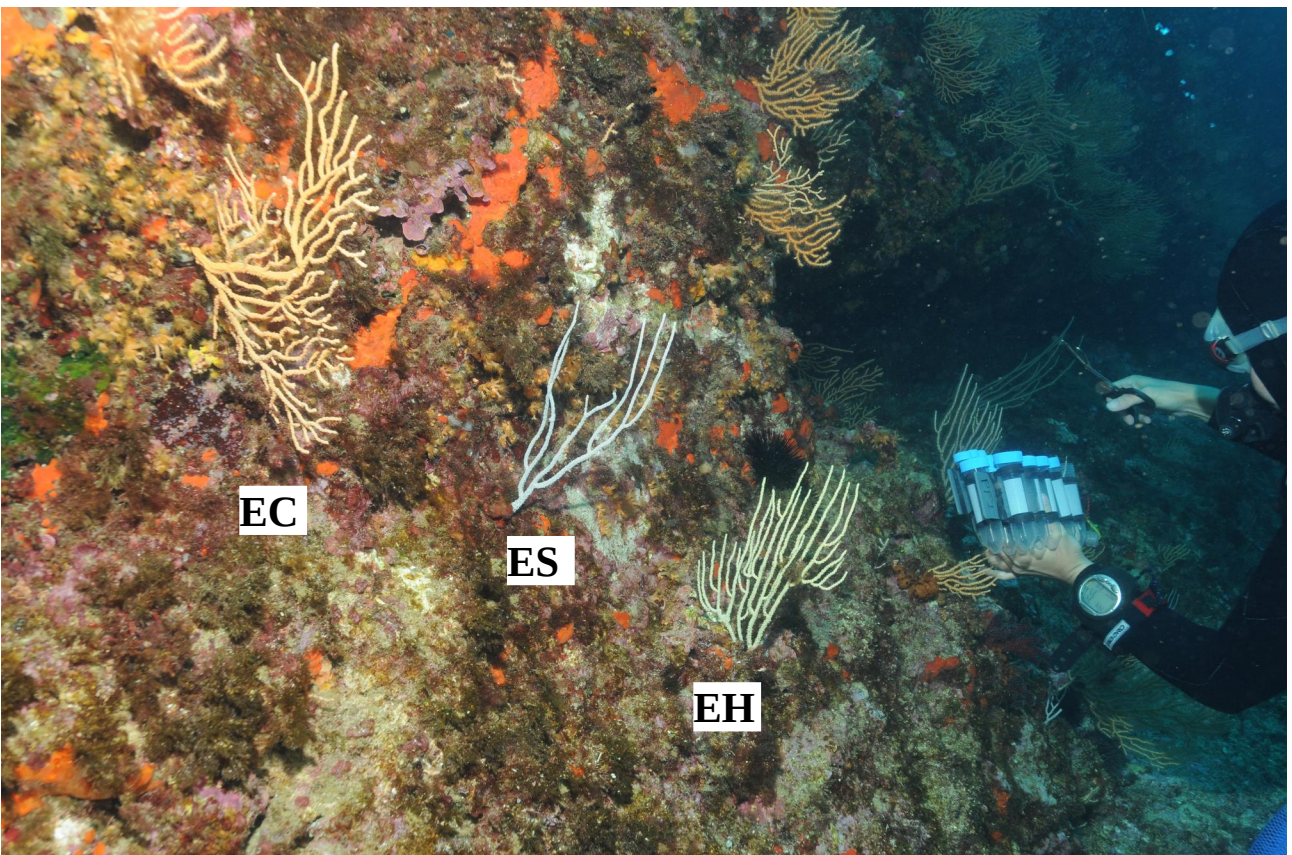
**B) example of a typical *E. singularis***



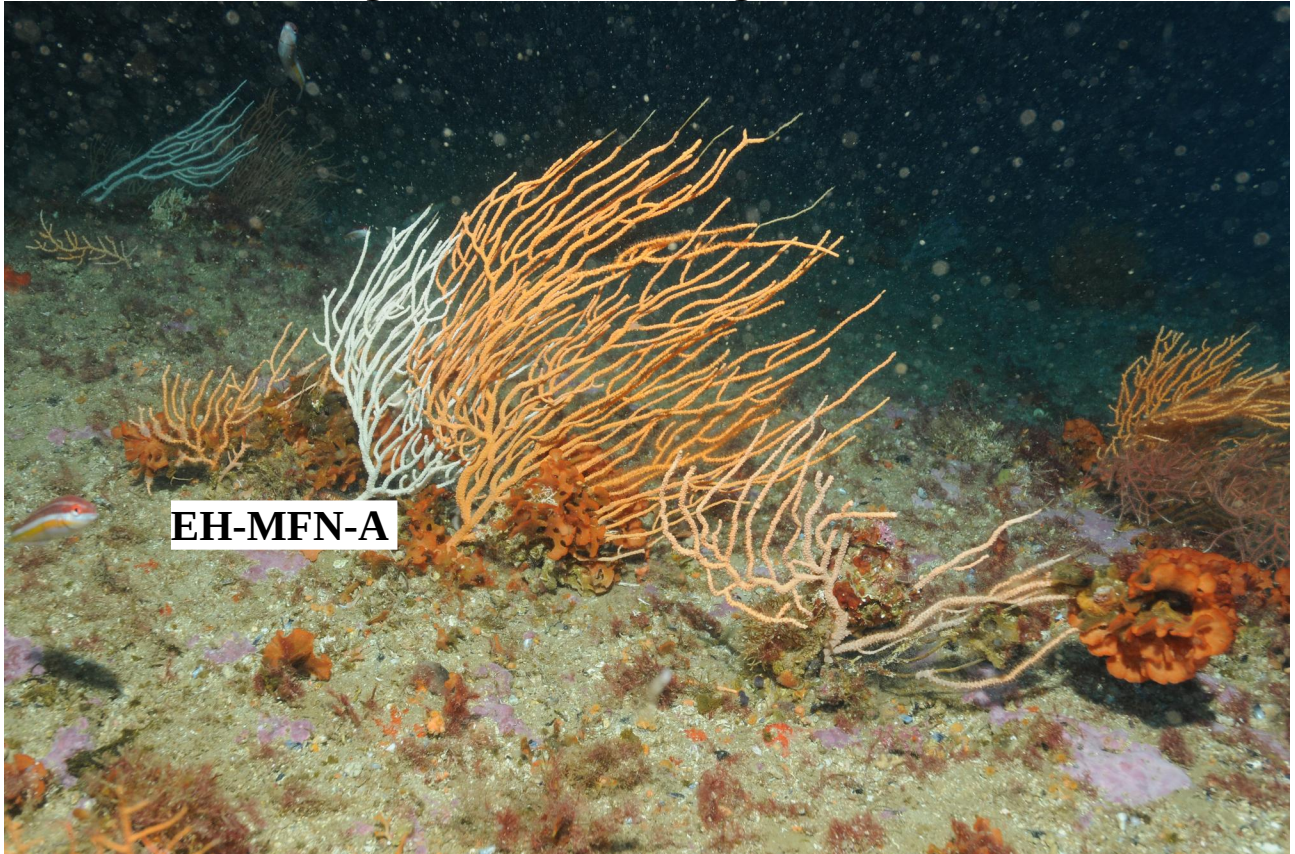
C) example of a typical *E. verrucosa*:



