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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 (Brener-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 *Phlozoon* (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

267 **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

295 **Genetic differentiation and analysis of hybrids**

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

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

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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

680 **References**

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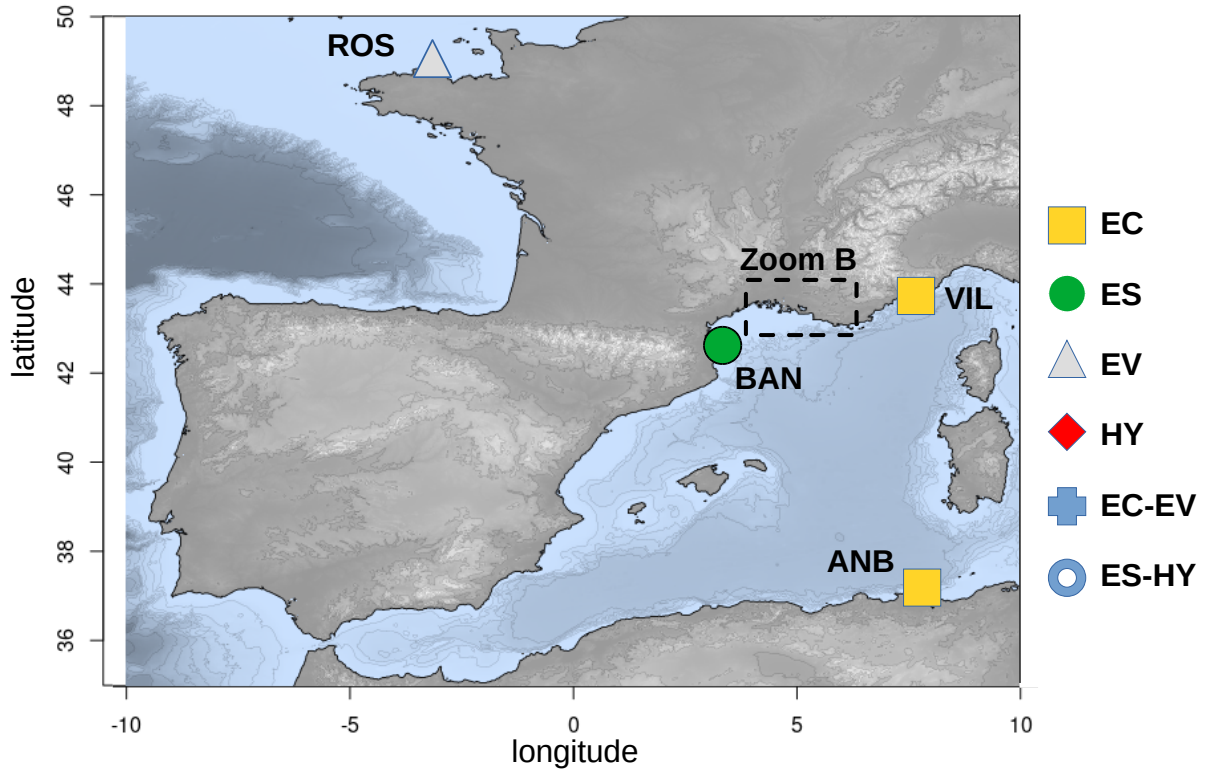
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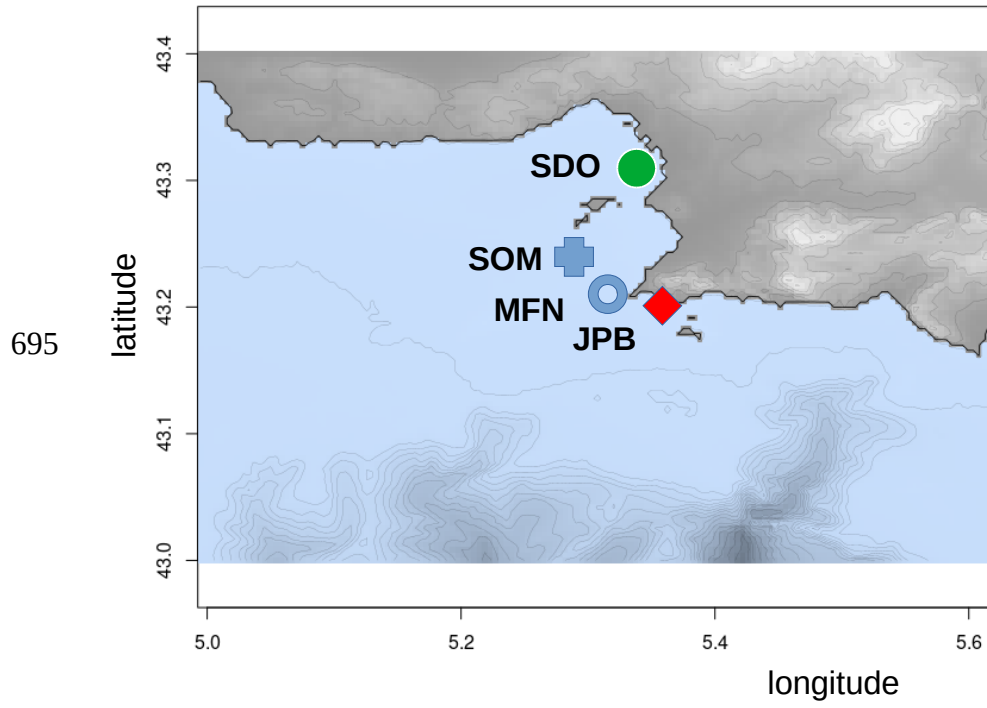
684 **Figure 1:** map of sampling sites; A) general view, B) zoom on the area of
685 Marseille. The symbols present the different samples: EC *E. cavolini*, ES
686 *E. singularis*, EV *E. verrucosa*, HY hybrids, EC-EV *E. cavolini* and *E. verrucosa* in
687 sympatry, ES-HY *E. singularis* and hybrids 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)**



