Symbiosis, hybridization and speciation in Mediterranean octocorals (Octocorallia, Eunicellidae)

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ABSTRACT

Understanding how species can form and remain isolated in the marine environment still stimulates active researches. Here we study the differentiation and the possibility of hybridization among three temperate octocorals: *Eunicella cavolini*, *E. singularis* and *E. verrucosa*. Morphologically intermediate individuals have been observed between them. Among these three species, *E. singularis* is the only one described in mutualistic symbiosis with photosynthetic Symbiodiniaceae. The symbiosis between Symbiodiniaceae and scleractinian corals is well studied, especially in the context of the response to anthropogenic climate change. Nevertheless, the potential role of symbiotic interactions in speciation processes remains unknown in cnidaria. 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 on-going 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. Despite current gene flow, these two species appear genetically well differentiated. Our data also suggest an intermediate abundance of Symbiodiniaceae in the hybrids of the two parental populations. We discuss the evolution of the Symbiodiniaceae / cnidarian symbiosis in the light of our results.

Keywords: speciation, hybridization, symbiosis, transcriptome, RAD sequencing, octocoral

Introduction

As corner stones of evolutionary biology, species and speciation still raise a wealth of questions fuelled by the technological and conceptual advancements in genomics. Genomic data allow testing hypotheses about species boundaries and origins. Named species are indeed hypotheses, built on available data, that can be rejected or validated through the integration of additional data and / or the use of additional criteria based on evolutionary concepts (Pante *et al.*, 2015b). Sound species delimitations are useful, among others, to better estimate species range and biodiversity patterns (Muir *et al.*, 2022; Coelho *et al.*, 2023), to avoid biases in studies of connectivity (Pante et al., 2015b), and of adaptive abilities (Brener-Raffalli *et al.*, 2022). However, proposing sound species delimitation can be problematic because different delimitation criteria may bring contradictory conclusions about species boundaries (the Grey Zone of de Queiroz, 2007). This grey zone corresponds to puzzling cases such as the absence of gene flow among morphologically undifferentiated sets of organisms (i.e. cryptic species, Cahill *et al.*, 2024), or conversely, the detection of gene flow among sets of organisms recognized, based on morphological distinctiveness, as distinct species (Leroy *et al.*, 2020). Evolutionary inferences, based on genomic data, allow testing scenarios of speciation and current gene flow: this provides a better understanding on the origin and persistence of species at the light of genomic divergence (Roux *et al.*, 2016; De Jode *et al.*, 2023).

In the marine realm, the question of speciation is considered as particularly confusing. Notably, how new species can originate from populations with large effective size associated to high level of gene flow is still abundantly debated in the literature (e.g. Palumbi, 1992; Mayr, 2001; Faria *et al.*, 2021). Difficulties in sampling and rearing organisms also hamper experiments to test reproductive isolation (Faria *et al.*, 2021). Important progresses in methodologies now allow to better understand spatial patterns of genetic structure in marine organisms, for example through the study of oceanographic connectivity (Reynes *et al.*, 2021), clines in allele frequencies (Gagnaire *et al.*, 2015), and hybrid zones (Bierne *et al.*, 2003).

In this context, the role of symbiotic interactions in reproductive isolation remains poorly investigated. There are various examples of the involvement of microbial species in reproductive isolation, especially in insects (Brucker and Bordenstein, 2012). For marine species, microbial communities have been mainly explored in light of adaptative evolution (Rosenberg and Zilber-Rosenberg, 2018). Shallow water scleractinian corals (hexacorals) are usually associated with various species of photosynthetic zooxanthellae, in the family Symbiodiniacae (Cairns, 2007; LaJeunesse *et al.*, 2018). Changes in associated Symbiodiniaceae can impact the thermotolerance of the coral holobiont, and the possibility

of adaptation facing climate change (Berkelmans and van Oppen, 2006; van Oppen & Medina, 2020). Inferences from the phylogeny of Anthozoans (hexacorals and octocorals) have shown multiple acquisitions of the symbiotic state throughout evolution (Cairns, 2007; Campoy *et al.*, 2020, Mc Fadden *et al.*, 2021). The symbiotic interactions between Anthozoans and Symbiodiniaceae provide important mutualistic benefits especially from a nutritional point of view (Furla *et al.*, 2005). These interactions require specific adaptations for the animal host, as for example protection against oxygen produced by photosynthesis (Furla *et al.*, 2005). Therefore, one can hypothesize that in hybrids such adaptations could be modified and a breakdown of symbiosis could occur, leading to reduced fitness. The association with Symbiodiniaceae can range from mutualism to parasitism (Sachs and Wilcox, 2006; Lesser *et al.*, 2013; see also Matz, 2024), and a change in the genomic background in hybrid hosts could modify the nature of symbiosis as well. The presence of Symbiodiniaceae could then be involved in genetic incompatibilities with the host genome, as previously observed with bacterial species (Bordenstein, 2003; Brucker and Bordenstein, 2012). All these observations raise the question of the potential role of Symbiodiniaceae in speciation and reproductive isolation in Anthozoans. This topic has been poorly explored up to now. In *Plexaura* octocorals, two incompletely isolated species have been shown to present different populations of Symbiodiniaceae, questioning their role in species boundaries (Pelosi *et al.*, 2020).

