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1 **Fuzzy species limits in Mediterranean gorgonians (Cnidaria, Octocorallia) :**  
2 **inferences on speciation processes**

3

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24

25 **Running title:** Species limits in Mediterranean octocorals

26 Aurelle *et al.*

27

28 **Abstract**

29 The study of the interplay between speciation and hybridization is of primary

30 importance in evolutionary biology. Octocorals are ecologically important species

31 whose shallow phylogenetic relationships often remain to be studied. In the

32 Mediterranean Sea, three congeneric octocorals can be observed in sympatry: *Eunicella*

33 *verrucosa*, *E. cavolini* and *E. singularis*. They display morphological differences and *E.*  
34 *singularis* hosts photosynthetic *Symbiodinium*, contrary to the two other species. Two  
35 nuclear sequence markers were used to study speciation and gene flow between these  
36 species, through network analysis and Approximate Bayesian Computation (ABC).  
37 Shared sequences indicated the possibility of hybridization or incomplete lineage  
38 sorting. According to ABC a scenario of gene flow through secondary contact was the  
39 best model to explain these results. At the intra-specific level neither geographical nor  
40 ecological isolation corresponded to distinct genetic lineages in *E. cavolini*. These  
41 results are discussed in the light of the potential role of ecology and genetic  
42 incompatibilities in the persistence of species limits.

43

44 **Keywords:** octocorals, speciation, hybridization, *Eunicella*, intron, ABC

45

46

## 47 **Introduction**

48

49 Since Darwin's (1859) seminal work, the question of species formation has remained  
50 central in evolutionary biology. The role of ecological differentiation in promoting and  
51 maintaining speciation has received increasing attention over the past several years  
52 (Bierne *et al* 2013; Nosil, Harmon & Seehausen 2009; Roy *et al* 2016). In particular,  
53 recent reappraisals of gene flow between species have led to the proposal that  
54 speciation with gene flow, or of secondary contact between well-differentiated species  
55 might be more common than previously thought (Hey & Pinho, 2012; Roux *et al* 2013,  
56 2016). The development of new molecular markers, as well as improved analytical  
57 tools, such as Isolation with Migration models and Approximate Bayesian  
58 Computations (ABC, Beaumont 2010; Hey 2010), allowed novel insights about the  
59 dynamics of speciation. For instance such approaches have shown that the levels of  
60 gene flow between species can be very different between loci (Roux *et al* 2013). These  
61 studies confirm that speciation is a continuous process ranging from intra-specific  
62 differentiation to complete reproductive isolation (Feder *et al* 2012). They also allow the  
63 re-evaluation of the role of ecology in speciation: are ecological differences drivers of

64 speciation or do they highlight genetic incompatibilities that accumulated in allopatry  
65 (Bierne *et al* 2013)?

66 The problem of species delimitation in light of ecological differentiation is particularly  
67 important in corals (i.e. hexa- and octocorals). Phenotypic plasticity and cryptic species  
68 are frequent in corals, and genetic markers are often helpful to study species limits  
69 (Marti-Puig *et al* 2014; McFadden *et al* 2010; Sanchez *et al* 2007). As corals are deeply  
70 impacted by climate change (Garrabou *et al* 2009; Hoegh-Guldberg 2014), accurate  
71 species delimitation is also important to study the response of coral communities to  
72 climate change. Morphologically similar coral species can correspond to distinct genetic  
73 entities with potentially different responses to climate change (Boulay *et al* 2014). For  
74 example, the adaptation to different depths in the octocoral *Eunicea flexuosa* has been  
75 linked to the existence of two distinct genetic lineages (Prada & Hellberg 2013), and  
76 distinct lineages of the endosymbiont dinoflagellate (*Symbiodinium*) are tightly linked  
77 with the different *Eunicea* lineages (Prada *et al* 2014). Conversely, hybridization can be  
78 a source of evolutionary novelty and new adaptation (Rieseberg *et al* 2003; Thomas *et*  
79 *al* 2014). Several cases of hybridization have been demonstrated in hexacorals (Thomas  
80 *et al* 2014; Vollmer & Palumbi 2004) and in octocorals (McFadden & Hutchinson  
81 2004). Additionally, the analysis of genetic connectivity, an important driver of  
82 evolution, must be based on sound delimitation of species (Pante *et al* 2015b).