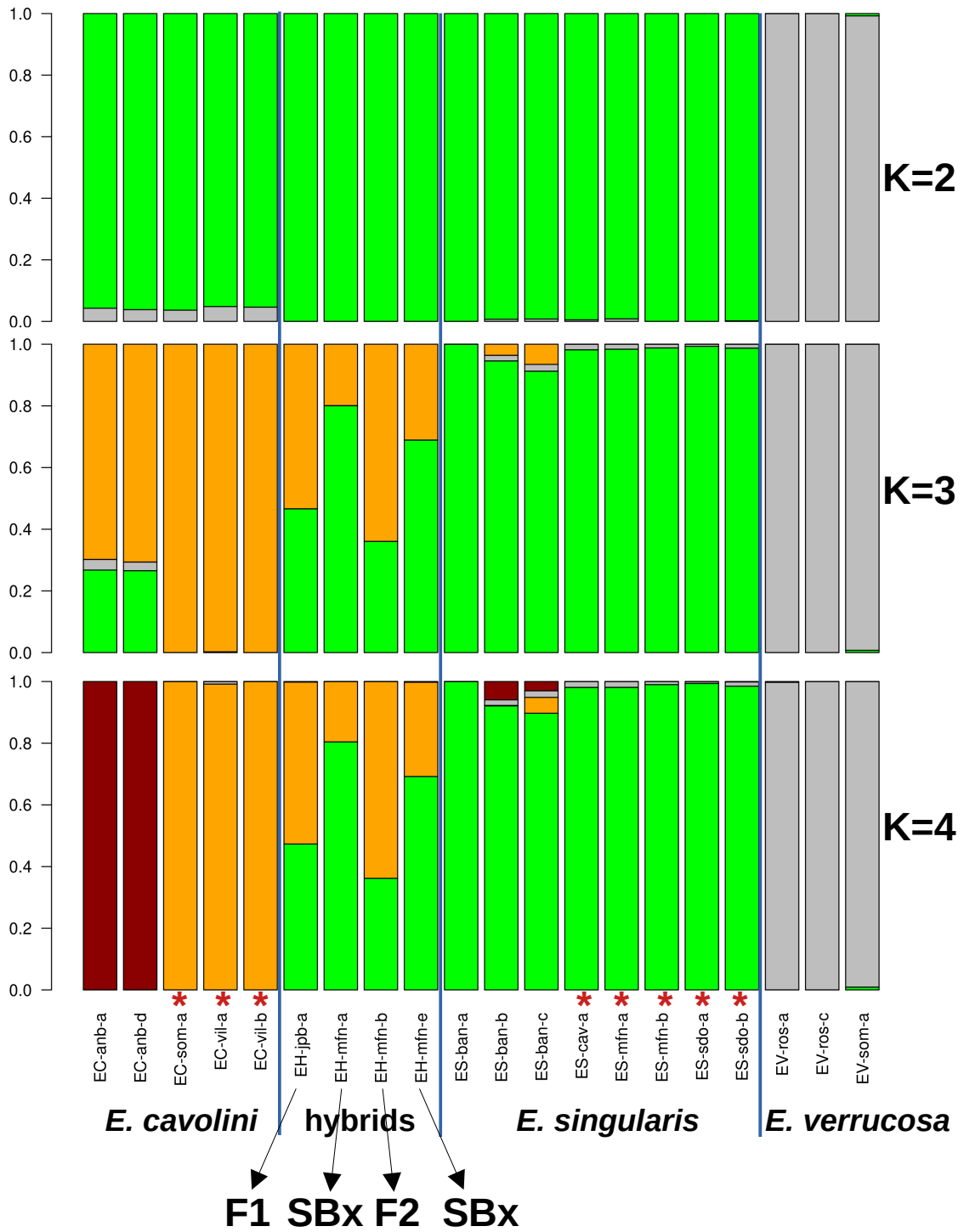
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B)



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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, 1st generation; F2, 2nd 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.

Accession number	Genus	species
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 _{ST} , LEA, PCA
1 % SNPs	without <i>verrucosa</i>	polymorphic sites ; no missing data ; 1 % highest F _{ST} <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} .

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

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

Sample	Species	Raw reads	Transcriptome
e-cavol-anb-a	<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*

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

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

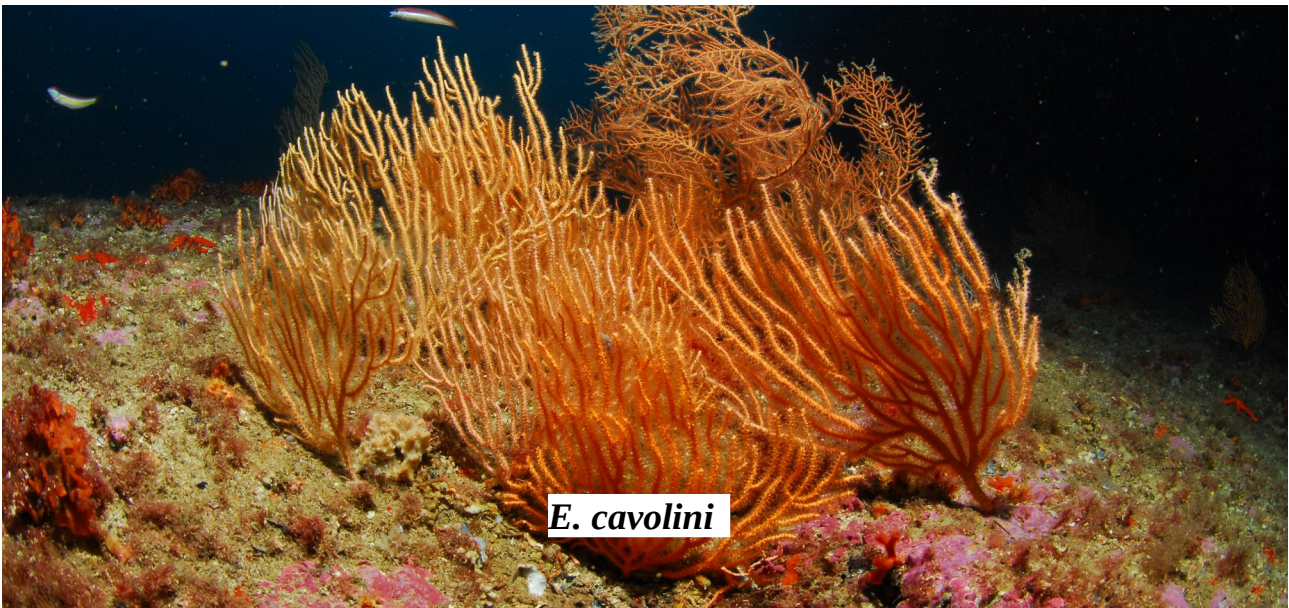
	HPD 0.025	HPD median	HPD 0.0975
constant size, AM			
N_C	630969	744556	875220
N_V	648850	755298	920095
N_A	698501	784512	879664
T_{split}	909392	1054488	1225792
T_{AM}	840920	991118	1147073
M_{CV}	4	6	7
M_{VC}	9	12	14
variable size, AM			
N_C	777526	1099410	1694348
N_V	871210	1230360	1803231
N_A	692366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

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

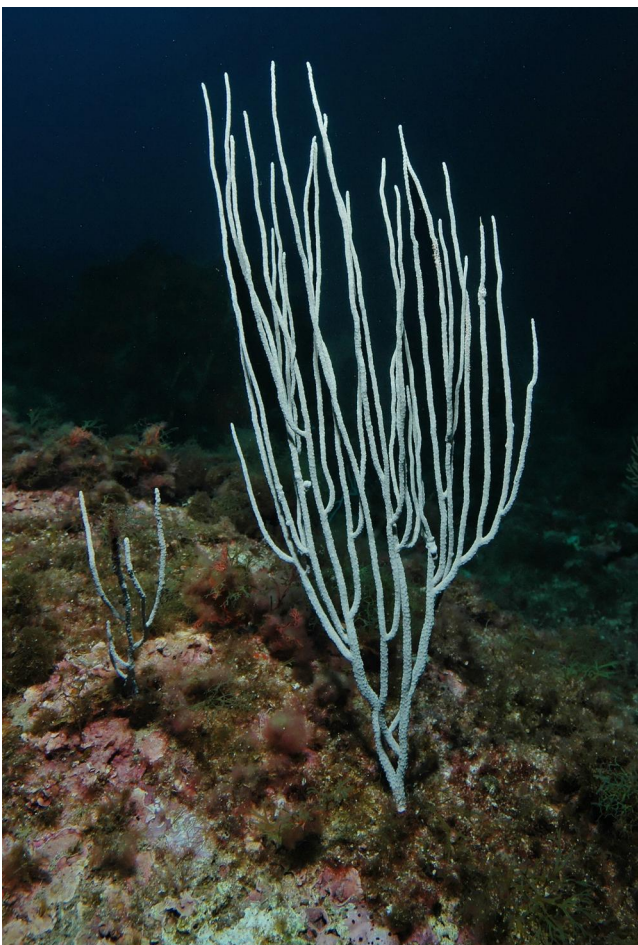
	HPD 0.025	HPD median	HPD 0.0975
constant size, AM			
N_S	263390	298162	336536
N_V	490519	592796	715930
N_A	632004	708517	790246
T_{split}	741840	899098	1091610
T_{AM}	698891	811827	934655
M_{SV}	10	14	17
M_{VS}	21	27	33
variable size, AM			
N_S	281023	386388	494606
N_V	856542	1165039	1566087
N_A	592428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