Here we explore the robustness of species limits between named species of the gorgonian genus *Eunicella* (Octocorallia, Eunicellidae) documented as displaying different symbiotic relationships. In shallow conditions (above 50 m depth), three *Eunicella* species are mainly present in the Mediterranean Sea: *Eunicella cavolini* (Koch, 1887), *E. singularis* (Esper, 1971), and *E. verrucosa* (Pallas, 1766). These three species have partially overlapping ranges, and they can be observed in sympatry in the area of Marseille (France). *Eunicella singularis* hosts Symbiodiniaceae corresponding to the *Philozoon* genus (Forcioli *et al.*, 2011; LaJeunesse *et al.*, 2018, 2022; Porro, 2019), whereas the two other gorgonian species are devoided of these symbionts (Carpine and Grasshoff, 1975). The Symbiodiniaceae contribute to the carbon metabolism of *E. singularis*, but a non-symbiotic *aphyta* morph has already been observed (Gori *et al.*, 2012). The lack of variability in mitochondrial DNA does not allow to distinguish these three species (Calderón *et al.*, 2006), and a study using two nuclear introns suggested the possibility of hybridization between *E. cavolini* and *E. singularis* (Aurelle *et al.*, 2017). Moreover, demographic inferences based on a large number of nuclear loci in *E. cavolini* and *E. verrucosa* indicated the possibility of current gene flow between these two species (Roux *et al.*, 2016). However, these data are incomplete because neither individuals identified as *E. singularis*, nor individuals that are morphologically difficult to attribute to a named species (potential hybrids) have been

analysed. Here, we will go further on these topics with the following objectives: i) estimate the genomic differentiation among these three species and test for species limits, ii) test whether symbionts are present or absent in the hybrids, to look for a possible breakdown in symbiosis, and iii) infer scenarios of speciation. Studying the history of speciation is useful to infer how divergence happened, and to test the possibility of current and past gene flow. Analysing the hybrid status on morphologically intermediate individuals allows to further test if hybridization is still on-going. We used restriction sites associated DNA sequencing (RAD-sequencing; Baird *et al.*, 2008) to test species limits and hybridization. We complementary used transcriptome data for demographic inferences, for the analysis of putative hybrids, and to test for the presence of Symbiodiniaceae. The results will be useful to better understand the evolution of these species in different environments and particularly the possible impact of hybridization in adaptation to changing environment.

Material and methods

Species distribution

Eunicella verrucosa is present both in the Eastern Atlantic Ocean and the Mediterranean Sea (Carpine and Grasshoff, 1975). In the Atlantic, *E. verrucosa* can be found from Ireland and West coasts of Britain, to Angola (Grasshoff, 1992; Readman and Hiscock, 2017). *Eunicella verrucosa* has been observed in the North Western Mediterranean Sea, in Sardinia (Canessa et al., 2022), and in the Adriatic and Aegean Seas (Chimienti, 2020). In the Mediterranean Sea, *E. verrucosa* can be observed from shallow conditions (20-40 m) up to 200 m depth (Sartoretto and Francour, 2011; Fourt and Goujard, 2012; Chimienti, 2020). *Eunicella singularis* and *E. cavolini* are only present in the Mediterranean Sea. *Eunicella cavolini* can be observed in the Western Mediterranean, Adriatic and Aegean Seas, from 5 to 200 m depth (Sini *et al.*, 2015; Carugati *et al.*, 2022). *Eunicella singularis* can be found in the Western Mediterranean and Adriatic Seas, and, less frequently, in the Eastern Mediterranean (Gori *et al.*, 2012). It is usually observed up top 40 m depth. *Eunicella singularis* is the only Mediterranean octocoral known to harbour Symbiodiniaceae (but see Bonacolta *et al.*, 2024). These Symbiodiniaceae belong to the temperate clade A (Forcioli *et al.*, 2011; Casado-Amezúa *et al.*, 2016), now corresponding to the *Philozoon* genus (LaJeunesse *et al.*, 2018, 2022). Deep occurrences (up to 70 m) of *E. singularis* have been mentioned, and assigned to the *aphyta* morph, without Symbiodiniaceae (Gori et al., 2012). In the area of Marseille, these three species can be observed in sympatry and at the same depth (Sartoretto and Francour, 2011).

Sampling

The sampling for RAD sequencing included 25 specimens identified as *E. cavolini*, 23 *E. singularis*, seven *E. verrucosa*, and 12 morphologically intermediate individuals (potential hybrids). These latter individuals displayed intermediate colors and branching patterns between *E. cavolini* and *E. singularis (*Figure S1), and they were analysed to test their hybrid status (Aurelle *et al.*, 2017). The specimens have been sampled by scuba diving at different periods in the area of Marseille, where the three species are present in sympatry (Figure S2; Table S1).

For transcriptome sequencing, specimens attributed to *E. cavolini*, *E. singularis*, and *E. verrucosa* have been collected in the Mediterranean (for the three species), and in the Atlantic (*E. verrucosa* only; Table S2; Figure 1) in 2016. The final sampling for transcriptomics included five *E. cavolini*, eight *E. singularis*, three *E. verrucosa*, and four potential hybrids.

Sampling was non-destructive, with authorizations from the local authorities, including Marine Protected Areas.

Mitochondrial MutS

To test the genetic proximity of three *Eunicella* species studied here, we built a tree with mitochondrial MutS sequences (McFadden *et al.*, 2011), available in GenBank. The methods and sequences are detailed in supplementary Figure S3, and Table S3.

RAD sequencing

DNA has been extracted with the Macherey-Nagel NucleoSpin DNA RapidLyse kit. RAD library preparation (with the PstI restriction enzyme) and sequencing (Illumina NovaSeq600 with 150 nucleotides paired-end sequencing) have been performed at the MGX platform (CNRS). The MGX platform performed control quality, demultiplexing and removal of PCR duplicates with unique molecule identifiers. Potential contaminants have been removed with kraken2 (Wood *et al.*, 2019; Lu *et al.*, 2022). RAD loci have been assembled with ipyrad (Eaton and Overcast, 2020). We tested four assembly strategies to test the robustness of the results: a *de novo* assembly, with a clustering threshold of 0.85, and assembly on a reference genome, with each of the three available genomes: for *E. cavolini*, *E. singularis*, and *E. verrucosa* (Ledoux *et al.*, in prep).