83 Mediterranean octocorals of the genus *Eunicella* provide an interesting case study of  
84 speciation processes. Six *Eunicella* species are found in the Mediterranean Sea, but only  
85 three are abundant: *E. verrucosa* (Pallas, 1766), *E. cavolini* (Koch, 1887), *E. singularis*  
86 (Esper, 1791) (Carpine & Grasshoff 1975). *E. cavolini* and *E. singularis* are endemic to  
87 the Mediterranean Sea whereas *E. verrucosa* is also found in the Atlantic Ocean, as far  
88 north as southwestern England, where it is more abundant. In some parts of the North  
89 Mediterranean, these three species are observed in sympatry. They can be distinguished  
90 on the basis of colony architecture and calcareous sclerites (Carpine & Grasshoff 1975).  
91 Nevertheless these morphological characters may be plastic, and can vary along a depth  
92 gradient in *E. singularis* (Gori *et al* 2012). From an ecological point of view, *E.*  
93 *singularis* is generally observed at shallower sites than the two other species. *Eunicella*  
94 *singularis* is the only Mediterranean octocoral harbouring the photosynthetic

95 endosymbiont *Symbiodinium*, although asymbiotic individuals have been observed in  
96 deep water (Gori *et al* 2012). *Eunicella* species have been affected by mass mortality  
97 events linked with positive thermal anomalies (Garrabou *et al* 2009). Different  
98 responses to thermal stress have been observed between *E. singularis* and *E. cavolini*  
99 which raises the question of the evolution of thermotolerance along with speciation  
100 (Ferrier-Pagès *et al* 2009; Pey *et al* 2013; Pivotto *et al* 2015).

101 From a genetic point of view, the phylogeny and delimitation of *Eunicella* species  
102 remain poorly studied, partially because of the lack of suitable markers. As observed in  
103 other octocorals, mitochondrial DNA has a very slow evolution rate (Shearer *et al*  
104 2002). As a consequence, no difference has been observed for the mitochondrial genes  
105 COI and mtMutS between these three *Eunicella* species (Calderón *et al* 2006; Gori *et al*  
106 2012). Similarly, ITS 1 and 2 did not allow species delimitation, potentially because of  
107 incomplete concerted evolution (Calderón *et al* 2006; Costantini *et al* 2016). Single  
108 copy nuclear markers are then required for an accurate analysis of species limits in  
109 octocorals (e.g. Concepcion *et al* 2008; Wirshing & Baker 2015). The comparison of  
110 sympatric and allopatric *Eunicella* samples would allow testing if the lack of divergence  
111 is the consequence of recent divergence, slow molecular evolution or hybridization. In  
112 order to investigate these questions, we used one mitochondrial marker, the COI – *igr1*  
113 (intergenic region; McFadden *et al* 2011) and two nuclear Exon Priming Intron  
114 Crossing (EPIC) markers. COI – *igr1* might be more variable and efficient for species  
115 delimitation than COI alone or mtMutS. The objectives of this study were to analyse the  
116 phylogenetic relationships and divergence levels between *Eunicella* species, and to test  
117 the possibility of gene flow between them. In addition, we tested if geographical or  
118 ecological isolation could correspond to distinct, cryptic, genetic lineages in *E. cavolini*,  
119 by analysing samples from distant areas in the Mediterranean Sea, and from different  
120 depths at the same site.

121

## 122 **Materials and methods**

123

### 124 *Sampling*

125 Samples of *Eunicella* spp. were collected by scuba diving in the Mediterranean Sea and

126 Atlantic Ocean (Figure 1; Table S1) with a particular focus on the area of Marseille,  
127 where our three focal species can be found in sympatry. Here, *E. cavolini* and *E.*  
128 *singularis* were sampled together at three sites (Maïre, Sormiou, Méjean) . *E. verrucosa*  
129 was sampled along with *E. cavolini* at one site (Somlit) located near Maïre. In three  
130 locations in Marseille, we also sampled *E. cavolini* at two depths (20 and 40 m) in order  
131 to test for species homogeneity along depths which correspond to different  
132 thermotolerance levels (Pivotto *et al* 2015). Colonies with morphologies intermediate  
133 between *E. cavolini* and *E. singularis* were also sampled at two sites in Marseille:  
134 Sormiou and Maïre (Figure S1). At the sampling depths of *E. singularis*, the *aphyta*  
135 morphotype (without *Symbiodinium*) is very rare, so all colonies were considered as  
136 symbiotic (Gori *et al* 2012).