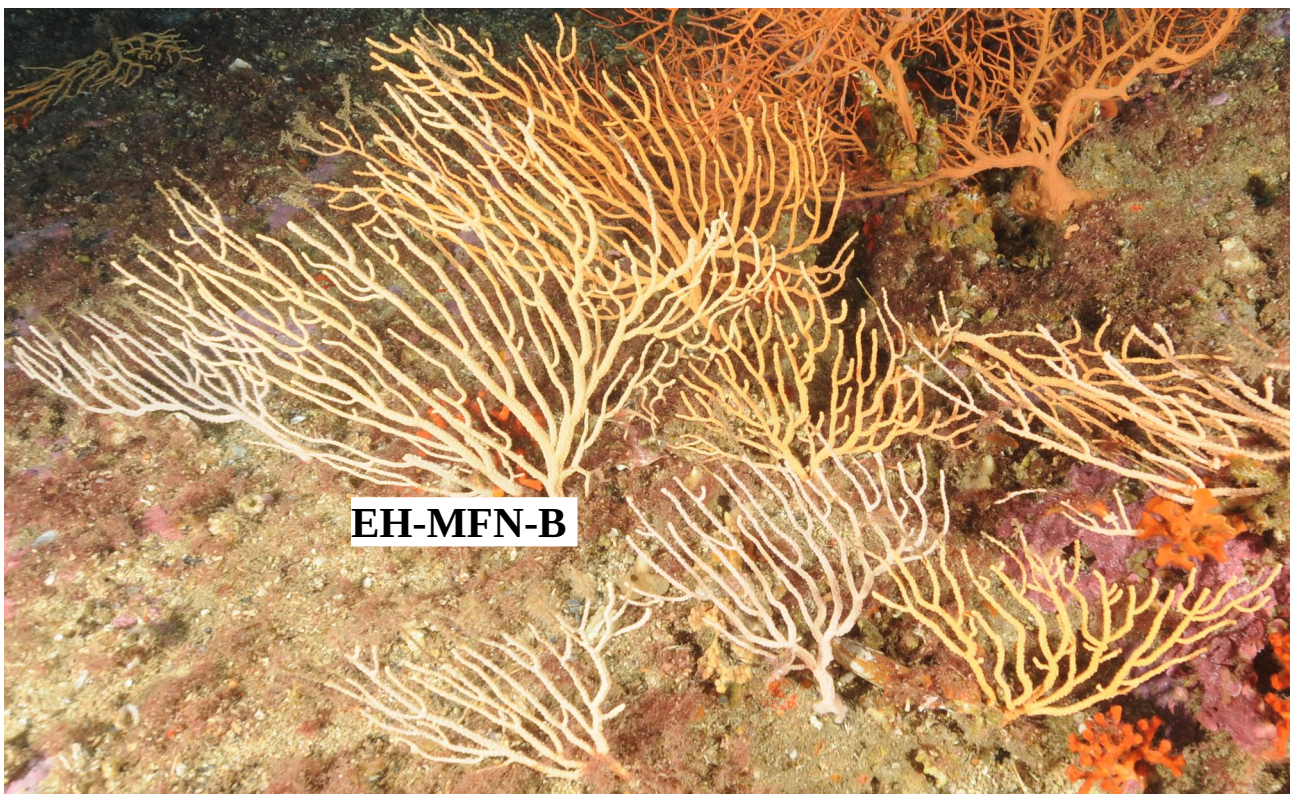
D) sampling with the presence of *E. cavolini* (EC), *E. singularis* (ES) and a potential hybrid (EH)