From these datasets, we built four datasets focused on the differentiation between *E. cavolini* and *E. singularis*: we excluded *E. verrucosa* samples and we retained the first

percent of the loci with the highest F_{ST} between *E. cavolini* and *E. singularis*. These last datasets will be labelled as “1%” (see characteristics of the different datasets are summarised in Table S4).

Transcriptome sequencing and SNPs calling

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

We mapped the reads on the meta transcriptome filtered for Symbiodiniaceae sequences with bwa option mem (Li and Durbin, 2009). The obtained sam files were converted in bam format with samtools 1.9 (Li *et al.*, 2009), and sorted with Picard tools (‘Picard Toolkit’, 2019). The SNPs calling has been performed with reads2snp 2.0 with default parameters (Tsagkogeorga *et al.*, 2012; Gayral *et al.*, 2013). The obtained dataset, including variable and non variable sites, will thereafter be referred as the “all sites” dataset. We performed separate SNP calls with reads2snp for pairwise comparisons among species and without the potential hybrid samples. These three datasets have been used for demographic inferences, and will be referred as “all-CS” for the *E. cavolini* / *E. singularis* comparison, “all-CV” for the *E. cavolini* / *E. verrucosa* comparison, and “all-SV” for the *E. singularis* / *E. verrucosa* comparison.

For an analysis of genetic differentiation, we filtered the “all sites” vcf file with vcftools (Danecek *et al.*, 2011). We retained biallelic sites, without missing data, and separated by at least 1 kb: this is the “polymorphic sites” dataset. As for RAD sequencing, we built a dataset focused on the differentiation between *E. cavolini* and *E. singularis*, retaining the 1% loci with the highest differentiation between *E. cavolini* and *E. singularis* (Table S4).

Presence of Symbiodiniaceae

We analysed the presence of Symbiodiniaceae in *Eunicella* gorgonians with transcriptome data. First, we counted the number of reads corresponding to the Symbiodiniaceae transcriptome type A1 with Salmon (Patro *et al.*, 2017). Second, we used the percentage of

assembled sequences (contigs) in the *Eunicella* transcriptomes corresponding to Symbiodiniaceae following the BLAT analysis. We used a Kruskal-Wallis test in R to test for differences among the four groups of samples (the three *Eunicella* species and the potential hybrids) for each metric. Additionally, we performed a BLAST analysis with the LSU, ITS and psbA sequences of *Philozoon* (LaJeunesse *et al.*, 2022) to try to identify the Symbiodiniaceae genera present in the different samples.

As our data pointed to the unexpected presence of Symbiodiniaceae in *E. cavolini* (see Results), we further explored this topic with preliminary data from another experiment dedicated to studying the microbiome of *E. cavolini* and *E. singularis*. This pilot study involved an analysis of microeukaryotic communities through 18S rDNA metabarcoding on two colonies of *E. cavolini*, and one *E. singularis (*Supplementary File S2).

Genetic differentiation and analysis of hybrids

With RAD sequencing data, we performed the analysis of genetic diversity with the four datasets including all loci. With transcriptomes, we performed the same analyses with the "polymorphic sites" dataset. We used the LEA R package to estimate ancestry coefficients (Frichot *et al.*, 2014; Frichot and François, 2015). We tested K values from 1 to 10, with 10 replicates for each K. To analyse the genetic differences among individuals, we performed a Principal Component Analysis (PCA) with the R package adegenet (Jombart, 2008). The pairwise F_{ST} (Weir and Cockerham, 1984) estimated among species were computed with the R package Genepop (Rousset, 2008; Rousset *et al.*, 2020), after conversion of the vcf file with PGDSpider (Lischer & Excoffier, 2012). The distribution of F_{ST} among loci was obtained with vcftools.

The hybrid status (e.g. first generation hybrids) of morphologically intermediate individuals was analysed with the NewHybrids software (Anderson and Thompson, 2002). We used the genepopedit R package to prepare the input file from genepop format (Stanley *et al.*, 2017). Following the results of the LEA and PCA analyses, we compared *E. cavolini*, *E. singularis* and potential hybrids. The NewHybrids analysis has difficulties to converge with a high number of loci compared to the number of individuals (https://github.com/eriqande/newhybrids/issues/5). We therefore used the different "1% SNP" datasets of RAD sequencing and transcriptome datasets (i.e. the most differentiated loci) for the NewHybrids analysis. As a prior, we used individuals with the lowest levels of admixture in LEA as potential parental individuals. For the RAD datasets, this corresponded to ten individuals of each species as priors. For transcriptome sequencing this corresponded to three individuals for *E. cavolini*, and six individuals for *E. singularis*. Each NewHybrids analysis was repeated ten times to test the robustness of the results.

Scenarios of speciation

We tested scenarios of speciation with the Demographic Inferences with Linked Selection (DILS) pipeline (Csilléry, *et al.*, 2012; Pudlo *et al.*, 2016; Fraïsse *et al.*, 2021) on transcriptome data only. Note that with the high number of loci recovered with transcriptomes, the numbers of specimens used here are adequate for robust inferences (Roux *et al.*, 2016). The DILS pipeline allows the analysis of two species scenarios only: we therefore performed separate analyses for the three two-species comparisons, with the “all-CS”, “all-CV”, and “all-SV” pairwise datasets. We did not include the potential hybrids in the analysis, which would have required the consideration of a separate population. The tested scenarios are presented in Figure S4 (see Fraïsse *et al.*, 2021 for details). Briefly, DILS allows testing scenario with current migration (i.e. gene flow), such as isolation / migration or secondary contact, versus scenarios of current isolation (no gene flow), such as complete or ancestral migration (gene flow among ancestral populations).

We used the same priors for all analyses, with different numbers of sequences per gene and per sample according to the dataset (Table S5). For all pairwise comparisons, we performed two DILS analyses: one with constant population sizes, and one with variable population sizes.