137

### 138 *Molecular analyses*

139 Total genomic DNA was extracted with the Qiagen DNeasy kit according to the  
140 manufacturer's instructions or with Macherey-Nagel's NucleoSpin kit on an epMotion  
141 5075 VAC automated pipetting system (Eppendorf). We amplified the mitochondrial  
142 marker COI-igr1 with primers defined in McFadden *et al* (2011) on a subset of 37  
143 individuals (Table S2). Two nuclear loci were amplified for all individuals. These  
144 markers were developed from transcriptome sequences obtained from *Paramuricea*  
145 *clavata* (Mokhtar-Jamaï *et al* unpublished). The putative function of two genes was  
146 identified through a search in the Uniprot database: Ferritin (hereafter FER) and  
147 Apoptosis Induction Factor (hereafter AIF). Degenerate primers were defined by  
148 aligning these sequences with Metazoan sequences obtained from a Blast search in  
149 Genbank. We could then amplify specifically these genes in *Eunicella* spp. and we  
150 retained primer pairs allowing the amplification of introns (i.e. EPIC PCR).  
151 The PCR conditions for a 25 µL final volume and for all markers were: Promega PCR  
152 buffer 1X, MgCl<sub>2</sub> 2.5 mM, 0.25 mM of each dNTP, 0.5 µM of each primer, Flexigotaq  
153 polymerase (Promega) 0.625 U, and 2.5 µL of DNA. The PCR program was 5 min at  
154 94°C, 30 cycles of [1 min at 94°C, 1 min at annealing temperature, 1 min at 72°C], and  
155 a final extension step of 10 min at 72°C. The primer sequences and annealing  
156 temperature for each marker and species are indicated in Table S3. For COI-igr1, PCR

157 products were directly sequenced. For EPIC markers the PCR products of four *E.*  
158 *cavolini* individuals were cloned with the pGEM®-T Easy Vector (Promega) according  
159 to the manufacturer's instructions, and ten clones were sequenced for each individual to  
160 check for the potential presence of paralogous loci. As there was no evidence of  
161 paralogous genes, two clones per individual and per population were sequenced as  
162 references. All other PCR products were directly sequenced. Sequencing was performed  
163 by Eurofins (Hamburg, Germany) and by Genoscope under the framework of the  
164 “Bibliothèque du Vivant” project. The sequences are available in Genbank under the  
165 following accession numbers: COI-igr1: KP190916 – KP190919; AIF: KP190656 –  
166 KP190915; FER: KP190338 – KP190655.

167

#### 168 *Sequence analyses*

169 The sequences were aligned in BioEdit (Hall 1999) with ClustalW (Thompson *et al*  
170 1994). After direct sequencing the double sequences induced by indels at heterozygous  
171 state were discarded. Singleton mutations were discarded from the dataset as they may  
172 correspond to PCR or cloning errors (Faure *et al* 2007). For sequences heterozygous for  
173 more than one SNP, SeqPHASE and then Phase 2.1 were used to infer the  
174 corresponding haplotypes (Flot 2010; Stephens & Donnelly 2003; Stephens *et al* 2001).  
175 The final alignment was comprised of two sequences per individual for each marker.  
176 The alignments have been deposited in Dryad (doi:10.5061/dryad.495hk).

177

178 DNAsp 5.10 (Librado & Rozas 2009) was used to compute the statistics describing the  
179 molecular polymorphism: nucleotide diversity ( $\pi$ ), haplotype diversity (Hd), number of  
180 segregating sites (S) and haplotype number (h). The average number of nucleotide  
181 substitutions per site between species Dxy (Nei 1987) was computed with DNAsp.

182

#### 183 *Genetic differentiation*

184 The pairwise genetic differentiation between species and between all samples was tested  
185 with permutation tests ( $n = 1000$ ) on  $F_{ST}$  and  $\Phi_{ST}$  (proportion of differences) with  
186 Arlequin 3.5 (Excoffier & Lischer 2010). An Analysis of Molecular Variance (AMOVA)

187 was performed for each locus with Arlequin 3.5 using both  $F_{ST}$  and  $\Phi_{ST}$ . The samples  
188 were grouped per species in order to study the genetic differentiation between and  
189 within species.

190

191 *Phylogenetic trees and networks reconstructions and tests of evolutionary scenarios*