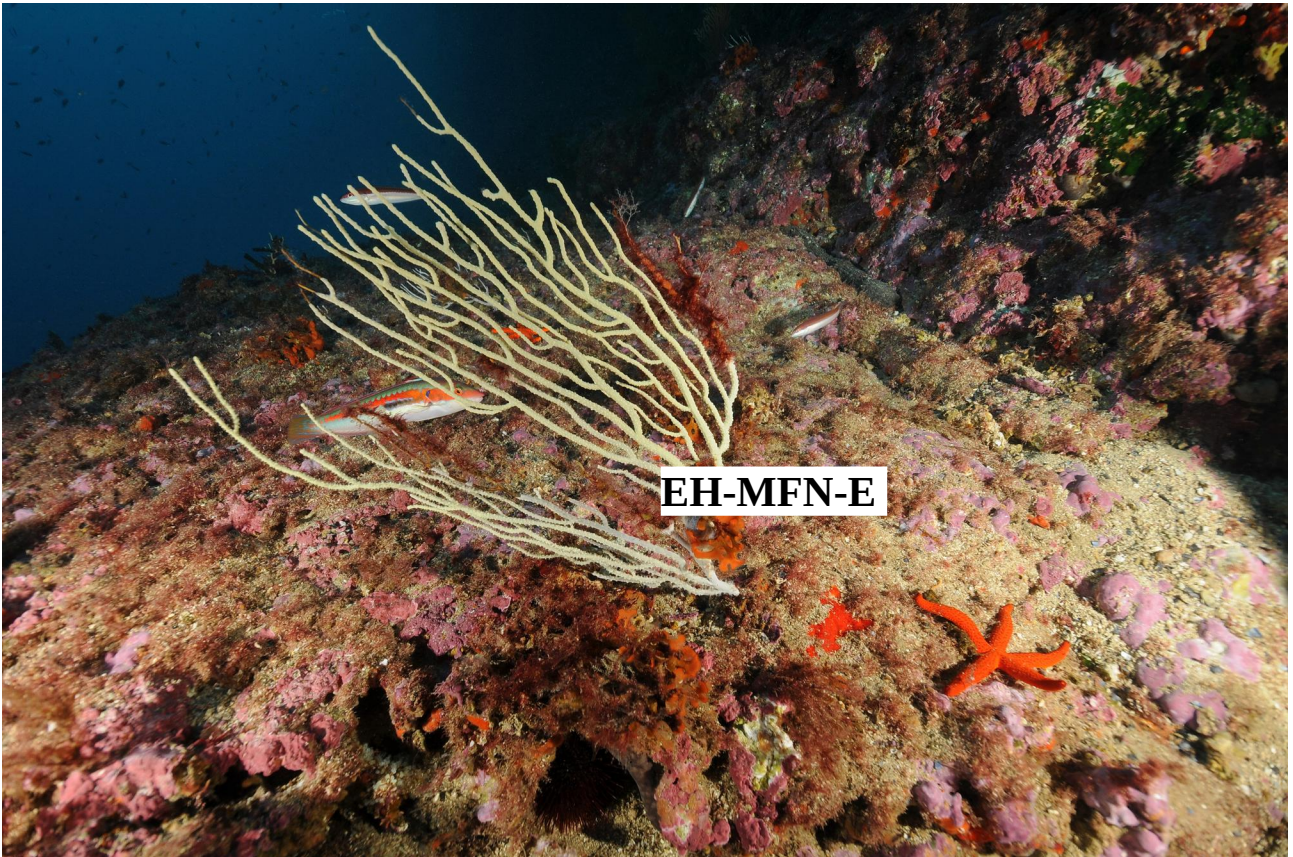
**E) morphology of the colony EH-MFN-A (white, in the background) with white color as *E. singularis* but branching more similar to *E. cavolini*.**



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



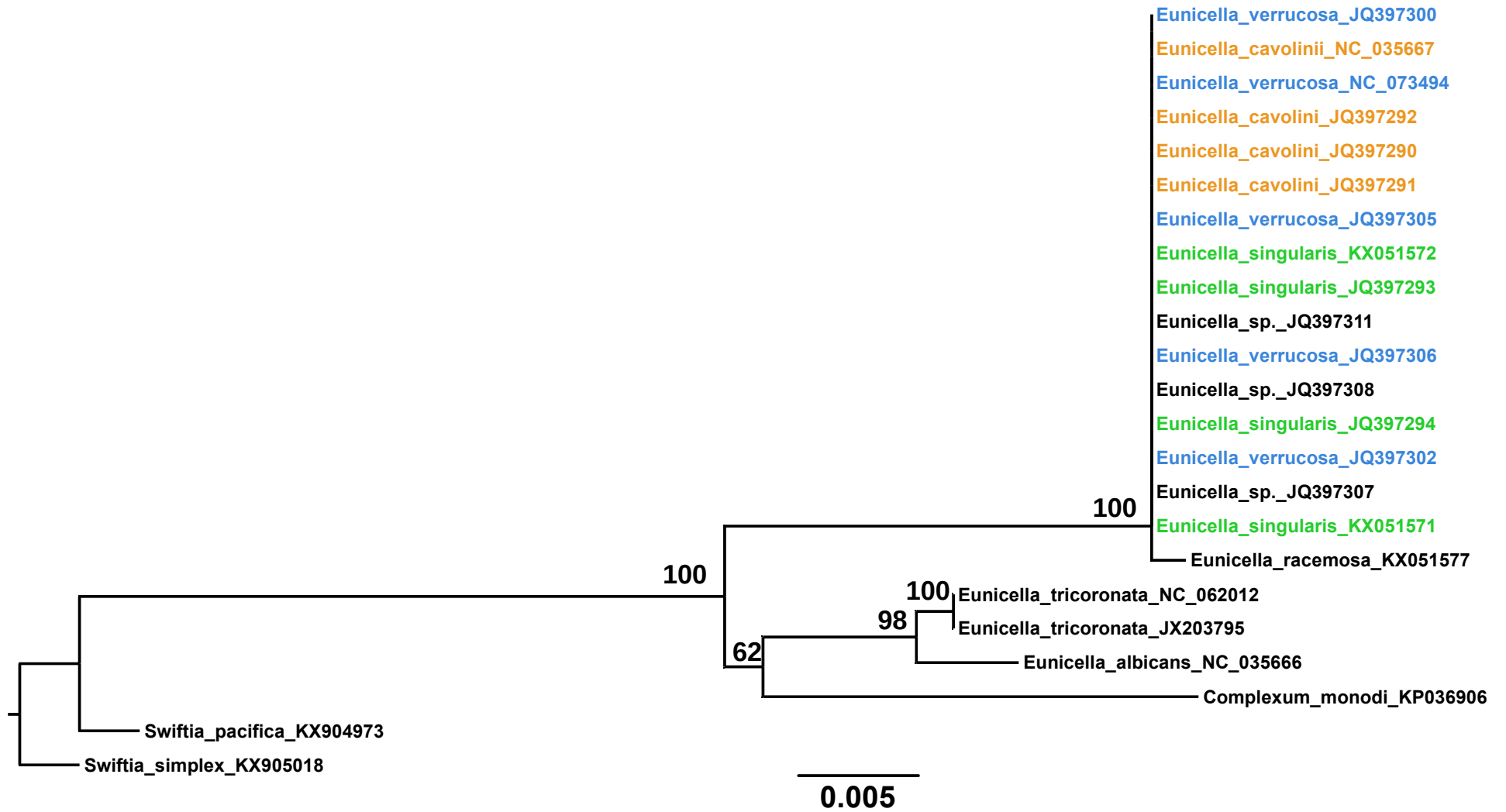
**F) morphology of the colony EH-MFN-E with intermediate branching and color between *E. cavolini* and *E. singularis***