Results

Mitochondrial MutS

The mitochondrial MutS sequences available in GenBank confirmed the proximity of the three *Eunicella* species analysed here: all sequences were identical for these three species, as well as for three other sequences deposited in GenBank as unidentified *Eunicella* (Figure S3). The closest species to this group was *Eunicella racemosa*. All other *Eunicella* MutS sequences (*E. tricoronata* and *E. albicans*) grouped separately with *Complexum monodi*, but with low bootstrap support.

Presence of Symbiodiniaceae

The transcriptomes showed low numbers of reads counts aligning on the Symbiodiniaceae transcriptome (1868 to 58406 reads; Table S6). The proportion of contigs corresponding to Symbiodiniaceae with BLAT was also very low (between 0.00276 and 0.03686; Table S6). Significant differences were observed among species in both cases (Kruskal-Wallis test, p =

0.047 for reads counts, and p = 0.002 for the proportions of contigs). The pairwise Wilcoxon-Test showed significant differences only for the comparisons of proportions of contigs involving *E. singularis*, which was higher than in other species (Table S7; Figure 2). The mean values of reads counts and contigs for the Symbiodiniaceae in the hybrids were lower than in *E. singularis* and *E. cavolini* but higher than in *E. verrucosa*, although pairwise tests were not significant.

The BLAST analysis with the LSU, ITS and psbA sequences of *Philozoon* only retrieved corresponding sequences in the transcriptomes of *E. singularis*. Regarding the pilot study of 18S rDNA metabarcoding, a diversity of 92 Operational Taxonomic Units (OTUs) corresponding to Symbiodiniaceae in the Silva database was observed in *E. singularis*, with a single OTU largely dominant in abundance (Supplementary file S2). The same OTU was also observed in *E. cavolini* with a low abundance of reads, but still representing 99% of all 12 to 13 Symbiodiniaceae OTUs detected in the two analysed colonies. A BLAST search in GenBank identified a subset of Symbiodiniaceae sequences related to this OTU. Phylogenetic inference based on these data indicated that this OTU was related to clade A of the Symbiodiniaceae.

Genetic differentiation and analysis of hybrids

With RAD sequencing we obtained between 12 952 and 29 061 SNPs for the assembly on *E. cavolini* and *E. verrucosa* genomes respectively (Table S4). The F_{ST} estimates from RAD sequencing were highest for the comparisons between *E. verrucosa* and all other samples (F_{ST} between 0.51 and 0.66 depending on dataset; Table S8). The F_{ST} between *E. cavolini* and *E. singularis* was lower (F_{ST} between 0.29 and 0.38), and the lowest F_{ST} values were observed for hybrids compared to these two species (F_{ST} between 0.09 and 0.13). The cross-entropy analysis using LEA with RAD sequencing showed a minimum at K = 3 for the four datasets (results not shown). The barplots of coancestry coefficients were very similar for the four datasets, with a separation of the three species, and an admixture between *E. cavolini* and *E. singularis* for the morphologically intermediate individuals (Figure S5). The PCAs on RAD sequencing were very similar for all datasets, with a separation between *E. verrucosa* and all other samples on the first axis (Figure S6). The second axis separated *E. cavolini* and *E. singularis*, with the potential hybrids in intermediate position between them. Projections on axes 3 and 4 resulted mainly in the separations *of E. verrucosa* samples from each other.

With transcriptomes, we obtained 31 369 SNPs for the “polymorphic sites” dataset. With this dataset, the highest F_{ST} values were observed for the comparisons between *E. verrucosa* and all other samples (F_{ST} > 0.43; Table S9). The F_{ST} between *E. cavolini* and *E. singularis* was much lower (0.21), and the lowest F_{ST} values were observed for hybrids compared to

these two species (F_{ST} around 0.07 in both cases). These differences corresponded to different distributions of F_{ST} over SNPs (Figure S7). For the 1% SNPs with the highest F_{ST} estimates, 52 SNPs were shared by both comparisons involving *E. cavolini* (i.e. *E. cavolini* vs *E. singularis* and *E. cavolini* vs *E. verrucosa*), 116 top 1% SNPs were shared by both comparisons involving *E. singularis*, and 1042 top 1% SNPs were shared by both comparisons involving *E. verrucosa*.

The cross-entropy analysis using LEA with transcriptomes indicated a best clustering solution corresponding to K = 2 or K = 3 clusters (Figure S8). At K = 2, the first distinction was observed between *E. verrucosa* and all other samples (Figure 3). The K = 3 analysis further separated *E. cavolini* and *E. singularis*, with morphologically intermediate individuals admixed between these two species. Conversely the individuals representative of *E. cavolini* and *E. singularis* presented low levels of admixture, apart from the *E. cavolini* of the site in Algeria (code ANB), and, at a small level, two *E. singularis* individuals from Banyuls (BAN). At K = 4, the two *E. cavolini* individuals from Algeria separated from other *E. cavolini* from the northern part of the Mediterranean.

As with RAD sequencing, the PCA on transcriptome SNPs separated *E. verrucosa* from other samples on the first axis (Figure 4). The second axis separated *E. cavolini* and *E. singularis*, with the potential hybrids in intermediate position between them. The third axis separated the *E. cavolini* samples from Algeria (ANB site) from all other samples (Figure S6).