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

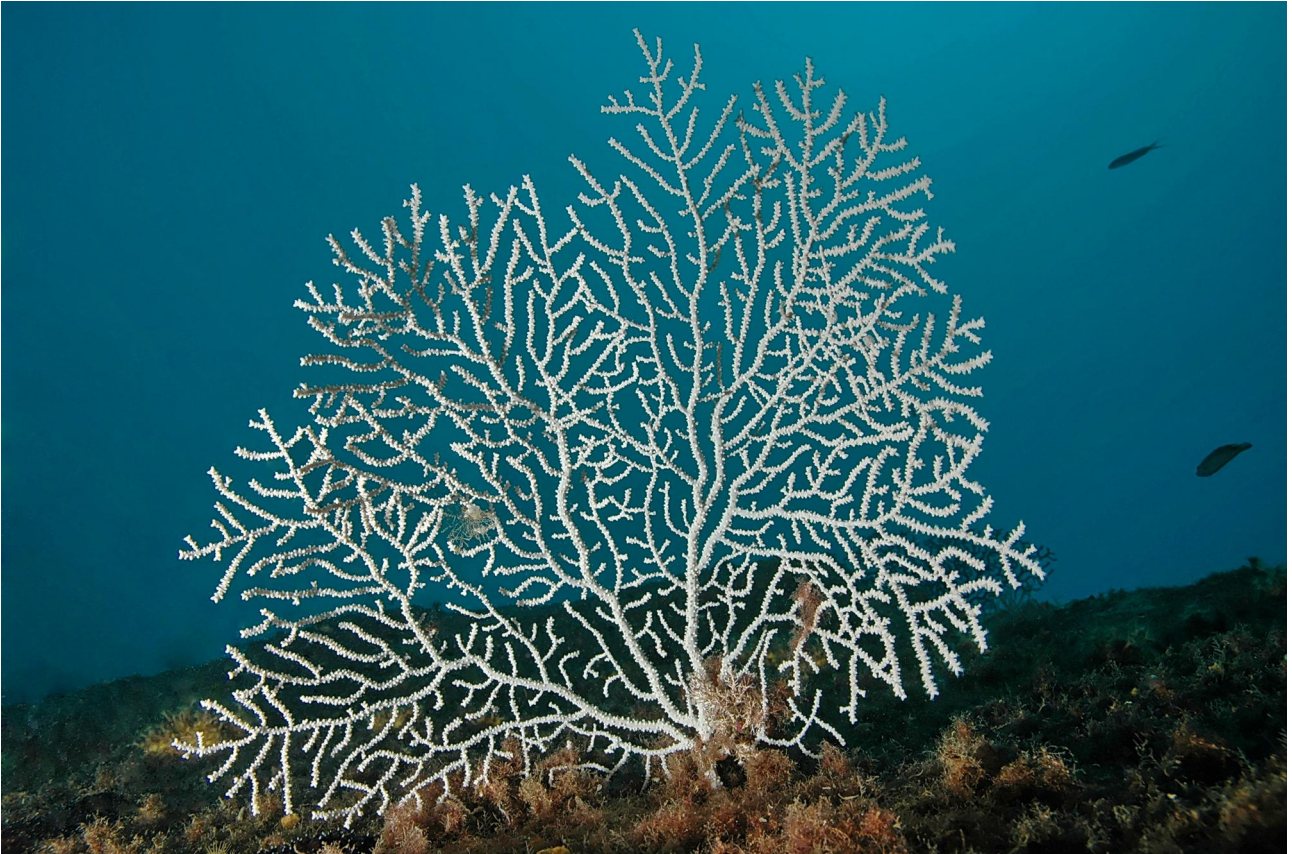
A) example of typical *E. cavolini* colonies (in the foreground)