**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 bootstraps. The Genbank accession numbers are listed in table S1.

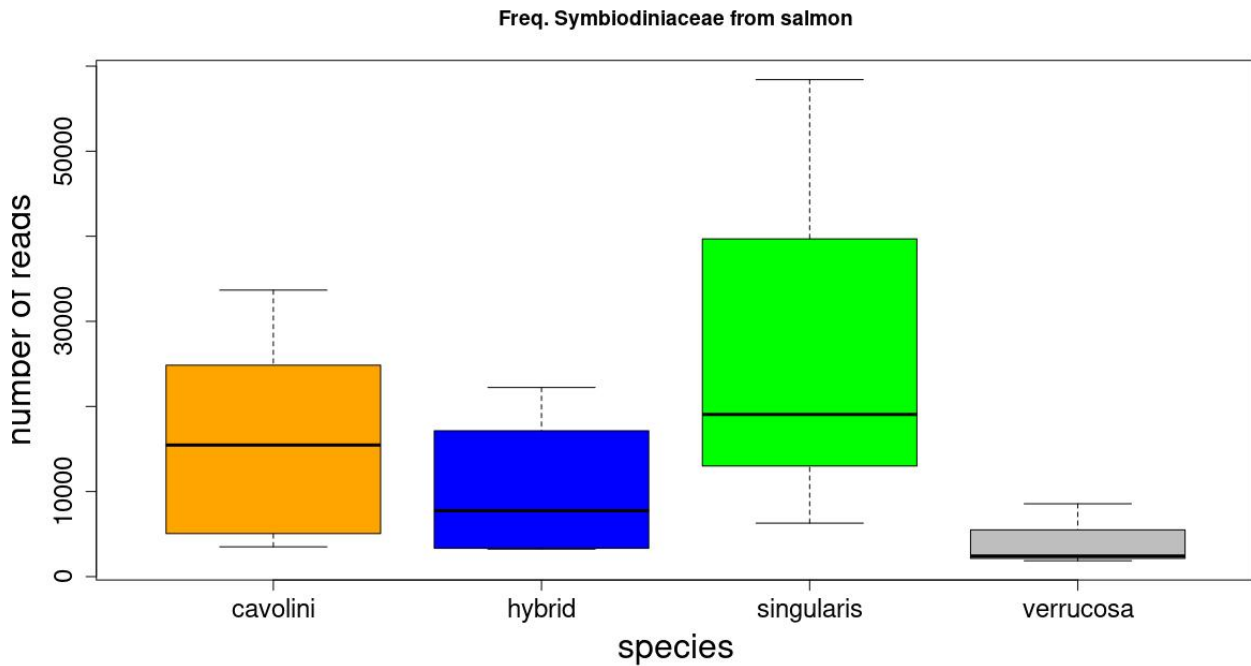
#### References:

- 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.
- Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Jermini, L. S. (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods*, 14(6), 587–589.  
<https://doi.org/10.1038/nmeth.4285>
- McFadden, C. S., van Ofwegen, L. P., & Quattrini, A. M. (2022). Revisionary systematics of Octocorallia (Cnidaria: Anthozoa) guided by phylogenomics. *Bulletin of the Society of Systematic Biologists*, 1(3).
- Nguyen, L.-T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Molecular Biology and Evolution*, 32(1), 268–274.  
<https://doi.org/10.1093/molbev/msu300>
- 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/>