The NewHybrids analysis on RAD sequencing indicated that all morphologically intermediate individuals, except one, appeared as hybrids: first generation (F1), second generation (F2), or backcrosses with *E. singularis* or *E. cavolini* (Table 1). These samples also appeared admixed on the basis of LEA (Figure S5). One individual identified as a potential hybrid *in situ*, was inferred as a parental *E. singularis*. For four individuals, the hybrid status varied according to the dataset: F2 or backcross with *E. cavolini* in two cases, F1 or F2 in two cases. Potential parental individuals not included in the priors were well inferred as parental with NewHybrids. The NewHybrids analysis with transcriptomes indicated that the morphologically intermediate individuals were hybrids with a probability of one in all ten iterations of the analysis. One individual was a F1 hybrid, another one was F2 hybrid, and the two other ones corresponded to backcrosses with *E. singularis* (Figure 3; Table 1). In the same analysis, the *E. cavolini* and *E. singularis* individuals not included as priors for parental species (see Figure 3 for the individuals used as priors), were indeed inferred as parental with a probability of one, including the *E. cavolini* individual from Algeria (ANB).

Scenarios of speciation

The average pairwise net divergence estimated from DILS was 0.0018 between *E. cavolini* and *E. singularis,* and around 0.007 for the two comparisons with *E. verrucosa* (Table S9, https://zenodo.org/records/12532817/files/results_DILS_suppl_file.ods?download=1). The DILS analysis indicated the existence of current gene flow between *E. cavolini* and *E. singularis* with high probability, both with constant and variable population sizes ($p = 0.87$ and 0.88 respectively; Table 2). This possibility of gene flow corresponded to a scenario of secondary contact. Conversely, a model of current isolation was inferred for the comparisons between *E. verrucosa* and each of the two other species, with a probability $p \geq 0.87$: in these two cases, the inferred scenario included a period of ancestral migration, though with moderate support (p between 0.61 and 0.69). A genomic heterogeneity in effective size (i.e. variations among loci) was inferred with strong support ($p \geq 0.99$) for all analyses. In the case of current gene flow (between *E. cavolini* and *E. singularis*), a genomic heterogeneity in migration rates was inferred ($p \geq 0.82$). We repeated the DILS analysis without including the two divergent samples of *E. cavolini* from Algeria: this led to similar results, with inference of secondary contact for the comparison with *E. singularis*, and ancestral migration for the comparison with *E. verrucosa* (results not shown). For parameters inferences, we used the complete datasets, with all *E. cavolini* samples. The inferred parameters for the different scenarios are presented in Supplementary Table S9. We will first present the results obtained for the constant population sizes models. The divergence time between *E. cavolini* and *E. singularis* (median 403 273 generations) was much lower than between *E. cavolini* and *E. verrucosa* (median 1 054 488 generations), and between *E. singularis* and *E. verrucosa* (median 899 098 generations). For the comparison between *E. cavolini* and *E. singularis,* the time of secondary contact was estimated after around 85% of time spent in isolation since divergence. Following secondary contact, the gene flow was similar in both directions for these two species. The duration of ancestral migration roughly corresponded to 6% and 8% of the total time since divergence for the comparison between *E. cavolini* and *E. verrucosa*, and for the comparison between *E. singularis* and *E. verrucosa*, respectively. For these last two cases, the gene flow (forward in time) during ancestral migration was higher towards *E. verrucosa* than in the opposite direction. The estimated effective sizes were of similar order for *E. cavolini* and *E. verrucosa*. Similar results were obtained for the models including variations in effective size, except for the estimate of current gene flow between *E. cavolini* and *E. singularis*: with variable population size, gene flow from *E. singularis* to *E. cavolini* was higher than in the opposite direction.

Discussion

The three named *Eunicella* species studied here have been previously described with differences in colony morphology, sclerites shape, and in the presence of photosynthetic Symbiodiniaceae (Carpine and Grasshoff, 1975). Our results demonstrate a continuum between *E. cavolini* and *E. singularis*, with morphologically intermediate individuals, on-going gene flow, and hybrids characterised by a reduced frequency of Symbiodiniaceae compared to *E. singularis*. On the other hand, *E. verrucosa* appears genetically isolated from these two species. We will discuss here the differences observed among markers, the outcome of hybridization, the speciation scenarios, and what can be learnt on the evolution of symbiosis.

Discordances between molecular markers

As previously observed (Aurelle *et al.*, 2017), mitochondrial DNA did not allow to discriminate the three species due to the usually slow evolution of mitochondrial DNA in octocorals (McFadden *et al.*, 2011; Muthye *et al.*, 2022). The use of transcriptome sequences first confirmed the closer proximity between *E. cavolini* and *E. singularis* than with *E. verrucosa*. This had been previously suggested with two intron sequences, but with incomplete lineage sorting (Aurelle *et al.*, 2017). The Mediterranean *Eunicella* then add a new example of the lack of power of mitochondrial DNA to discriminate genetically differentiated octocoral species, as demonstrated in other genera (Erickson *et al.*, 2021; Pante *et al.*, 2015a). The slow rate of evolution of mitochondrial DNA in octocorals has been linked to the presence of the mitochondrial locus MutS, an homolog of a bacterial gene involved in DNA repair. However, there are contradictory examples showing that the presence of this locus is not the only factor explaining the slow evolution of mitochondrial DNA in octocorals (Muthye *et al.*, 2022). More generally, as hybridization can lead to the sharing of mitochondrial DNA among species, the use of multiple independent nuclear loci is required for species discrimination in such cases.

Incomplete reproductive isolation among two named species

Inferences of genetic ancestry and hybrid status confirmed that morphologically intermediate individuals are indeed hybrids between *E. singularis* and *E. cavolini*, with the identification of F1, F2 and backcrosses with both parental lineages: first generation hybrids can then be fertile. The fact that gene flow indeed goes further than the hybrid levels is confirmed by the DILS analysis, which did not include hybrid individuals. Reproductive isolation is therefore at least partial between these lineages. The ease to find hybrids in the area studied here, as well as similar observations in other sites (S. Sartoretto, pers. com.) indicate that hybridization is not rare on an evolutionary scale. Similarly, transcriptome sequencing has

led to infer hybridization among *Plexaura* species on the basis of a small number of samples (Pelosi *et al.*, 2022).