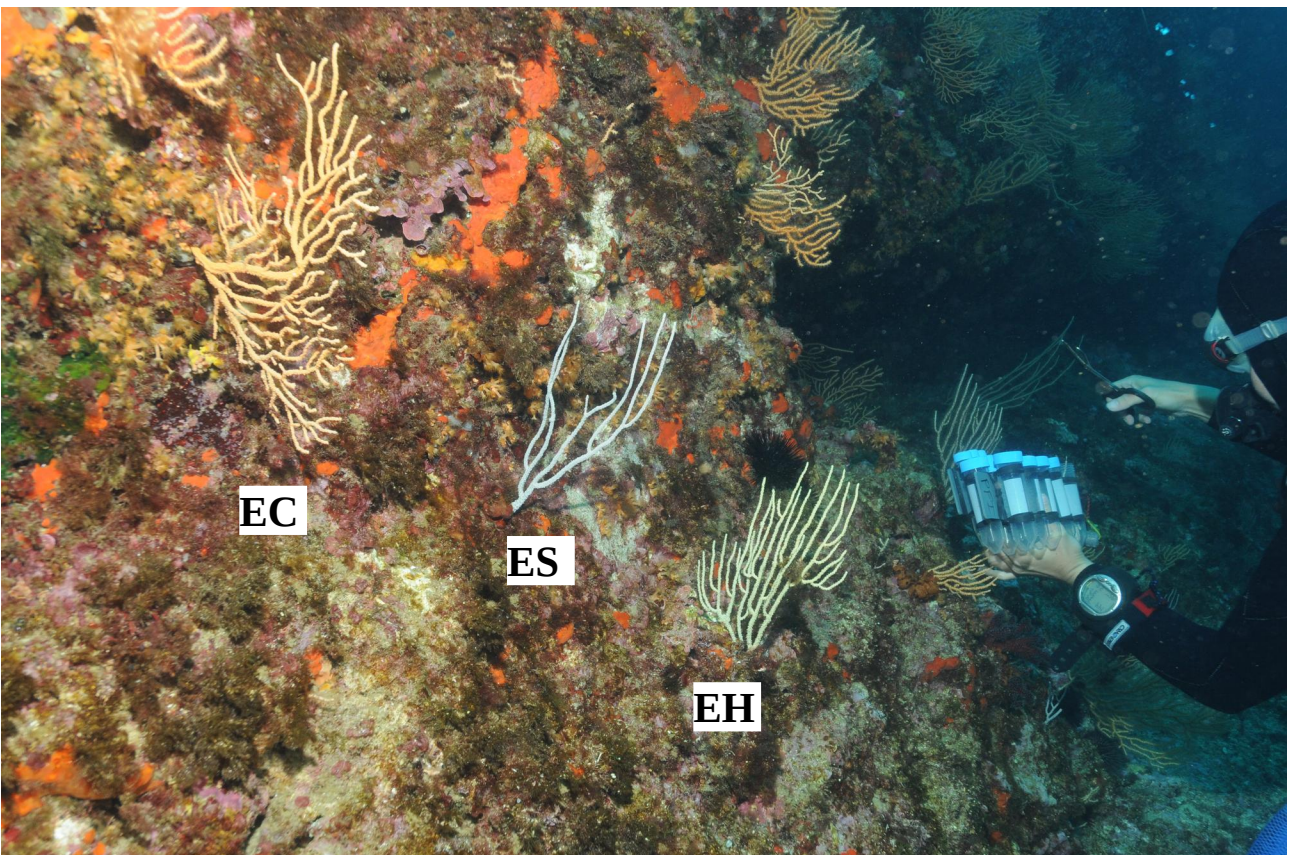
B) example of a typical *E. singularis*



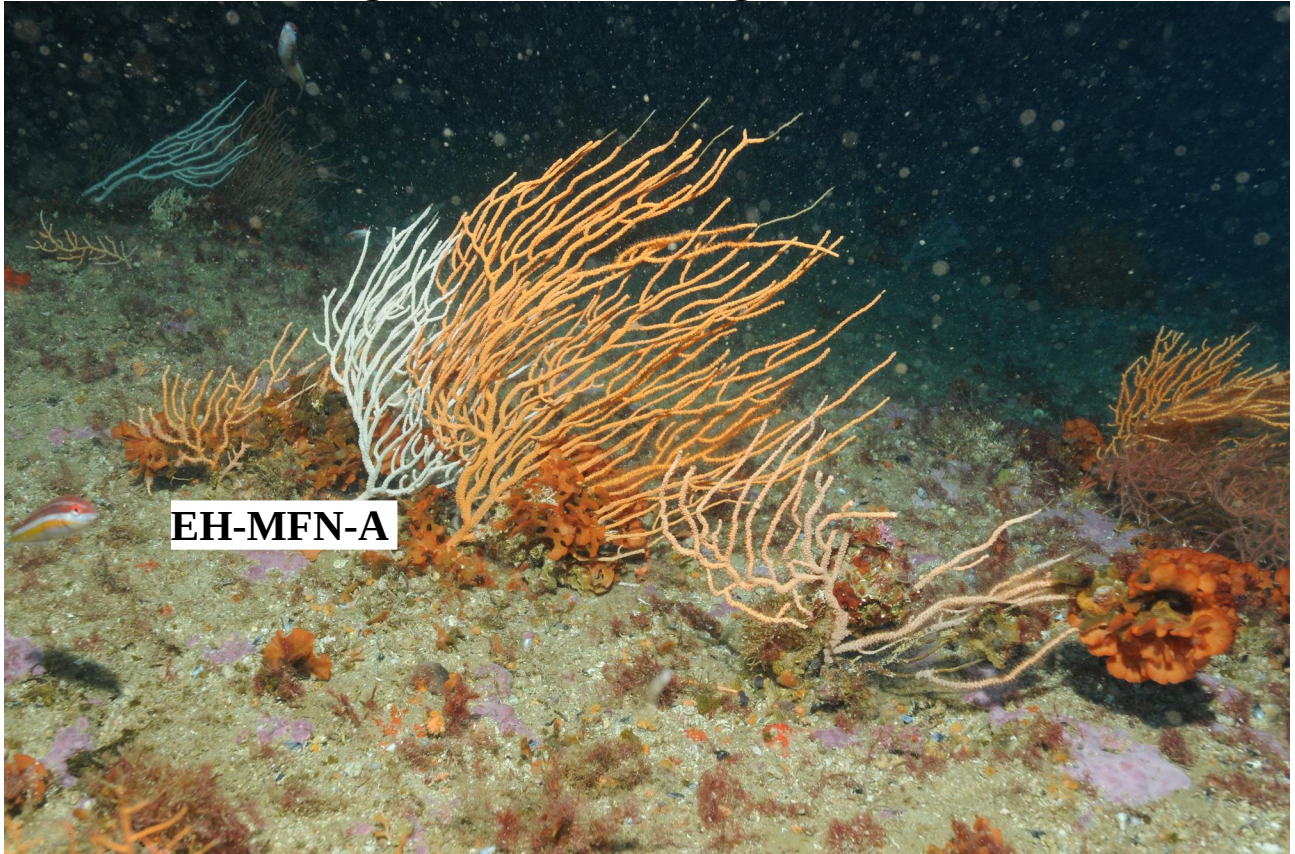
C) example of a typical *E. verrucosa*:



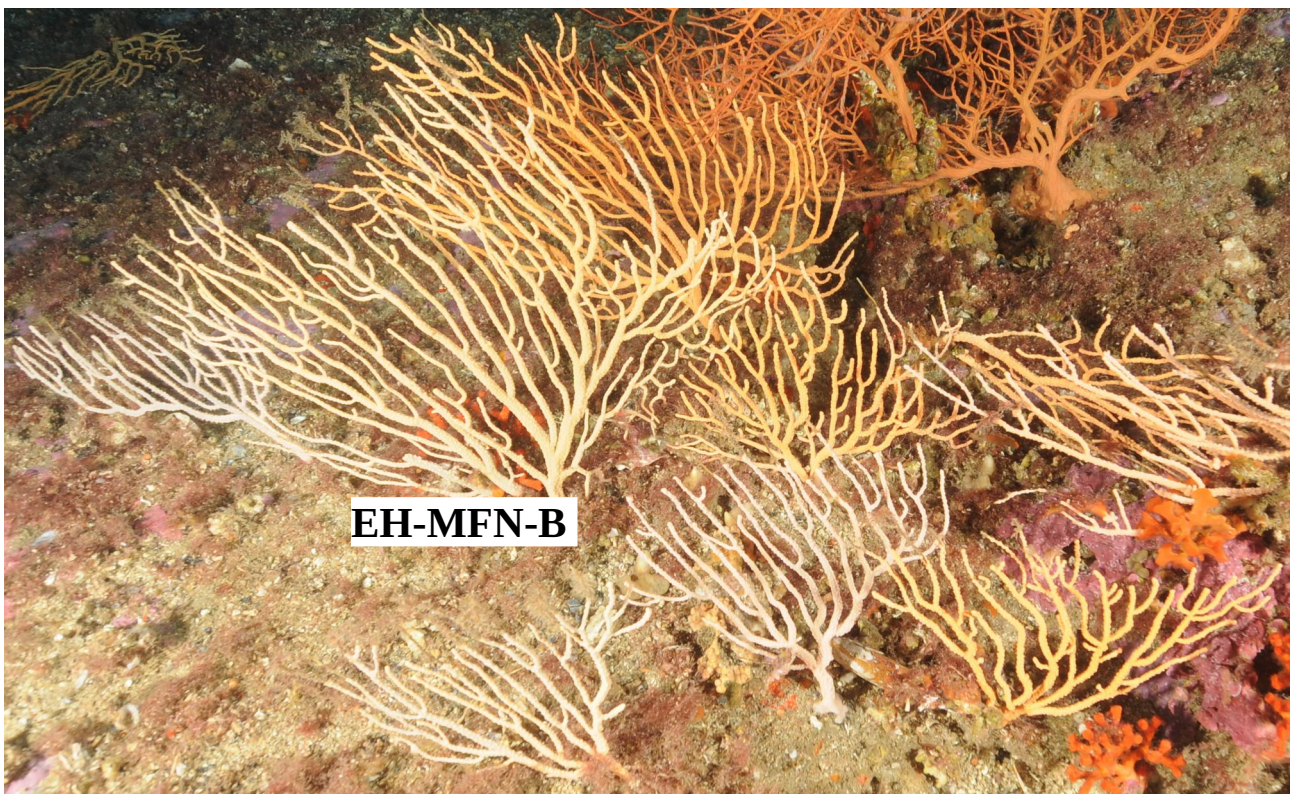
D) sampling with the presence of *E. cavolini* (EC), *E. singularis* (ES) and a potential hybrid (EH)