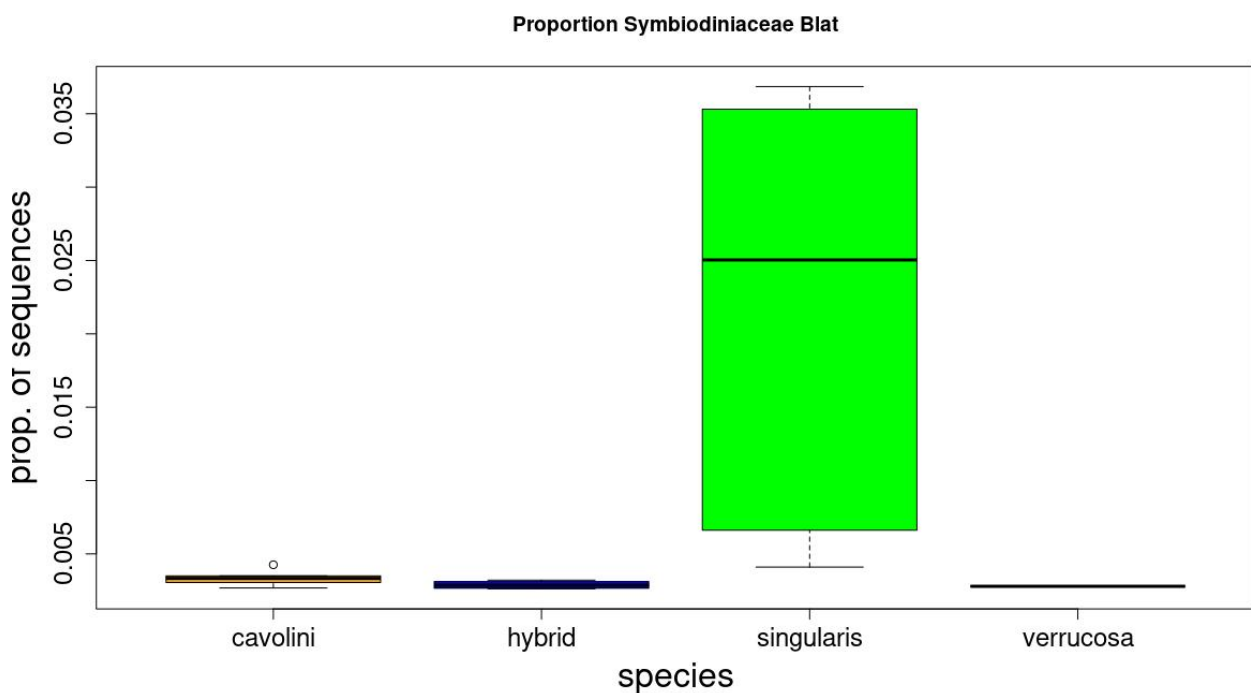


**Figure S3:** distribution of the frequency of Symbiodiniaceae sequences in the individual transcriptomes according to the species based A) on the number of reads estimated with Salmon, and B) on the proportion of assembled sequences with the BLAT analyses.

**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.

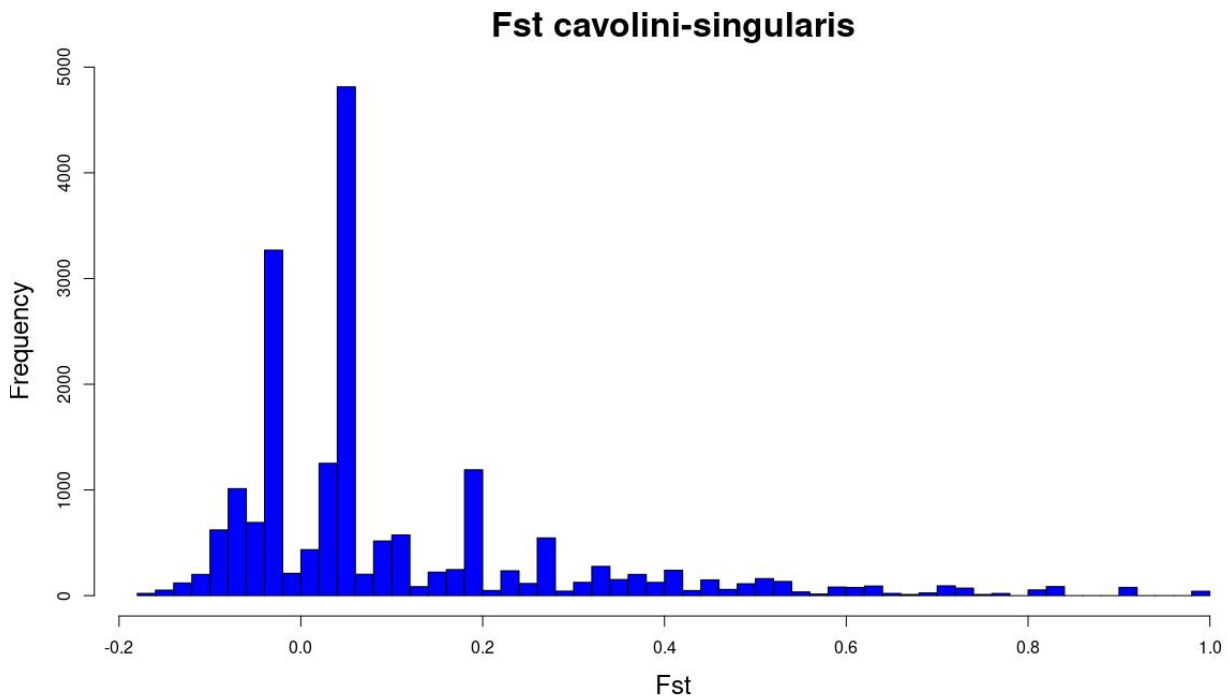


**B)** Assembled sequences with BLAT: Mean values per group: *E. cavolini*: 0.0034; hybrids: 0.0029; *E. singularis*: 0.0219; *E. verrucosa*: 0.0028. Kruskal-Wallis test of the differences among groups: chi-squared = 14.352, df = 3, p-value = 0.002.