The alternation of populations with and without hybrids would point to a mosaic hybrid zone (Bierne *et al.*, 2003), where hybrids could form in different areas and from different parental populations. As, or because, hybridization between *E. cavolini* and *E. singularis* had not been reported before, the presence of hybrids has probably been overlooked up to now. This may be the consequence of previously focusing on colonies with “typical” morphologies. The frequency of hybridization therefore remains to be studied.

Our results allow discussing the evolution of genomic divergence among these species. The persistence of genomic differentiation between these lineages in sympatry, despite current gene flow, indicates that intrinsic (i.e. genomic incompatibilities) or extrinsic (e.g. ecology) factors can maintain partial isolation. Difference or overlap in the timing of reproduction should also be considered in contributing to pre-mating isolation (Pelosi *et al.*, 2022). A better characterization of the ecological range of parental and hybrid populations would be useful to test if local adaptation is involved in their distribution. Intrinsic factors such as genetic incompatibilities, potentially coupled with differences in adaptation to local environments, can be present as well (Bierne *et al.*, 2011). A genome wide analysis of differentiation is required to investigate whether divergence between *E. cavolini* and *E. singularis*, is homogeneous along the genome (as suggested by the DILS analysis which inferred a homogeneity of gene flow), or whether genomic islands of differentiation exist (Peñalba *et al.*, 2024). We could then better understand to what stage of divergence the *E. cavolini* / *E. singularis* split corresponds: from intra-specific polymorphism to species separated by semipermeable barriers to gene flow.

One interesting question in this context is whether changes in selection regimes induced by human activities can change the outcome of hybridization (Ålund *et al.*, 2023). For example, Mediterranean octocorals are impacted by mortality events linked with climate change (Sini *et al.*, 2015; Estaque *et al.*, 2023), and the impact of these events could be different for hybrids and parental individuals. In scleractinian corals, interspecific hybridization has been reported to enhance the survival under elevated temperature conditions (Chan *et al.*, 2018) .

Regarding *E. verrucosa*, the more ancient divergence corresponded to much more loci with high F_{ST}. Among the list of the most highly differentiated loci, more overlap was also observed for the two comparisons involving *E. verrucosa* than for the other pairwise comparisons: this may indicate that few genomic areas of potential incompatibilities with *E. verrucosa* are involved in the divergence between *E. cavolini* and *E. singularis*.

Scenarios of speciations

The scenarios of speciations inferred with DILS supported the current isolation (no gene flow) of *E. verrucosa* with the two other species with high posterior probability. Conversely current gene flow was strongly supported versus isolation between *E. cavolini* and *E. singularis*. The posterior probabilities for ancestral migration (for *E. verrucosa* versus the two other species), and secondary contact (*E. cavolini* and *E. singularis*), were lower than for inferences on current gene flow. These scenarios were indeed the best ones among those tested here but they might not provide the best possible representation of the evolutionary history. Other models of evolution could be tested for better inferences, for example by including the three species and hybrids, or gene flow from unsampled taxa (Tricou *et al.*, 2022). The current isolation of *E. verrucosa* from *E. cavolini* is also at odds with previous results which showed the possibility of current gene flow between these two species despite an important divergence (Roux *et al.*, 2016). It will be useful to explore the reasons for the discrepancy between this last study and the present one, which are both based on transcriptome datasets but obtained from different samples and sequencing platforms.

Eunicella verrucosa is currently widely distributed in the North Eastern Atlantic Ocean, and less frequent in the Mediterranean Sea, whereas both other species are only present in the Mediterranean Sea. The Atlantic / Mediterranean Sea transition does not seem to act as a phylogeographic barrier for *E. verrucosa* (Macleod *et al.*, 2024). We can propose a scenario where the split between *E. verrucosa* and both other species occurred in allopatry between the Atlantic Ocean and the Mediterranean Sea, followed by the colonization of the Mediterranean Sea by *E. verrucosa*. The generation time remains unknown for the *Eunicella* species, and previous studies have shown important variation in the age at first reproduction in gorgonians, from 2 to 13 years (see references in Munro, 2004). If we use a generation time of two years for *Eunicella* species, with a median estimate of divergence time around 900 000 generations for *E. verrucosa* / *E. singularis* and 1 000 000 for *E. verrucosa* / *E. cavolini*, and based on a mutation rate set at 3.10^{-9}, this would indicate a divergence at least around 2 000 000 years (2 Ma). The divergence time between *E. cavolini* and *E. singularis* would be 2.5 times more recent, around 800 000 years, with a median time of secondary contact around 60 000 generations, corresponding to 15% of the time spent since divergence. It is difficult to infer past distributions of *E. singularis* and *E. cavolini*, but one can note that even if they are currently found in sympatry in different areas, their range do not completely overlap. For example *E. cavolini* is nearly absent at the West of the Rhone estuary on the French coast, whereas *E. singularis* is present there. The ecological range of *E. singularis* and *E. cavolini* is also not completely overlapping, as *E. cavolini* can be observed deeper than *E. singularis* (Gori *et al.*, 2012; Carugati *et al.*, 2022). Therefore one can envision an historical separation of these two species either geographically or ecologically, followed by a secondary contact where gene flow took place. In any case,

additional information on generation time, mutation rate and past demographic fluctuations are required to be more precise on the history of these species.

