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

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Running title: Species limits in Mediterranean octocorals
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Abstract

The study of the interplay between speciation and hybridization is of primary importance in evolutionary biology. Octocorals are ecologically important species whose shallow phylogenetic relationships often remain to be studied. In the Mediterranean Sea, three congeneric octocorals can be observed in sympatry: *Eunicella*

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

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

Introduction

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

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

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

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

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

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

Materials and methods

Sampling

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

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

Molecular analyses

Total genomic DNA was extracted with the Qiagen DNeasy kit according to the manufacturer's instructions or with Macherey-Nagel's NucleoSpin kit on an epMotion 5075 VAC automated pipetting system (Eppendorf). We amplified the mitochondrial marker COI-igr1 with primers defined in McFadden *et al* (2011) on a subset of 37 individuals (Table S2). Two nuclear loci were amplified for all individuals. These markers were developed from transcriptome sequences obtained from *Paramuricea clavata* (Mokthar-Jamaï *et al* unpublished). The putative function of two genes was identified through a search in the Uniprot database: Ferritin (hereafter FER) and Apoptosis Induction Factor (hereafter AIF). Degenerate primers were defined by aligning these sequences with Metazoan sequences obtained from a Blast search in Genbank. We could then amplify specifically these genes in *Eunicella* spp. and we retained primer pairs allowing the amplification of introns (i.e. EPIC PCR).

The PCR conditions for a 25 µL final volume and for all markers were: Promega PCR buffer 1X, MgCl2 2.5 mM, 0.25 mM of each dNTP, 0.5 µM of each primer, Flexigotaq polymerase (Promega) 0.625 U, and 2.5 µL of DNA. The PCR program was 5 min at 94°C, 30 cycles of [1 min at 94°C, 1 min at annealing temperature, 1 min at 72°C], and a final extension step of 10 min at 72°C. The primer sequences and annealing temperature for each marker and species are indicated in Table S3. For COI-igr1, PCR

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

Sequence analyses

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

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

Genetic differentiation

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

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

Phylogenetic trees and networks reconstructions and tests of evolutionary scenarios

For phylogenetic and network reconstructions, indels were recoded with SeqState (Müller 2005) following the Simple Indel Coding method (Simmons & Ochoterena 2000). The relationships between sequences (after indel coding) were reconstructed with the split decomposition network approach implemented in SplitsTree 4 and the robustness of the groups was tested with 1000 bootstraps (Huson & Bryant 2006). As a complementary approach, phylogenies of FER and AIF were constructed separately with a maximum likelihood (ML) approach using PhyML 3.1 (Guindon *et al* 2010) and a Bayesian inference (BI) with MrBayes 3.2 (Ronquist & Huelsenbeck 2003). The evolution model used in PhyML was determined with JModelTest 2.1.4 (Darriba, *et al* 2012) according to the Akaike information criterion (AIC) and the evolution model used in MrBayes was determined by MrModelTest 2.3 (Nylander 2008) according to the AIC. For FER, the GTR+I+G model was chosen for both approaches, and for AIF, GTR+I was retained for Mr Bayes, whereas HKY+I+G was retained for PhyML. The robustness of the trees obtained with PhyML was tested with 500 bootstraps. For MrBayes, different run lengths were chosen for each marker to reach an average standard deviation below 0.01 and a stabilization of log likelihood as recommended in the MrBayes Manual. For FER the total run length was comprised of $20x10^6$ generations with a burn-in of $5x10^6$, and for AIF $5x10^6$ generations and a burn-in of $1.5x10^6$. In both cases sampling was performed every 1000 generations. Trees were visualised and edited with FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). For AIF two sequences of an heterozygous *E. gazella* individual from the Atlantic (Arrábida, Portugal) were used as an outgroup to root the tree. Because we did not succeed in obtaining FER sequences for *E. gazella*, the tree was rooted at the midpoint.

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

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

Results

Genetic polymorphism

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

The final alignment for the nuclear markers FER and AIF were 638 bp and 720 bp long respectively. The statistics describing the levels of polymorphism for each marker and at the population and species levels are presented in Table S1. The sample sizes varied 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 Φ_{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 Φ_{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

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

Three and four haplotypes were shared between *E. cavolini* and *E. singularis* with AIF and FER respectively (Table S9). For AIF, the shared haplotypes were observed at frequencies varying from 0.21 to 0.47 in *E. singularis* and at frequencies around 0.01 in *E. cavolini*. In *E. cavolini*, the shared haplotypes were observed only in the area of Marseille. For AIF, one individual identified as *E. cavolini* from Marseille was heterozygous for two haplotypes otherwise observed in *E. singularis*. This was not observed for FER, where the haplotypes of this individual were characteristic of *E. cavolini* haplotypes. This individual displayed a rarely observed pink color (Fig. S1). Two individuals identified as *E. cavolini* were heterozygous for one *E. cavolini* and one *E. singularis* AIF haplotypes (according to the respective frequencies of these haplotypes). Their morphology did not appear different from other *E. cavolini* individuals. We did not obtain any FER sequence for these individuals.