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



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



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

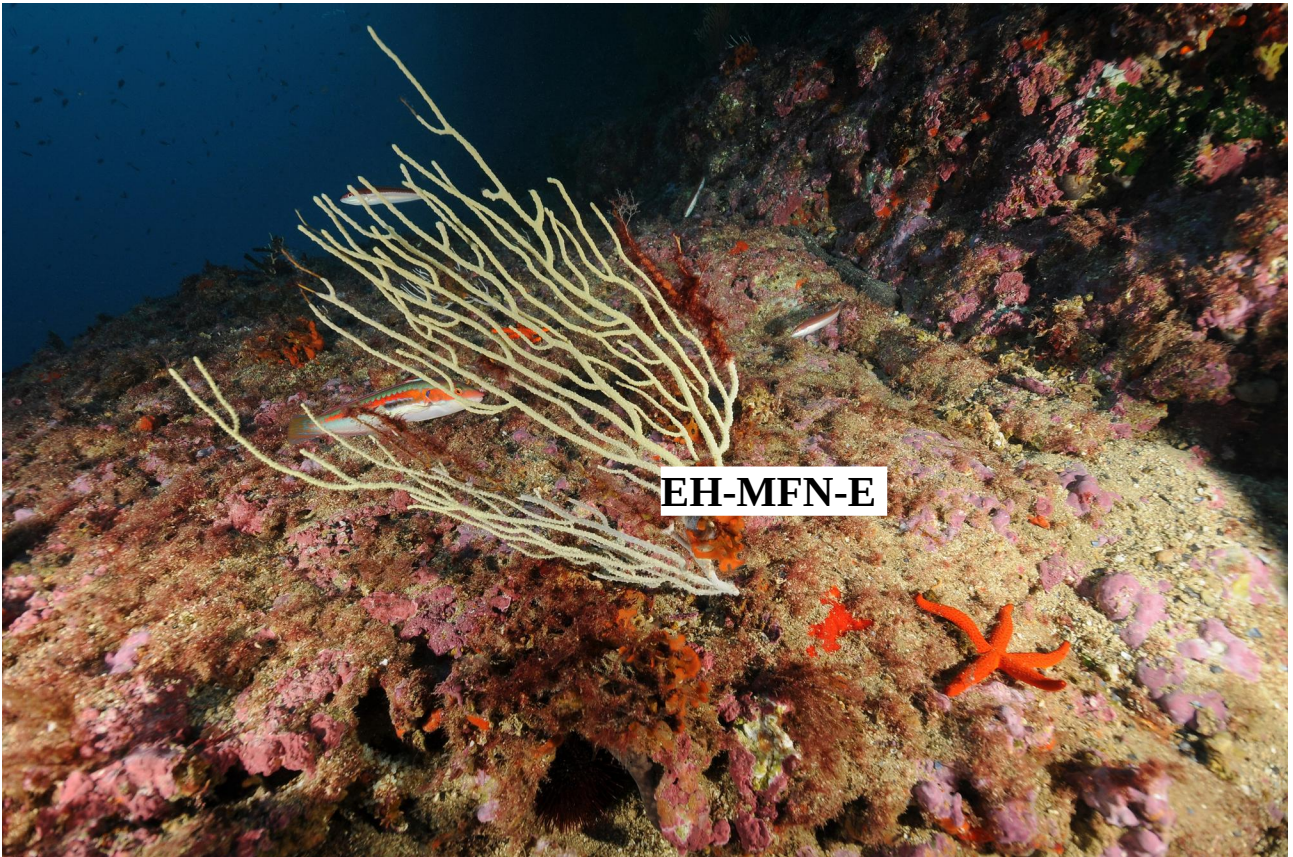


Figure S2: phylogenetic relationships among *Eunicella* species. The phylogenetic reconstruction has been performed with mitochondrial MutS sequences obtained from Genbank, with a search focused on *Eunicella* species. Sequences from the *Complexum* and *Swiftia* genera have been used as outgroups on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

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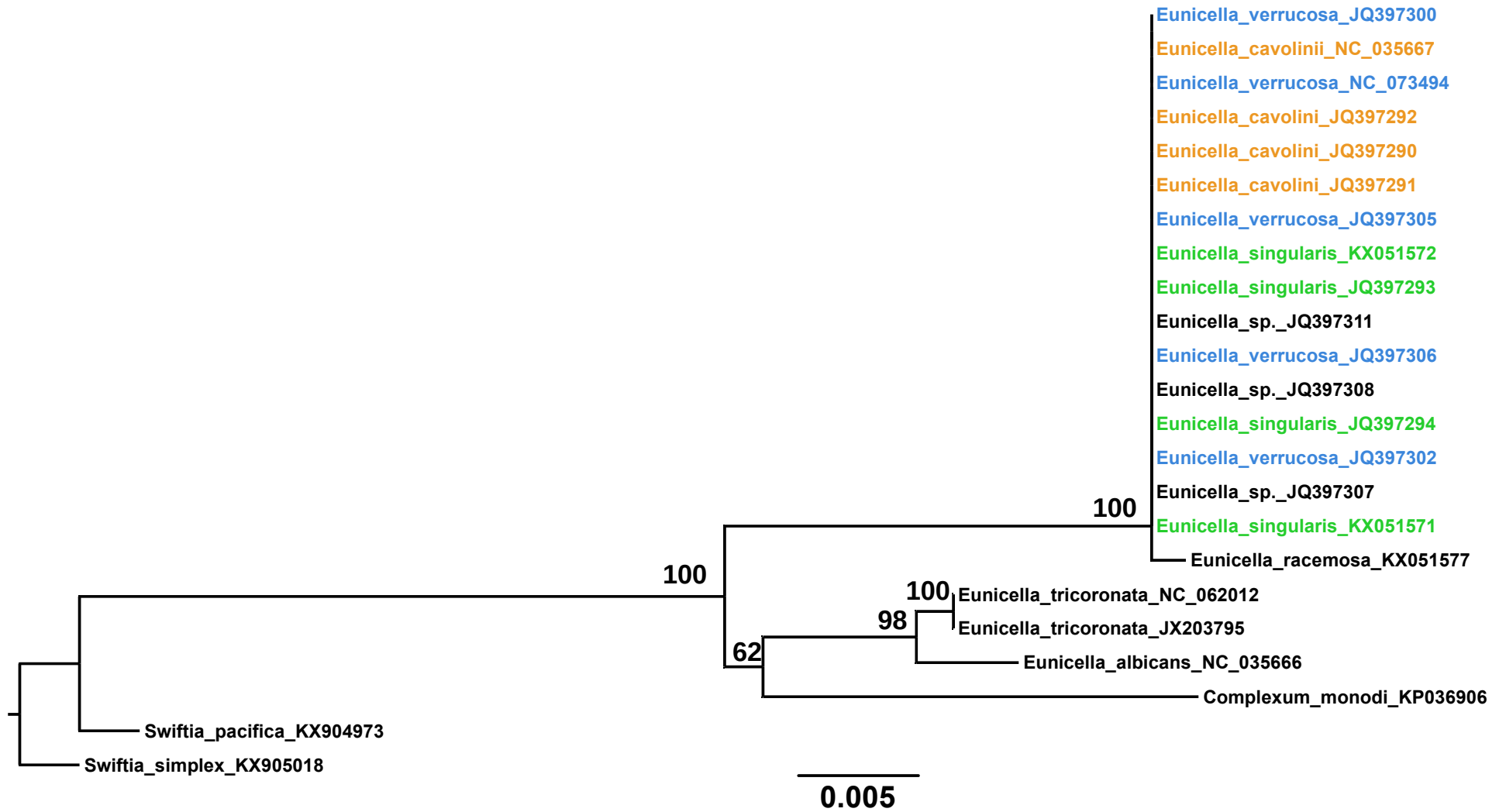
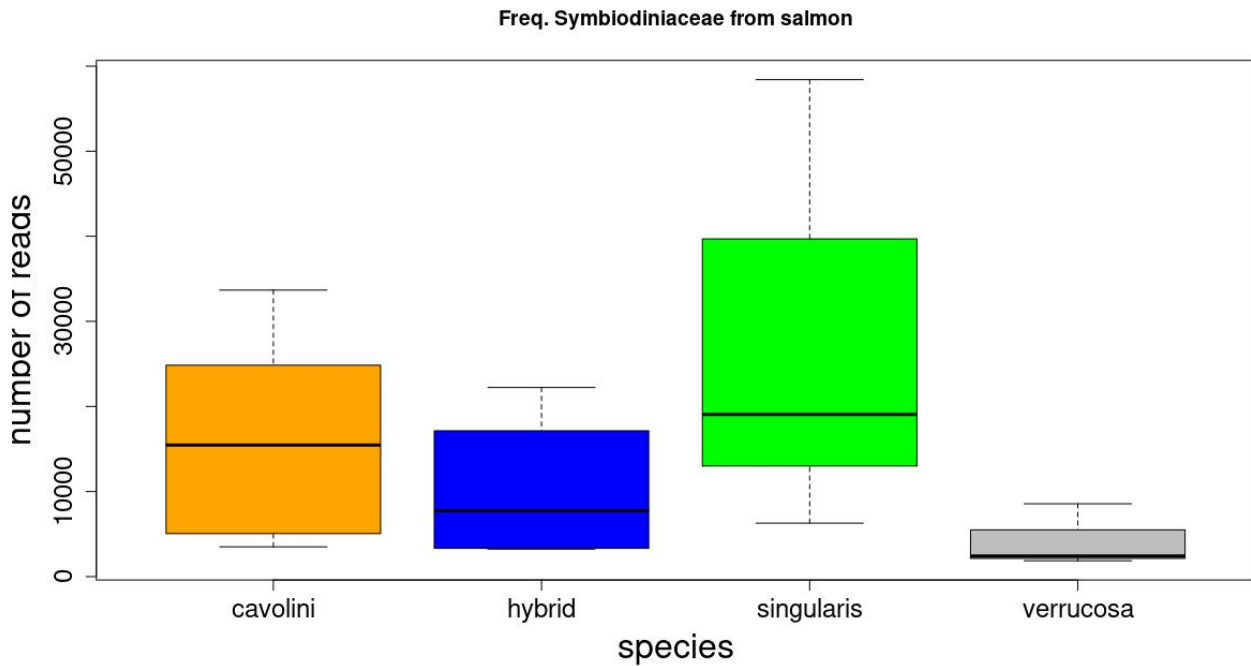