Evolution of symbiosis

As previously discussed, we clearly demonstrated here the possibility of gene flow between symbiotic (hosting Symbiodiniaceae) and non-symbiotic octocorals. Symbiodiniaceae could nevertheless be involved in genetic incompatibilities with the genome of some cnidarian hosts, but this would require additional analysis of symbiotic status in hybrids. The methods used here did not aim at a precise quantification of Symbiodiniaceae, and one can note the low levels of sequences corresponding to these symbionts, even in *E. singularis,* which may be due to difficulties in extracting the RNA of the symbionts (but see Guzman *et al.*, 2018; Rivera-García *et al.*, 2019). Despite these limits we observed, as expected, a higher Symbiodiniaceae concentration in *E. singularis* than in *E. cavolini* and *E. verrucosa.* Interestingly, the hybrids showed a lower frequency of Symbiodiniaceae than *E. singularis*, and possibly than *E. cavolini*, though this last result remains to be confirmed. In *E. singularis*, the transmission of Symbiodiniaceae seems to occur both vertically, through ovules, and horizontally, from the environment (Forcioli *et al.*, 2011). Both transmission modes did not restore the levels of Symbiodiniaceae in the hybrids to those of *E. singularis*. This suggests a breakdown of or a failure to establish symbiosis for hybrid genotypes, which may impact the fitness of hybrids and consequently the possibility of introgression. The *aphyta* type of *E. singularis* observed in deep conditions indicates a plasticity of symbiotic status apart from hybridization. Nevertheless, here the hybrids were sampled in shallow conditions (10-20 m depth) which underlines the role of hybridization in reducing the extent of symbiosis. More precise estimates of Symbiodiniaceae abundance, and of physiological parameters such as photosynthetic and respiration rates (Ezzat *et al.*, 2013). would help understanding the role of symbionts in hybrids fitness. It would also be interesting to study if the Symbiodiniaceae of the different samples belong to the same population (Pelosi *et al.*, 2022).

Our results also question the evolution and significance of octocoral / Symbiodiniaceae symbiosis. In scleractinians, the transition between symbiotic and non-symbiotic states happened repeatedly, but mostly in the direction of the acquisition of symbiosis, with very low rates of reversal (Campoy *et al.*, 2020). This could indicate that investing in such mutualistic interactions for the cnidarian would lead to increasingly relying on autotrophy for energetic supply, making reversal to heterotrophy difficult. In octocorals, an evolutionary versatility in symbiotic state seems possible, as in various families and genera, both symbiotic and non-symbiotic species are present (Van Oppen *et al.*, 2005). In the

Mediterranean Sea, all octocoral species are non-symbiotic, except for *E. singularis* (but see Bonacolta et al. 2024). The most parsimonious scenario here would be an acquisition of symbiosis in *E. singularis* during or following its divergence from *E. cavolini*. The symbiotic status of *E. singularis* nevertheless could be facultative as previously mentioned for the *aphyta* type (Gori *et al.*, 2012). Additionally, experimental physiological studies have demonstrated the nutritional plasticity of *E. singularis* which is able to use either heterotrophy or autotrophy for its metabolism (Ezzat *et al.*, 2013). Nevertheless, in natural conditions, autotrophy seems to provide an important contribution to the metabolism of *E. singularis*, and the collapse of photosynthetic capacities in too warm conditions can contribute to mortality events in this species (Coma *et al.*, 2015).

The question of symbiosis could be reversed as well: why are Symbiodiniaceae not more abundant in *E. cavolini*? This species can be observed in shallow conditions (less than 10 m depth) where there is enough light for photosynthesis, and in syntopy with *E. singularis*. The availability of preys or particulate organic matter may provide enough energy to *E. cavolini* in its habitat, but this species may have never engaged in mutualistic interaction with Symbiodiniaceae. Interestingly we observed a low rate of sequences related to Symbiodiniaceae in the transcriptomes of *E. cavolini* (and even lower, but not null in *E. verrucosa*). This could either correspond to a signal from free living Symbiodiniaceae, or to rare, transient, associations with the cnidarian. In addition, a Symbiodiniaceae OTU that is common to *E. singularis* and *E. cavolini* was identified among the microeukaryotes associated with the two species: this OTU is related to strains observed in symbiosis with *E. singularis* and other Mediterranean cnidarians. Molecular markers also allowed to evidence the presence of Symbiodiniaceae in species previously supposed to be asymbiotic, as in the Mediterranean octocoral *Paramuricea clavata*, and in several Hawaiian antipatharian species (Wagner *et al.*, 2011; Bonacolta *et al.*, 2024). These results, and our observations in *Eunicella* species, obviously underline the dynamic nature of interactions between Symbiodiniaceae and cnidarians: the establishment of symbiosis may be preceded by more or less stable, and more or less mutualistic interactions. The development of effective symbiosis, with stable relationships, and higher abundance of symbiont, would require specific adaptation from both partners. We can see here that even if on a macro-evolutionary scale the acquisition of symbiosis is much more frequent than its loss, on a micro-evolutionary scale the gene flow between the *Eunicella* species considered here has not led to the full development of symbiosis in *E. cavolini*.

Conclusions and perspectives

We demonstrated the lack of genetic isolation between octocorals with contrasted levels of mutualistic interaction with Symbiodiniaceae. Understanding the evolution and adaptation of these species in heterogeneous environments should then consider the possible impact of introgression. We also show that symbiosis is more flexible that previously envisioned in octocorals. For these species it will be useful to estimate the frequency and spatial extent of hybrid zones: does it correlate with particular environments with a coupling between endogenous and exogenous barriers to gene flow (Bierne *et al.*, 2011)? Characterizing the genomic landscape of introgression would help to look for the effects of introgression on adaptation or symbiosis for example. Indeed, even low levels of interspecific gene flow can have important consequences on the evolution of species (Arnold *et al.*, 1999). Finally, various cases of hybridization have been demonstrated in symbiotic anthozoans (e.g. Combosch and Vollmer, 2015; Pelosi *et al.*, 2022): it would then be interesting to study the dynamics of symbiosis in these cases, especially when different Symbiodiniaceae strains are involved.