192 For phylogenetic and network reconstructions, indels were recoded with SeqState  
193 (Müller 2005) following the Simple Indel Coding method (Simmons & Ochoterena  
194 2000). The relationships between sequences (after indel coding) were reconstructed  
195 with the split decomposition network approach implemented in SplitsTree 4 and the  
196 robustness of the groups was tested with 1000 bootstraps (Huson & Bryant 2006). As a  
197 complementary approach, phylogenies of FER and AIF were constructed separately  
198 with a maximum likelihood (ML) approach using PhyML 3.1 (Guindon *et al* 2010) and  
199 a Bayesian inference (BI) with MrBayes 3.2 (Ronquist & Huelsenbeck 2003). The  
200 evolution model used in PhyML was determined with JModelTest 2.1.4 (Darriba, *et al*  
201 2012) according to the Akaike information criterion (AIC) and the evolution model used  
202 in MrBayes was determined by MrModelTest 2.3 (Nylander 2008) according to the  
203 AIC. For FER, the GTR+I+G model was chosen for both approaches, and for AIF,  
204 GTR+I was retained for Mr Bayes, whereas HKY+I+G was retained for PhyML. The  
205 robustness of the trees obtained with PhyML was tested with 500 bootstraps. For  
206 MrBayes, different run lengths were chosen for each marker to reach an average  
207 standard deviation below 0.01 and a stabilization of log likelihood as recommended in  
208 the MrBayes Manual. For FER the total run length was comprised of  $20 \times 10^6$   
209 generations with a burn-in of  $5 \times 10^6$ , and for AIF  $5 \times 10^6$  generations and a burn-in of  
210  $1.5 \times 10^6$ . In both cases sampling was performed every 1000 generations. Trees were  
211 visualised and edited with FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). For  
212 AIF two sequences of an heterozygous *E. gazella* individual from the Atlantic  
213 (Arrábida, Portugal) were used as an outgroup to root the tree. Because we did not  
214 succeed in obtaining FER sequences for *E. gazella*, the tree was rooted at the midpoint.

215

216

217 In order to study the evolutionary histories that might have produced the observed



218 relationships between species, we used an ABC approach (see Csilléry *et al* 2012 for an  
219 introduction to ABC). Based on the phylogenetic trees and the obtained levels of  
220 differentiation, we considered *E. singularis* and *E. cavolini* as sister species, and *E.*  
221 *verrucosa* as sister to these two species for all the evolutionary scenarios tested. Four  
222 scenarios were considered (Fig. S2): 1) divergence without gene flow (Strict Isolation:  
223 SI); 2) divergence with gene flow (or Isolation / Migration: IM); 3) ancestral gene flow  
224 followed by isolation (or Ancestral Migration: AM); and 4) divergence and isolation  
225 followed by Secondary Contact (SC). The simulations (n = 100 000 per scenario) and  
226 computations of summary statistics were performed with ABCsampler in ABCtoolbox  
227 (Wegmann *et al* 2010). The prior distributions of the parameters and the observed  
228 summary statistics are detailed in Tables S4 and S5. We used the R package abc  
229 (Csilléry *et al* 2012) to estimate which scenario best fitted to the observed summary  
230 statistics. First, a cross-validation procedure was performed to test if the simulations and  
231 statistics could indeed distinguish the different scenarios. Then the posterior  
232 probabilities of each model and their ratios (the Bayes factors) were computed. Cross-  
233 validation and posterior probabilities were computed with a multinomial logistic  
234 regression method. A goodness of fit procedure was used to test the fit of the models to  
235 the observed data. Finally, parameters were inferred with the neural network procedure  
236 implemented in the R package abc.

237

## 238 **Results**

239

### 240 *Genetic polymorphism*

241 We obtained mitochondrial COI-igr1 sequences for 37 individuals: 19 *E. cavolini*, 14 *E.*  
242 *singularis*, 4 *E. verrucosa* (Table S2) with a 820 bp alignment. No polymorphism or  
243 difference between species was observed. Hence no further analysis was pursued with  
244 this marker.

245 The final alignment for the nuclear markers FER and AIF were 638 bp and 720 bp long  
246 respectively. The statistics describing the levels of polymorphism for each marker and at  
247 the population and species levels are presented in Table S1. The sample sizes varied  
248 because of different frequencies of overlapping sequences obtained after direct

249 sequencing for each marker and population. With FER we obtained 9 haplotypes for *E.*  
250 *singularis* and *E. verrucosa*, and (64 haplotypes for *E. cavolini* . With AIF we obtained  
251 6 haplotypes for *E. singularis*, 19 haplotypes for *E. verrucosa*, and 43 haplotypes for *E.*  
252 *cavolini*. Inside species, the FER haplotype diversity ranged between 0.4 and 1 for *E.*  
253 *cavolini*, between 0.39 and 0.89 for *E. singularis* and between 0 and 0.96 for *E.*  
254 *verrucosa*. With AIF the ranges of diversity were: 0.5-1 for *E. cavolini*, 0.36-0.68 for *E.*  
255 *singularis*, and 0-0.9 for *E. verrucosa*.