Figure S3: distribution of the frequency of Symbiodiniaceae sequences in the individual transcriptomes according to the species based A) on the number of reads estimated with Salmon, and B) on the proportion of assembled sequences with the BLAT analyses.

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.

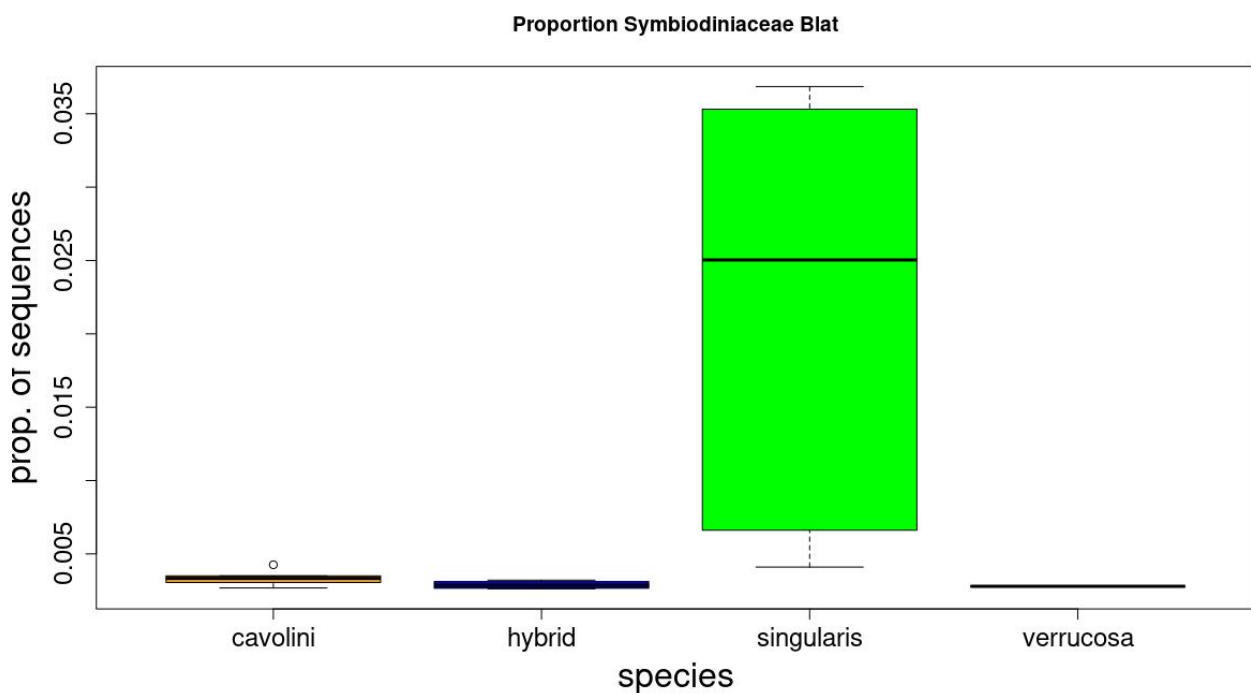
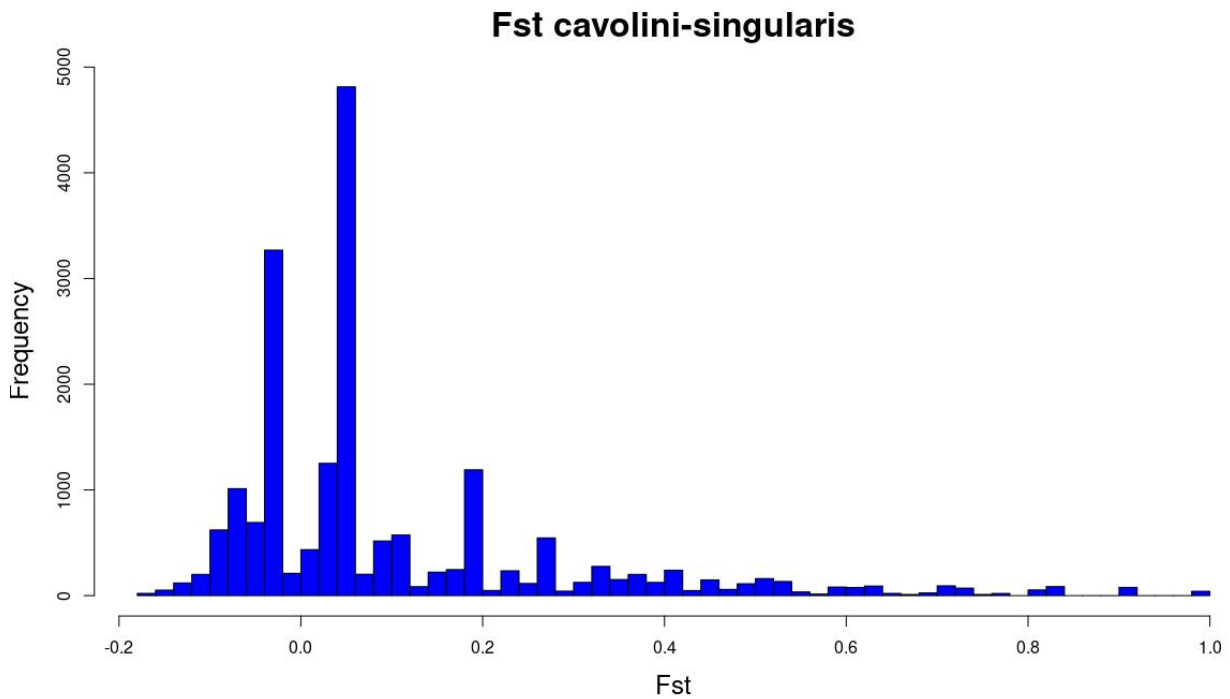
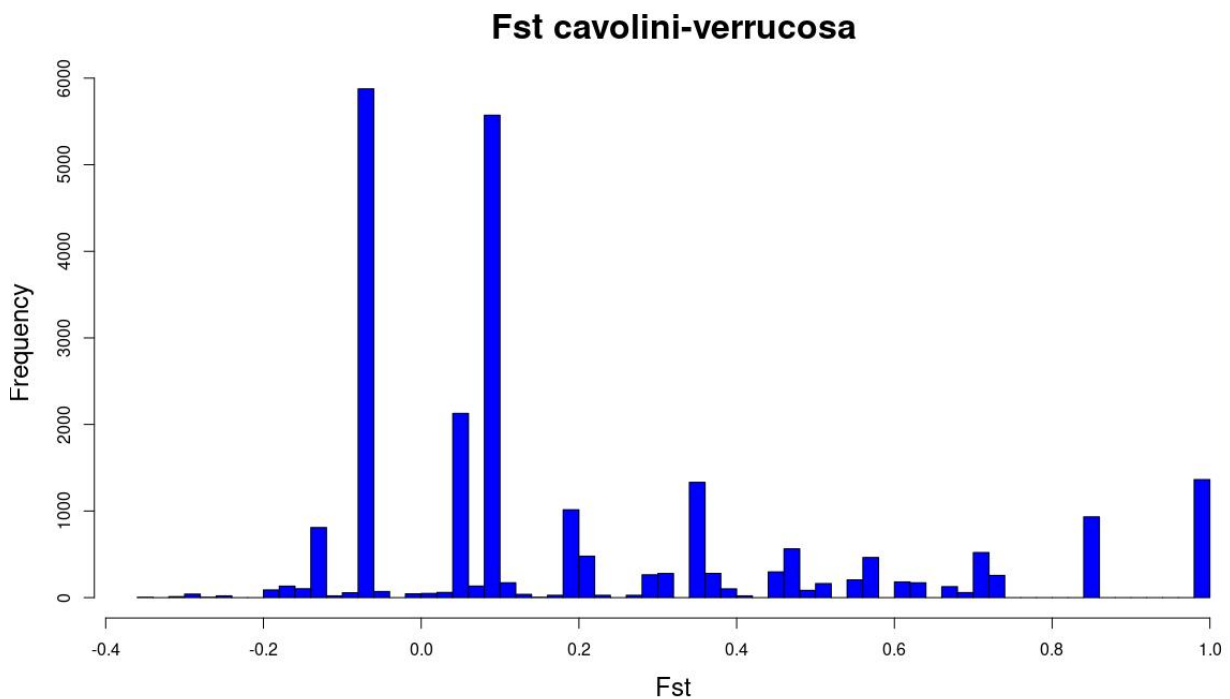