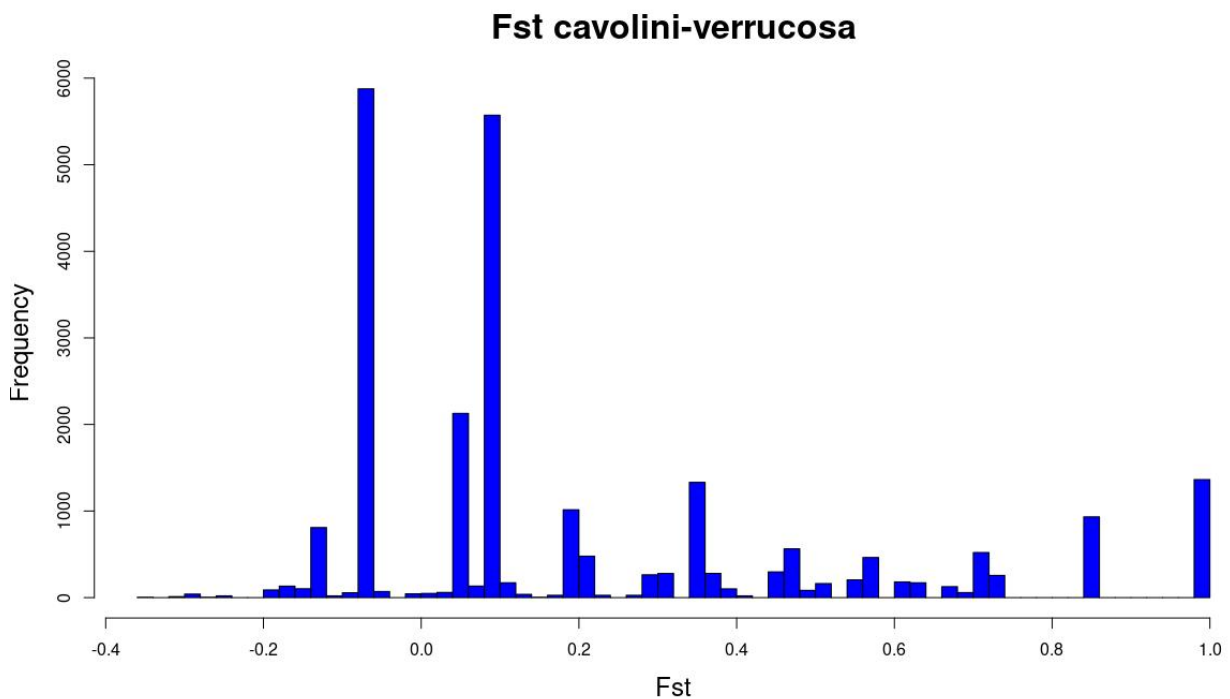


**Figure S6:** distribution of  $F_{ST}$  estimates over loci, for the pairwise comparisons among the three species, with the exclusion of potential hybrids.

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



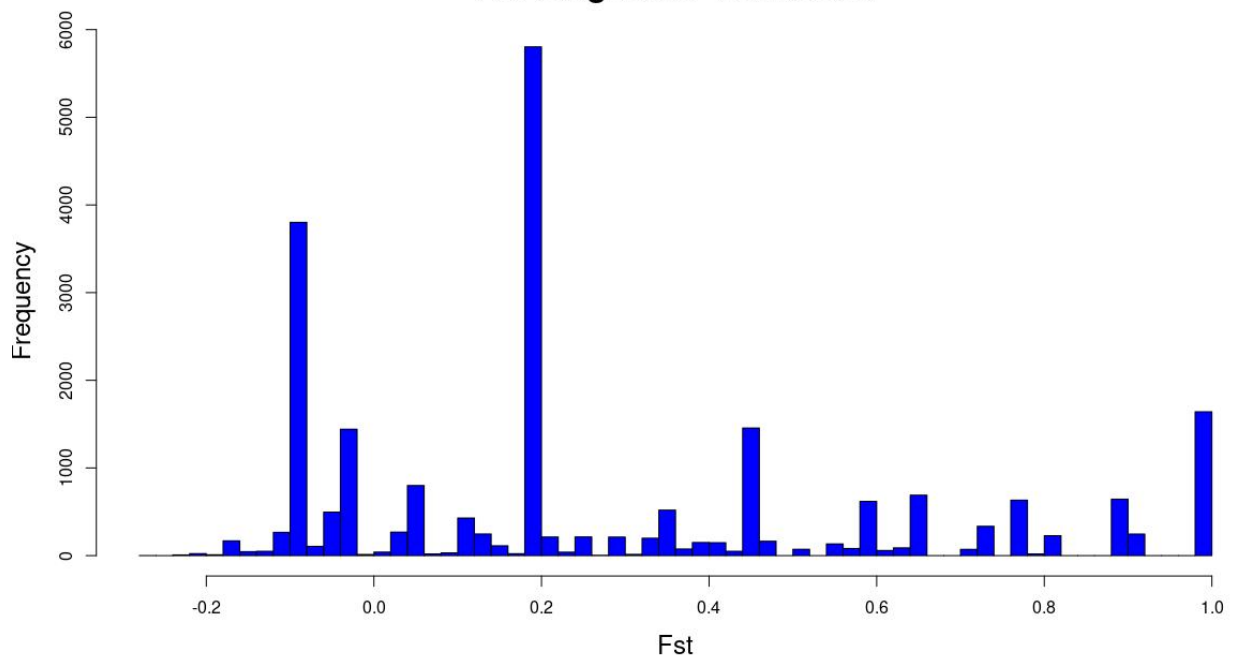
**B)** comparison between *E. cavolini* and *E. verrucosa*