256

### 257 *Relationships between species*

258 The network reconstructed with AIF sequences (Fig. 2A) separated sequences of *E.*  
259 *verrucosa* and *E. gazella* on one side, and *E. cavolini* and *E. singularis* on the other.  
260 Reticulation was observed for internal relationships among *E. verrucosa* and *E. gazella*  
261 sequences. The sequences of *E. cavolini* and *E. singularis* were intermixed, and did not  
262 form two separate groups. The intermixing of sequences from these two species was  
263 supported by high bootstrap values. The network reconstructed with FER sequences  
264 (Fig. 2B) also did not separate *E. cavolini* and *E. singularis* in different groups, with  
265 some *E. verrucosa* sequences from Marseille and the Atlantic mixing with sequences  
266 from these two species. An internal reticulation suggested different relationships  
267 between the main groups but none supported a separation between the three species.  
268 The Bayesian and ML approaches confirmed the polyphyletic relationships between *E.*  
269 *singularis* and *E. cavolini* (Fig. S3). *Eunicella verrucosa* appeared paraphyletic with  
270 AIF and polyphyletic with FER. The internal relationships were well supported which  
271 contrasted with the reticulation observed in the network.

272

### 273 *Differentiation between species*

274 The  $\Phi_{ST}$  between species varied between 0.41 and 0.80 for AIF and between 0.22 and  
275 0.80 for FER (Table 1a,b). All  $F_{ST}$  and  $\Phi_{ST}$  between species were significantly different  
276 from zero. The genetic differentiation was lower between *E. cavolini* and *E. singularis*  
277 than with *E. verrucosa*. Nevertheless the  $F_{ST}$  computed with AIF indicated a closer  
278 relationship between *E. singularis* and *E. verrucosa* than with *E. cavolini*. For sites  
279 where two species were sampled, most comparisons between species were also

280 significant, but small sample sizes could explain non-significant tests (Table S6 and  
281 S7). The results of the AMOVA confirmed the differentiation between species with  
282 significant values of  $\Phi_{CT}$  (0.69 for AIF and 0.55 for FER; Table S8). The Nei's genetic  
283 distance  $D_{xy}$  was much lower between *E. cavolini* and *E. singularis* than between *E.*  
284 *verrucosa* and the two other species (Table 2c).

285 Three and four haplotypes were shared between *E. cavolini* and *E. singularis* with AIF  
286 and FER respectively (Table S9). For AIF, the shared haplotypes were observed at  
287 frequencies varying from 0.21 to 0.47 in *E. singularis* and at frequencies around 0.01 in  
288 *E. cavolini*. In *E. cavolini*, the shared haplotypes were observed only in the area of  
289 Marseille. For AIF, one individual identified as *E. cavolini* from Marseille was  
290 heterozygous for two haplotypes otherwise observed in *E. singularis*. This was not  
291 observed for FER, where the haplotypes of this individual were characteristic of *E.*  
292 *cavolini* haplotypes. This individual displayed a rarely observed pink color (Fig. S1).

293 Two individuals identified as *E. cavolini* were heterozygous for one *E. cavolini* and one  
294 *E. singularis* AIF haplotypes (according to the respective frequencies of these  
295 haplotypes). Their morphology did not appear different from other *E. cavolini*  
296 individuals. We did not obtain any FER sequence for these individuals.

297 For FER the shared haplotypes were observed at frequencies varying from 0.02 to 0.63  
298 in *E. singularis* and from 0.004 to 0.44 in *E. cavolini* (Table S9). In *E. cavolini* the  
299 shared haplotypes were observed in the area of Marseille, three in Corsica, one in  
300 Turkey, and one in Algeria. Three individuals from Marseille identified as potential *E.*  
301 *singularis* were heterozygous for one *E. cavolini* haplotype and one *E. singularis*  
302 haplotype (according to the respective frequencies of these haplotypes). They were all  
303 observed at the Sormiou Figuier site (Marseille) and had a faint yellow color found in  
304 *E. cavolini*. We did not get any AIF sequence for these individuals.

305

306 Before choosing a model with ABC we first tested, with the cross-validation, if we were  
307 able to discriminate the models: the majority of simulations led to the choice of the right  
308 model but with a better distinction of SI and IM than for SC and AM (Table 2a). The  
309 test of goodness of fit indicated for the four models that the simulations agreed with the  
310 observed statistics (data not shown). The highest posterior probability was obtained for

311 the SC model (table 2b). The Bayes factors for the comparison of this model with the  
312 three other ones were all greater than five, indicating a strong support for secondary  
313 contact (Tables 2c). We estimated the parameters corresponding to the SC scenario:  
314 effective sizes, divergence times, migration and mutation rates. The tests of cross  
315 validation (data not shown) and the flat posterior histograms indicated a lack of  
316 information for a precise estimate of the parameters (Table S10 and Fig. S4).  
317 Nevertheless, one can note that the posterior distribution of the time of secondary  
318 contact ( $t_1$ ) appeared skewed towards the lower bound of the prior, suggesting recent  
319 gene flow. The migration rates seemed lower between *E. verrucosa* and the two other  
320 species (parameters  $m_{13}$  and  $m_{23}$ ) than between *E. cavolini* and *E. singularis*  
321 (parameter  $m_{12}$ ) but the distribution remained wide (Fig. S4).