Figure S6: distribution of F_{ST} estimates over loci, for the pairwise comparisons among the three species, with the exclusion of potential hybrids.

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

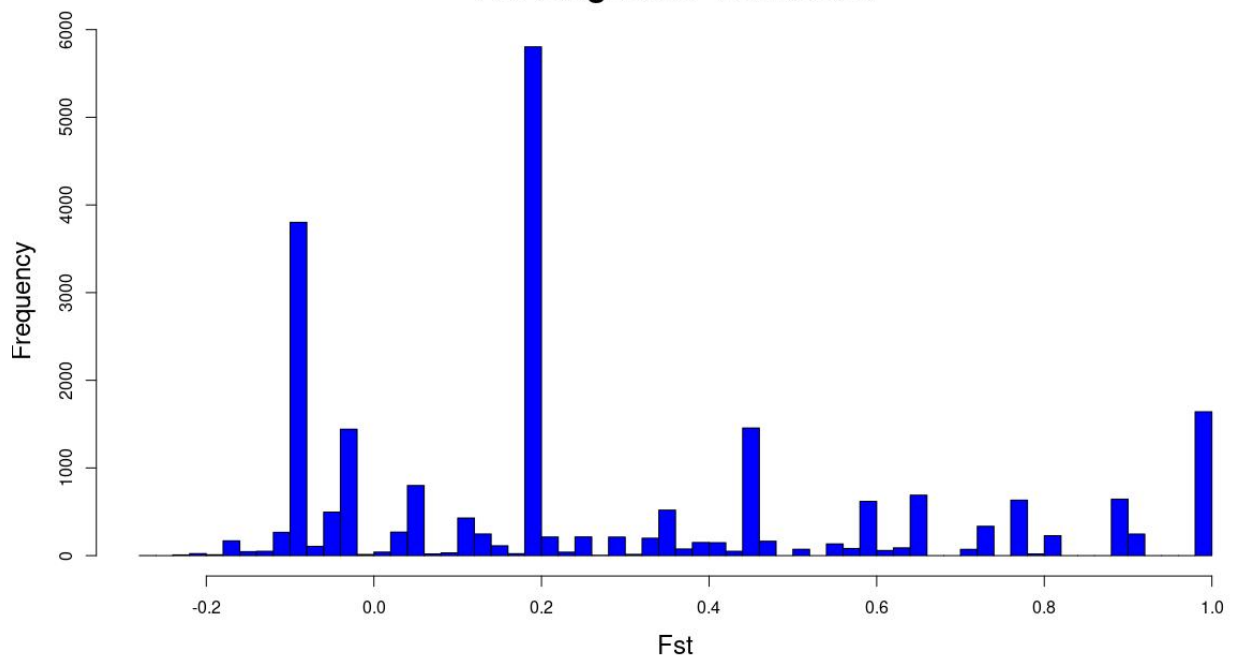


Figure S5: result of the cross-entropy analysis with the LEA R package

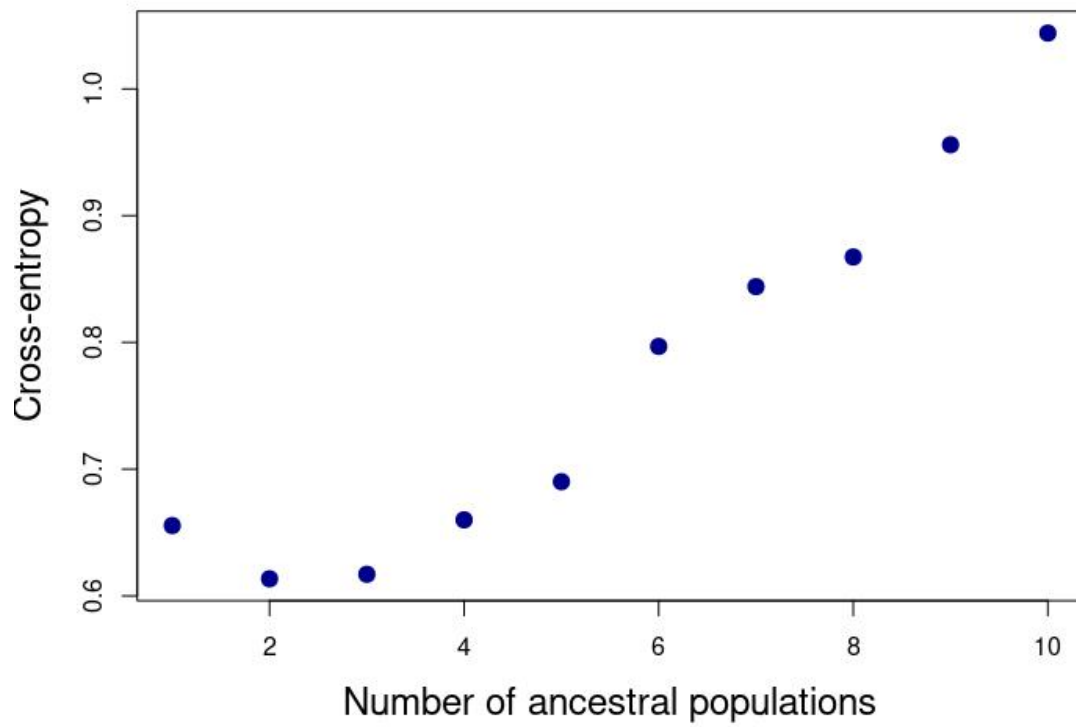
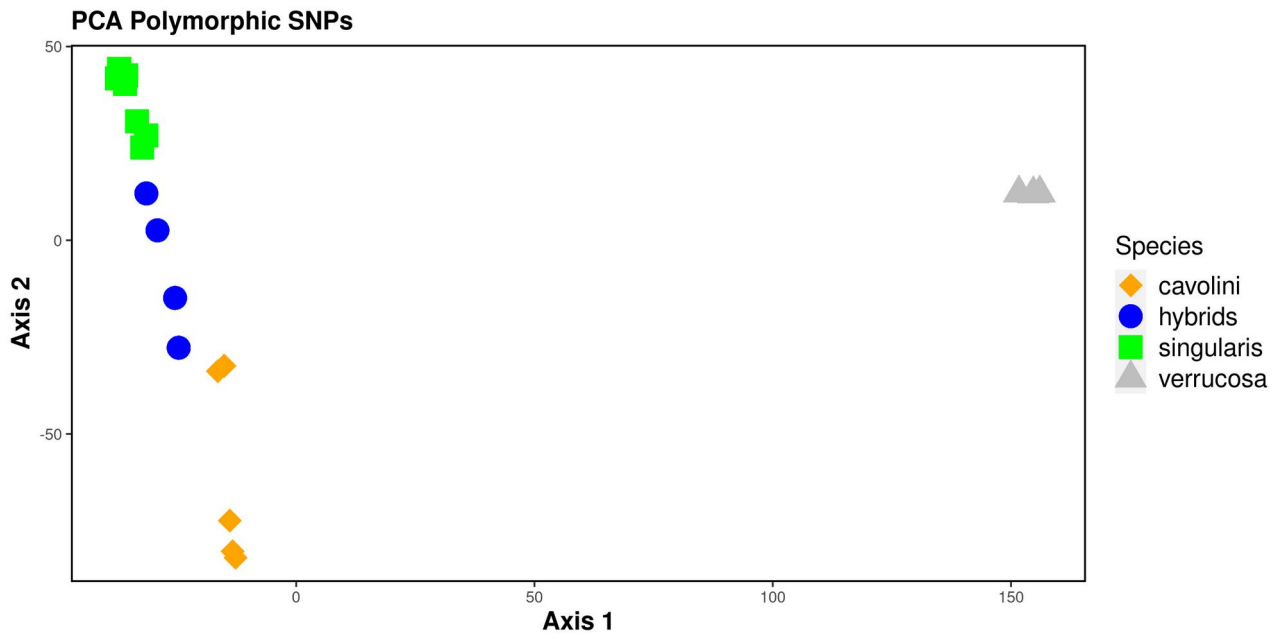


Figure S6: Principal Component Analysis based on the “polymorphic SNPs” dataset. The axis 1 corresponded to 33.2% of the variance, and axis 2 to 13% of the variance.