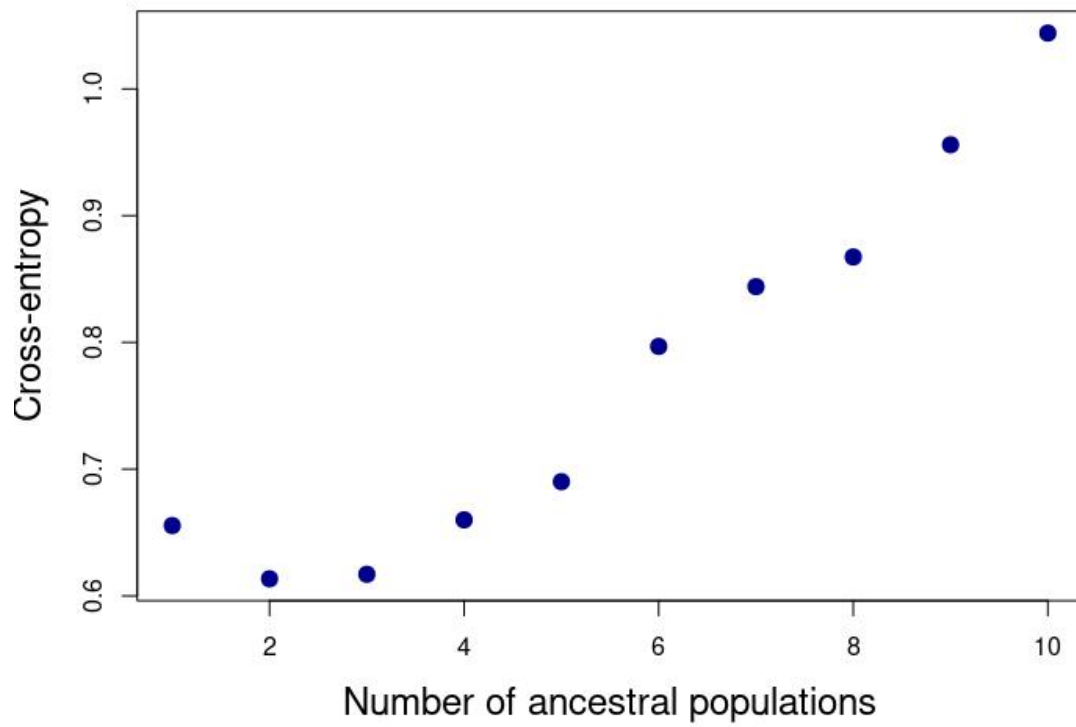


**C)** comparison between *E.singularis* and *E. verrucosa*

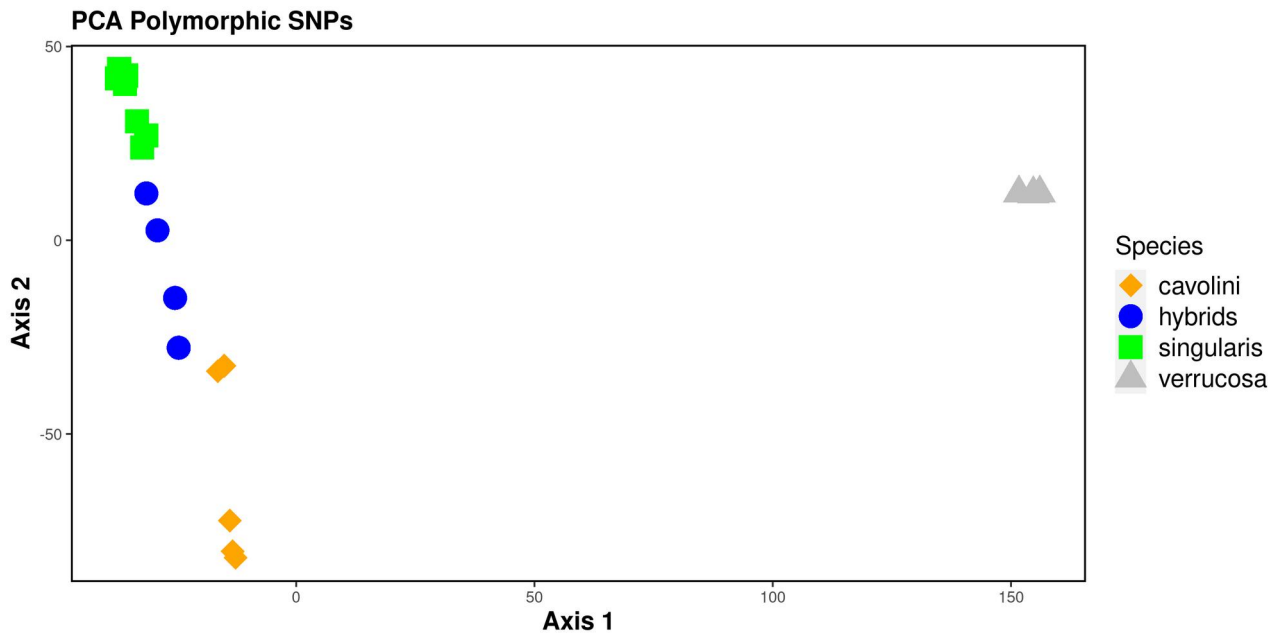
**Fst singularis-verrucosa**



**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'-CCAGCASCYGC GGTAATTCC-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