322

### 323 *Genetic differentiation in E. cavolini*

324 For AIF and FER, the pairwise  $F_{ST}$  and  $\Phi_{ST}$  between samples of *E. cavolini* indicated  
325 that the highest differentiation was observed between samples from the Marmara Sea  
326 and all other samples (Tables S6 and S7). At a local scale, near Marseille, a significant  
327 differentiation was observed between individuals sampled at 20 m and 40 m depths with  
328  $F_{ST}$  for FER (pairwise  $F_{ST}$  varying from 0.07 to 0.20), but not AIF (pairwise  $F_{ST}$  varying  
329 from -0.03 to 0.07), for the three site where we tested it (Veyron, Riou and Méjean).  
330 There was no clear separation of sequences according to geography or depth in the  
331 networks nor in the trees. For example sequences from Eastern (Turkey) and Western  
332 (Marseille, Corsica) Mediterranean were mixed together and usually displayed few  
333 differences.

334

## 335 **Discussion**

336

### 337 *Species relationships and history*

338 Mitochondrial data did not indicate any difference between the three *Eunicella* species,  
339 with three markers: mtMutS, COI and COI-igr1 (Calderón *et al* 2006; Gori *et al* 2012;  
340 our results). The lack of polymorphism of mitochondrial DNA is well known in  
341 octocorals (Calderón *et al* 2006; Shearer *et al* 2002). The proposed extended barcoding

342 (combination of COI-igr1 and mtMutS; McFadden *et al* 2011) did not distinguish  
343 *Eunicella* species. Nuclear markers can be more efficient in resolving octocoral  
344 phylogeny or delimiting species than mitochondrial ones (Concepcion *et al* 2008; Pante  
345 *et al* 2015a; Pratlong *et al* 2016). Here nuclear markers indicated a significant  
346 differentiation with incomplete phylogenetic separation of the three *Eunicella* species,  
347 as observed with ITS1 and 2 as well (Calderón *et al* 2006; Costantini *et al* 2016).  
348 However only a few haplotypes were shared between species, and only between *E.*  
349 *cavolini* and *E. singularis*: this resulted in a significant AMOVA outcome which  
350 indicated higher differentiation between species than within species. Inside species  
351 neither long distance isolation nor depth differences corresponded to deep genetic  
352 lineages. Different scenarios can be considered to explain the lack of monophyly despite  
353 a significant differentiation, such as a recent divergence with incomplete lineage sorting,  
354 or current or past interspecific gene flow following allopatric isolation. The high levels  
355 of diversity observed with EPICs suggests that homoplasy could blur the phylogenetic  
356 signal as well. Nevertheless several well supported internal nodes suggested the non  
357 monophyly of the three species. Concerning ITS one can note that non monophyly can  
358 also be the consequence of a lack of concerted evolution or of hybridization (Calderón  
359 *et al* 2006; Vollmer & Palumbi 2004).

360 In the present study the best scenario, according to ABC, was secondary contact. The  
361 models with gene flow (apart from the IM model) were all better supported than strict  
362 isolation: this indicates that incomplete lineage sorting alone could not explain our  
363 results. The cross validation analysis, based on simulated data, indicates that with two  
364 loci we can separate the main scenarios but the distinction was less clear between SC  
365 and AM and the possibility of current gene flow would require additional studies.

366 Recent transcriptome analyses on *E. cavolini* and *E. verrucosa* support current  
367 introgression at least between these two species (Roux *et al* 2016). Using two markers  
368 can also be misleading as the inter-specific migration rate can be very different between  
369 loci (Roux *et al* 2016), which can not be studied here. Gene flow following secondary  
370 contact has been demonstrated even between well differentiated species (Roux *et al*  
371 2013, 2016; Tine *et al* 2014). Other more specific scenarios, including partial (i.e. only  
372 between two species) or asymmetric gene flow, could be tested, but this would require

373 more markers to get enough information. Finally the reduced number of markers is  
374 probably a factor preventing precise estimate of the parameters with ABC.  
375 Both the  $F_{ST}$ 's and networks indicated a closer relationship between the two  
376 Mediterranean species (*E. cavolini* and *E. singularis*) than with the Atlantic-  
377 Mediterranean one (*E. verrucosa*). *Eunicella verrucosa* does not show a deep Atlantic –  
378 Mediterranean genetic break with the markers used here and with microsatellites  
379 (Holland 2013). This could indicate a relatively recent colonization of the  
380 Mediterranean by *E. verrucosa*, which might explain its more distant relationships with  
381 *E. singularis* and *E. cavolini*. Concerning *E. singularis* and *E. cavolini*, their initial  
382 divergence could have been linked to different Quaternary glacial refugia whose  
383 locations remain to be studied. Estimating the parameters of this evolutionary history is  
384 also interesting. Nevertheless, the flat posterior distributions were not helpful and only  
385 suggested a recent occurrence of gene flow for our markers.