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

Before choosing a model with ABC we first tested, with the cross-validation, if we were able to discriminate the models: the majority of simulations led to the choice of the right model but with a better distinction of SI and IM than for SC and AM (Table 2a). The test of goodness of fit indicated for the four models that the simulations agreed with the 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 (t1) 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 m13 and m23) than between *E. cavolini* and *E. singularis*
321 (parameter m12) but the distribution remained wide (Fig. S4).
322
323 *Genetic differentiation in* E. cavolini
324 For AIF and FER, the pairwise F_{ST} and Φ_{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

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

In the present study the best scenario, according to ABC, was secondary contact. The models with gene flow (apart from the IM model) were all better supported than strict isolation: this indicates that incomplete lineage sorting alone could not explain our results. The cross validation analysis, based on simulated data, indicates that with two loci we can separate the main scenarios but the distinction was less clear between SC and AM and the possibility of current gene flow would require additional studies. Recent transcriptome analyses on *E. cavolini* and *E. verrucosa* support current introgression at least between these two species (Roux *et al* 2016). Using two markers can also be misleading as the inter-specific migration rate can be very different between loci (Roux *et al* 2016), which can not be studied here. Gene flow following secondary contact has been demonstrated even between well differentiated species (Roux *et al* 2013, 2016; Tine *et al* 2014). Other more specific scenarios, including partial (i.e. only between two species) or asymmetric gene flow, could be tested, but this would require

more markers to get enough information. Finally the reduced number of markers is probably a factor preventing precise estimate of the parameters with ABC.

Both the F_{ST}'s and networks indicated a closer relationship between the two Mediterranean species (*E. cavolini* and *E. singularis*) than with the Atlantic-Mediterranean one (*E. verrucosa*). *Eunicella verrucosa* does not show a deep Atlantic – Mediterranean genetic break with the markers used here and with microsatellites (Holland 2013). This could indicate a relatively recent colonization of the Mediterranean by *E. verrucosa*, which might explain its more distant relationships with *E. singularis* and *E. cavolini*. Concerning *E. singularis* and *E. cavolini*, their initial divergence could have been linked to different Quaternary glacial refugia whose locations remain to be studied. Estimating the parameters of this evolutionary history is also interesting. Nevertheless, the flat posterior distributions were not helpful and only suggested a recent occurrence of gene flow for our markers.

Potential factors of isolation

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

Experimental crossing would be a complementary and direct test of hybridization (e.g. Isomura *et al* 2013).

Of particular interest is the potential link between speciation and symbiosis with *Symbiodinium*. We demonstrated here the close proximity between symbiotic (shallow *E. singularis*) and non symbiotic (*E. cavolini* and *E. verrucosa*) octocoral species with the possibility of gene flow between them. This demonstrates the possibility of changes in symbiotic interactions on short evolutionary timescales. The diversity of metazoans interacting with *Symbiodinium,* as well as the possibility of shift in *Symbiodinium* types observed in corals, illustrate the evolutionary flexibility of such associations (Baker 2003; Venn *et al* 2008). Conversely, the symbiotic state could contribute to reproductive isolation, and symbiosis has been proposed as a speciation factor in other contexts (Brucker & Bordenstein 2012). Here the genetic interactions with *Symbiodinium* and the associated physiological constraint can be the basis of an important constraint to introgression.

Geographical or ecological isolation in E. cavolini?

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

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

Conclusion

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

459 We acknowledge the staff of the diving service of the Pythéas Institute for sampling.
460 We acknowledge M. Libes and C. Yohia from the informatic service of the Pythéas
461 Institute and Katalin Csilléry for her advice with ABC. We thank P. Hammami for the
462 DNA extractions. We especially thank Alvaro Altuna and José Aldrey for the *Eunicella*
463 *verrucosa* samples from Atlantic. Part of the sequencing was completed thanks to the
464 Bibliothèque du Vivant project (http://bdv.ups-tlse.fr/index.php). We thank Aurélie
465 Blanfuné Thibault for the maps, Frédéric Zuberer for the pictures of *Eunicella* colonies,
466 and Nicolas Bierne and François Bonhomme for stimulating discussions. We thank
467 Abigail E. Cahill for the correction of the manuscript. We acknowledge the technical
468 support of the molecular biology service of the IMBE. M. Malfant was funded by the
469 French National Research Agency (ANR) program Adacni (ANR n° ANR-12-ADAP-
470 0016; http://adacni.imbe.fr). I.D. Pivotto was funded by a grant from the CNRS. The
471 Algeria – France collaboration was supported by a Hubert Curien "Tassili" program (n°
472 12MDU853). R.Castilho and M. Coelho were funded by CCMAR Strategic Plan (PEst-
473 C/MAR/LA0015/2011 and UID/Multi/04326/2013) from Fundação para a Ciência e a
474 Tecnologia - FCT (partially FEDER funded).

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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).

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

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

a) AIF

	E. cavolini	*E. singularis*	*E. verrucosa*
E. cavolini	-	0.33	0.27
E. singularis	0.41	-	0.22
E. verrucosa	0.80	0.58	-

b) FER

	E. cavolini	*E. singularis*	*E. verrucosa*
E. cavolini	-	0.22	0.29
E. singularis	0.22	-	0.41
E. verrucosa	0.80	0.60	-

c) Dxy

	E. cavolini	*E. singularis*	*E. verrucosa*
E. cavolini	-	0.0174	0.0544
E. singularis	0.0111	-	0.0504
E. verrucosa	0.0309	0.0285	-

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

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

b)

	SI	IM	SC	AM
Posterior probability	0.03	0.01	0.81	0.15

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