### Identification 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.

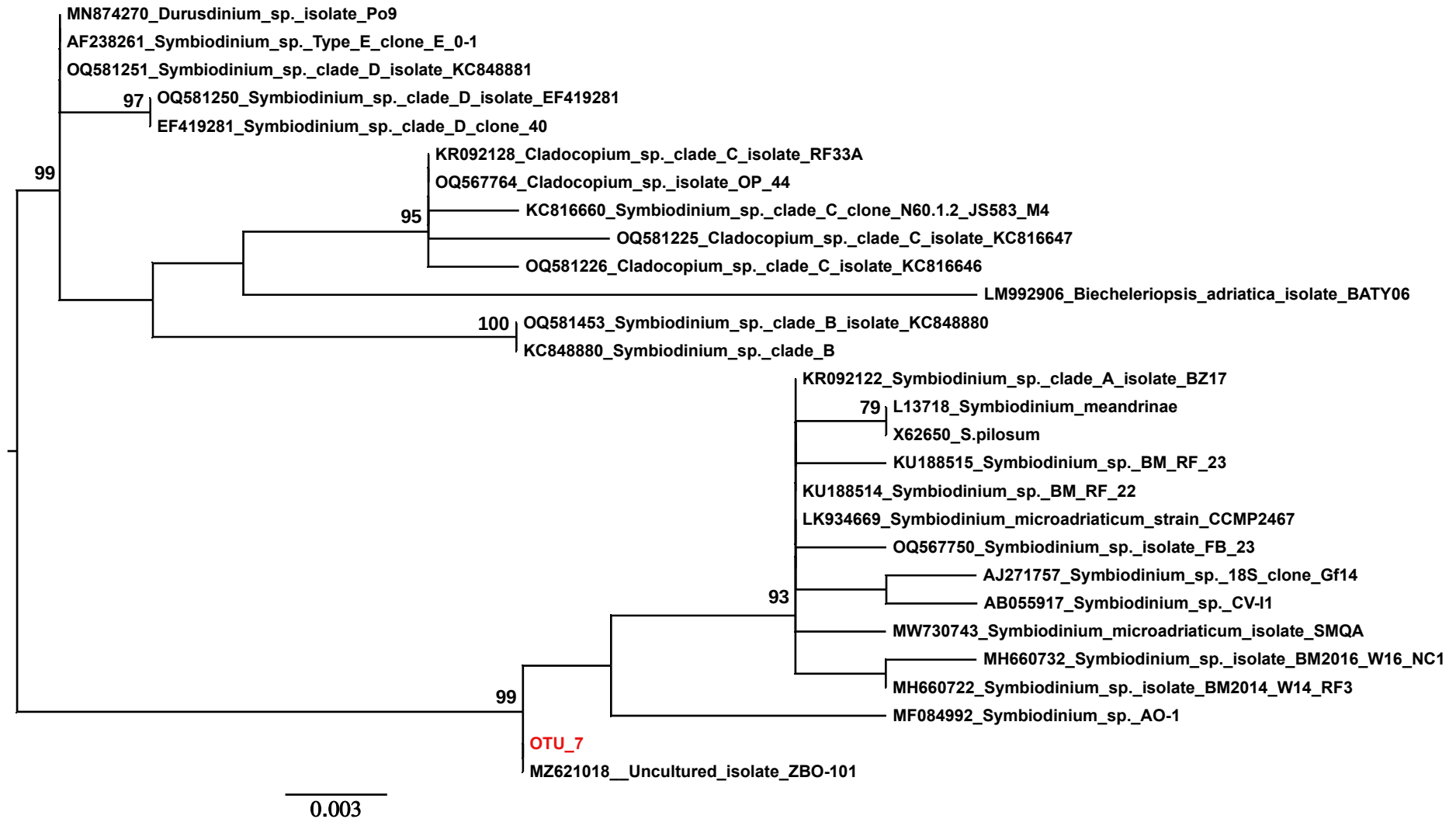
In *E. singularis*, we identified 92 OTUs belonging to the family Symbiodiniaceae, in good agreement with the intracolony diversity of zooxanthellae genotypes previously reported in this host species (Forcioli et al., 2011). Among these OTUs, a single OTU (OTU\_7) was highly dominant and contributed 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

- Clerissi, C., Brunet, S., Vidal-Dupiol, J., Adjeroud, M., Lepage, P., Guillou, L., ... & Toulza, E. (2018). Protists within corals: the hidden diversity. *Frontiers in Microbiology*, 9, 2043. <https://doi.org/10.3389/fmicb.2018.02043>
- Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., ... & Pascal, G. (2018). FROGS: find, rapidly, OTUs with galaxy solution. *Bioinformatics*, 34(8), 1287-1294. <https://doi.org/10.1093/bioinformatics/btx791>
- Forcioli, D., Merle, P. L., Caligara, C., Ciosi, M., Muti, C., Francour, P., ... & Allemand, D. (2011). Symbiont diversity is not involved in depth acclimation in the Mediterranean sea whip Eunicella singularis. *Marine Ecology Progress Series*, 439, 57-71. <https://doi.org/10.3354/meps09314>
- 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.
- Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Jermini, L. S. (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods*, 14(6), 587-589. <https://doi.org/10.1038/nmeth.4285>
- Lajeunesse, T. C., Wiedenmann, J., Casado-Amezúa, P., D'ambra, I., Turnham, K. E., Nitschke, M. R., Oakley, C. A., Goffredo, S., Spano, C. A., & Cubillos, V. M. (2022). Revival of Philozoon Geddes for host-specialized dinoflagellates, 'zooxanthellae', in animals from coastal temperate zones of northern and southern hemispheres. *European Journal of Phycology*, 57(2), 166-180. <https://doi.org/10.1080/09670262.2021.1914863>
- Nguyen, L.-T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Molecular Biology and Evolution*, 32(1), 268-274. <https://doi.org/10.1093/molbev/msu300>
- Okonechnikov, K., Golosova, O., Fursov, M., & Ugene Team. (2012). Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics*, 28(8), 1166-1167. <https://doi.org/10.1093/bioinformatics/bts091>
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1), D590-D596. <https://doi.org/10.1093/nar/gks1219>
- Rambaut, A. (2006). *FigTREE v1.4*. University of Edinburgh. <http://tree.bio.ed.ac.uk/software/figtree/>
- Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M. D., Breiner, H. W., & Richards, T. A. (2010). Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine

anoxic water. *Molecular ecology*, 19, 21-31.  
<https://doi.org/10.1111/j.1365-294X.2009.04480.x>

Vestheim, H., & Jarman, S. N. (2008). Blocking primers to enhance PCR amplification of rare sequences in mixed samples—a case study on prey DNA in Antarctic krill stomachs. *Frontiers in zoology*, 5, 1-11.  
<https://doi.org/10.1186/1742-9994-5-12>