386

#### 387 *Potential factors of isolation*

388 For most colonies, the morphological characteristics, such as colony shape, color and  
389 sclerites made it possible to separate these species (Carpine & Grasshoff 1975; Gori *et al*  
390 *al* 2012). For marine species with larval dispersal, efficient isolation mechanisms are  
391 required to maintain the integrity of the different genomes (Bierne *et al* 2002). Here, the  
392 persistence of differentiated phenotypes in sympatry suggests that reproductive barriers,  
393 either genetic or ecological, are efficient at preventing genetic homogenization despite  
394 the possibility of past or current sporadic gene flow. *Eunicella singularis* is found on  
395 rocky substrata ranging less than 10 m to more than 60 m, where it can be observed  
396 without photosynthetic *Symbiodinium* (Gori *et al* 2011, 2012). The depth range of *E.*  
397 *cavolini* is wider, from less than 10 m to over 220 m (Sini *et al* 2015). Therefore,  
398 although different responses to thermal stress have been demonstrated between *E.*  
399 *singularis* and *E. cavolini* (Pivotto *et al* 2015), ecological differences alone do not seem  
400 sufficient here to explain the limits to gene flow. Genetic isolation could be the main  
401 factor at stake here, and it would be interesting to test the possibility of current  
402 hybridization. A few individuals analysed in this study could be hybrids between *E.*  
403 *cavolini* and *E. singularis*, but data from two loci are not sufficient to draw conclusions.

404 Experimental crossing would be a complementary and direct test of hybridization (e.g.  
405 Isomura *et al* 2013).  
406 Of particular interest is the potential link between speciation and symbiosis with  
407 *Symbiodinium*. We demonstrated here the close proximity between symbiotic (shallow  
408 *E. singularis*) and non symbiotic (*E. cavolini* and *E. verrucosa*) octocoral species with  
409 the possibility of gene flow between them. This demonstrates the possibility of changes  
410 in symbiotic interactions on short evolutionary timescales. The diversity of metazoans  
411 interacting with *Symbiodinium*, as well as the possibility of shift in *Symbiodinium* types  
412 observed in corals, illustrate the evolutionary flexibility of such associations (Baker  
413 2003; Venn *et al* 2008). Conversely, the symbiotic state could contribute to reproductive  
414 isolation, and symbiosis has been proposed as a speciation factor in other contexts  
415 (Brucker & Bordenstein 2012). Here the genetic interactions with *Symbiodinium* and the  
416 associated physiological constraint can be the basis of an important constraint to  
417 introgression.

418

419 *Geographical or ecological isolation in E. cavolini?*

420 The second goal of our study was to test if geographical or ecological isolation could  
421 correspond to cryptic lineages in *E. cavolini*. We observed a significant differentiation  
422 between distant samples, but this did not correspond to deep phylogeographic break. In  
423 line with the incomplete lineage sorting among taxa, haplotypes from distant locations  
424 in *E. cavolini* were mixed together on the networks. This lack of deep phylogeographic  
425 differentiation has also been observed in the Mediterranean red coral (Aurelle *et al*  
426 2011) despite a clear regional structure (Ledoux *et al* 2010). Such pattern could be  
427 explained by sporadic gene flow between long-distance locations which would maintain  
428 the evolutionary cohesion of these species. A recent isolation along with low genetic  
429 drift could slow down the evolution of well separated lineages (Knowles & Carstens  
430 2007). At a local scale in *E. cavolini*, we did not observe any differentiation along

431 depth with AIF, but significant differences were observed with FER, for the three sites  
432 considered here. These differences did not correspond to deep genetic lineages  
433 contrarily to what has been observed in a Caribbean octocoral (Prada & Hellberg 2013).  
434 In *E. singularis* there was no significant differentiation above 30 m as well, but a  
435 restriction to vertical gene flow was observed around 30-40 m (Costantini *et al* 2016). A  
436 dedicated transcriptomic or genomic study would be necessary to test the link between  
437 genetic and adaptation to depth in *Eunicella* species (e.g. Pratlong *et al* 2015).