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).

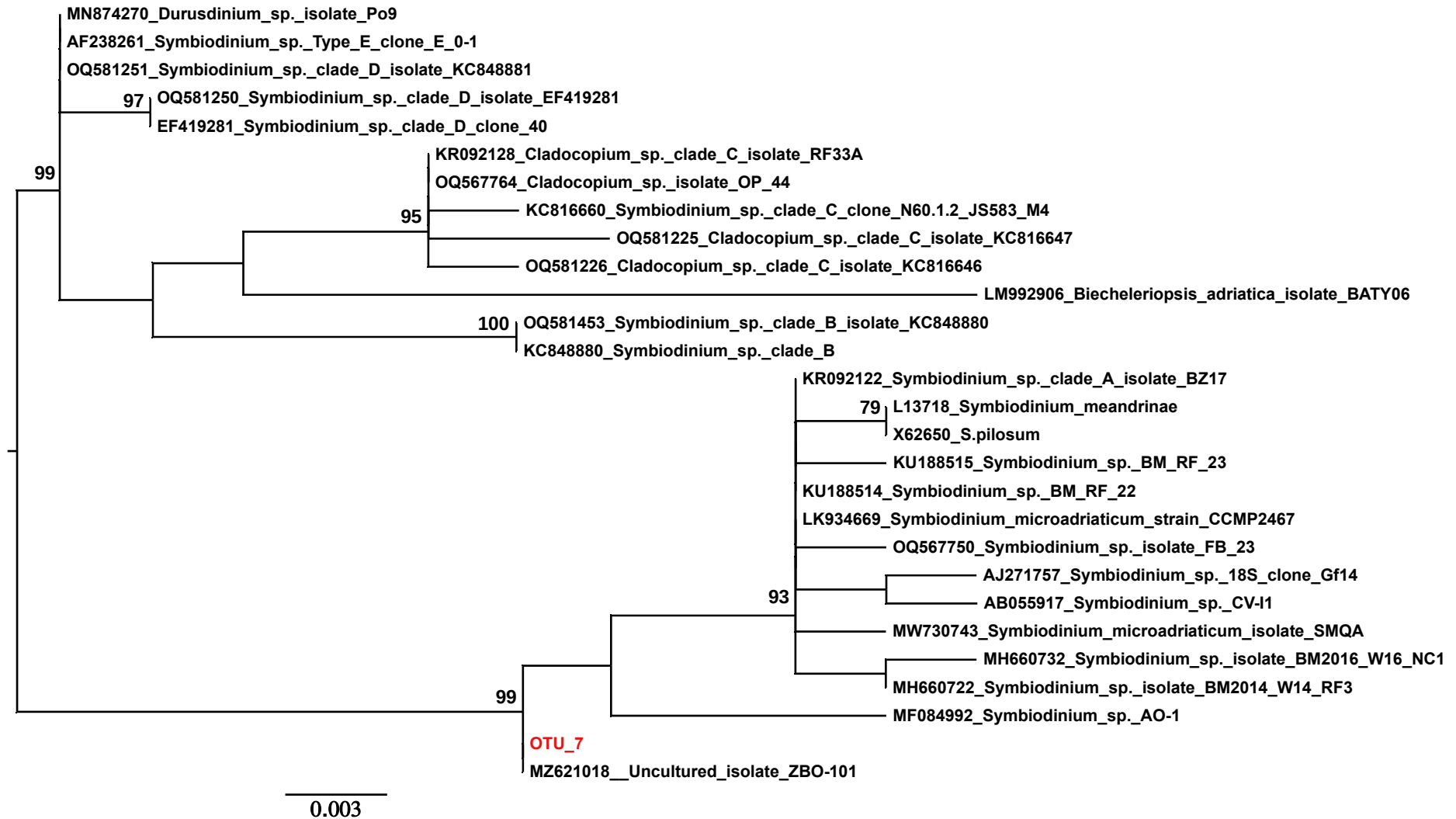


Figure S2.1. Maximum-likelihood phylogeny of Symbiodiniaceae based on the variable V4 region of the 18S rRNA gene, illustrating the relationship of OTU_7 sequence (in red) with Symbiodiniaceae spp. belonging to Clade A. The numbers to the left of the nodes indicate the percentages of bootstraps, for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

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