438

439

#### 440 **Conclusion**

441 Our results revealed complex phylogenetic relationships among the three *Eunicella*  
442 species, which was not visible with mitochondrial markers. Accordingly these species  
443 are in the grey zone of speciation and correspond to semi-isolated genetic backgrounds  
444 (Roux *et al.*, 2016). We did not identify a clear link between genetic differentiation and  
445 ecological differences. Even if this last point would require more dedicated studies, the  
446 observation of mixed populations of these species in the same sites stresses the role of  
447 endogenous (i.e. genetic) barriers to gene flow. It will be interesting to study more  
448 locations in order to infer the evolutionary history of the genus and potentially to  
449 identify different glacial refugia which may help understanding a potential allopatric  
450 speciation scenario. The development of population genomic approaches will then be  
451 necessary for i) studying the patterns of genomic differentiation and introgression, ii)  
452 testing the link between symbiosis and speciation, iii) testing for the presence of genetic  
453 x environment associations linked to thermal regime. This last point is important to



454 better understand how these species can live in very different thermal conditions. Apart  
455 from its fundamental interest this last question would be useful to study the potential  
456 response of these ecologically important species to climate change.

457

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Titles and legends to figures:

Figure 1. Map of the sampling sites for the three *Eunicella* species. The symbols indicate the different species sampled for each site. *Eunicella* spp. indicates that two or three species were sampled at the same site (see Table S1 for details).

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480

481 Figure 2. Split decomposition networks for the nuclear markers Apoptosis Induction  
482 Factor (AIF; A) and Ferritin (FER; B). The percentage of bootstraps support is indicated  
483 for values higher than 80% (based on 1000 bootstraps). The colors indicate the  
484 corresponding species: blue: *E. cavolini* (EC), red: *E. singularis* (ES), green: *E.*  
485 *verrucosa* (EV), purple *E. gazella* (EG). Numbers in parentheses indicate the number of  
486 sequences obtained for each species. See Table S1 for population codes. Red stars  
487 indicate shared sequences between *E. cavolini* and *E. singularis*; for FER, four sequence  
488 types were shared but their low divergence doesn't allow to clearly separate them on the  
489 figure.

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494 Table 1 : pairwise genetic differentiation between species estimated with  $\Phi_{ST}$  (below  
 495 diagonal) and  $F_{ST}$  (above diagonal) for AIF (2a) and FER (2b). All values are significant  
 496 with permutation tests (n = 1000). 2c: differentiation estimated with the average number  
 497 of nucleotide substitutions per site between populations Dxy. Above diagonal: FER,  
 498 below diagonal AIF.

499

500 a) AIF

	<i>E. cavolini</i>	<i>E. singularis</i>	<i>E. verrucosa</i>
<i>E. cavolini</i>	-	0.33	0.27
<i>E. singularis</i>	0.41	-	0.22
<i>E. verrucosa</i>	0.80	0.58	-

501

502 b) FER

	<i>E. cavolini</i>	<i>E. singularis</i>	<i>E. verrucosa</i>
<i>E. cavolini</i>	-	0.22	0.29
<i>E. singularis</i>	0.22	-	0.41
<i>E. verrucosa</i>	0.80	0.60	-

503

504 c) Dxy

505

	<i>E. cavolini</i>	<i>E. singularis</i>	<i>E. verrucosa</i>
<i>E. cavolini</i>	-	0.0174	0.0544
<i>E. singularis</i>	0.0111	-	0.0504
<i>E. verrucosa</i>	0.0309	0.0285	-

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508 Table 2 : results of model choice with ABC. The tested models were Strict Isolation  
509 (SI), Isolation Migration (IM), Secondary Contact (SC), Ancestral Migration (AM). See  
510 main text and Supplementary Material for descriptions of the models. a) results of the  
511 cross validation procedure using 100 samples and tolerance of 0.1. Each line indicates  
512 for the corresponding model the mean posterior probability of the four different models.  
513 b) posterior probabilities for each model. c) Bayes factors for the models considered on  
514 each line compared to models indicated in column.

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a)

	SI	IM	SC	AM
SI	0.79	0.11	0.01	0.09
IM	0.02	0.87	0.01	0.10
SC	0.12	0.34	0.47	0.07
AM	0.16	0.35	0.14	0.35

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b)

	SI	IM	SC	AM
Posterior probability	0.03	0.01	0.81	0.15

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c)

	SI	IM	SC	AM
SI	1	3.83	0.04	0.19
IM	0.26	1	0.01	0.05
SC	27.39	104.75	1	5.32
AM	5.15	19.69	0.19	1

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