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Data availability

The transcriptome raw sequences are available in Genbank under BioProject ID PRJNA1037721. The RAD raw sequences are available in Genbank under BioProject ID PRJNA1122331.

The scripts used in this study, the vcf files from RAD sequencing, and the detailed results of the DILS analysis are available at https://doi.org/10.5281/zenodo.14007931

Conflict of interest disclosure

The authors declare that they have no conflict of interest in relation to the content of the article

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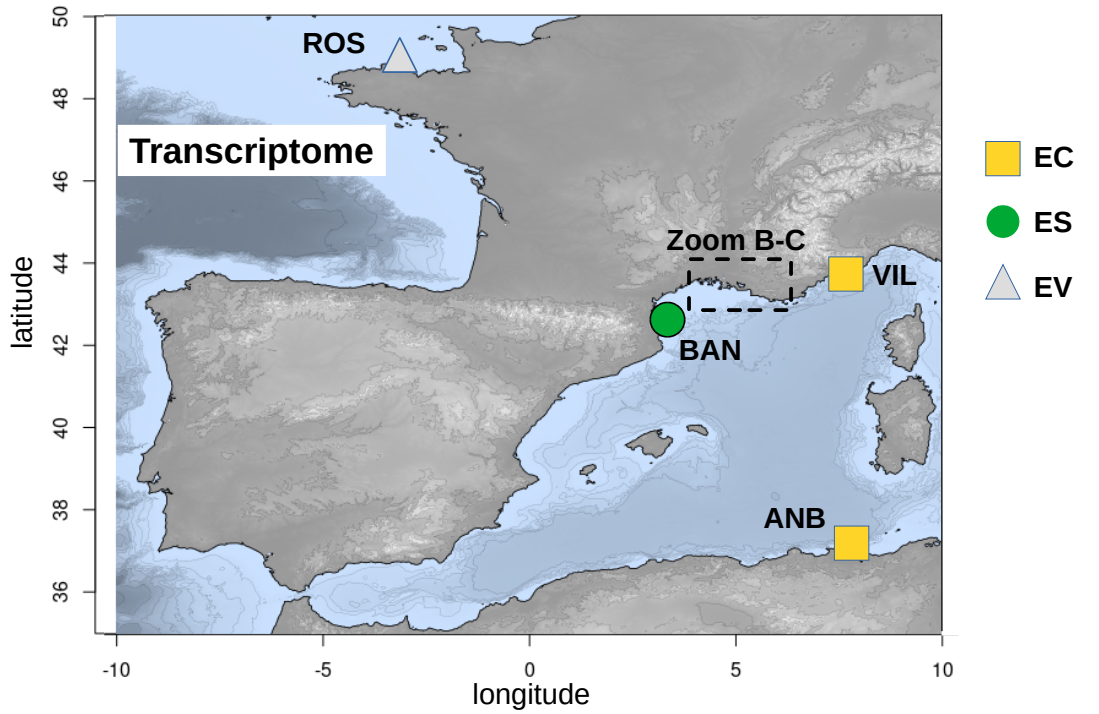
Figure 1: map of sampling sites for transcriptomes: A) general view, B) zoom on the area of Marseille. The symbols correspond to different samples: EC *E. cavolini*, ES *E. singularis*, EV *E. verrucosa*, HY potential hybrids. The three letters correspond to the codes of the sampling. The maps have been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

A)



B)

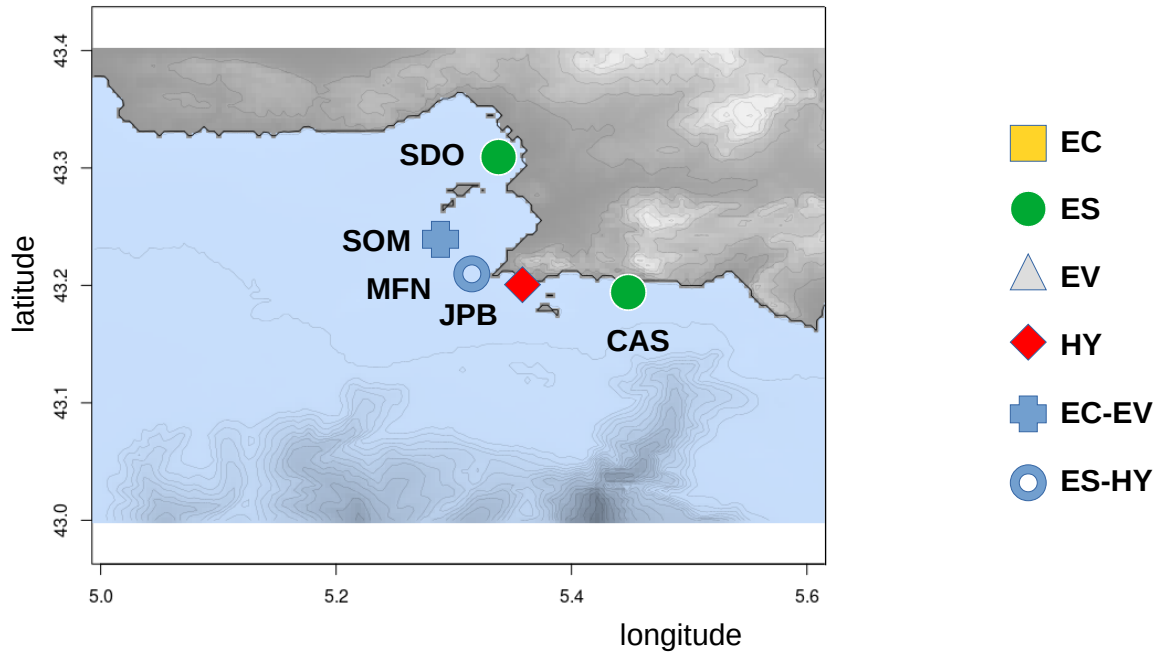
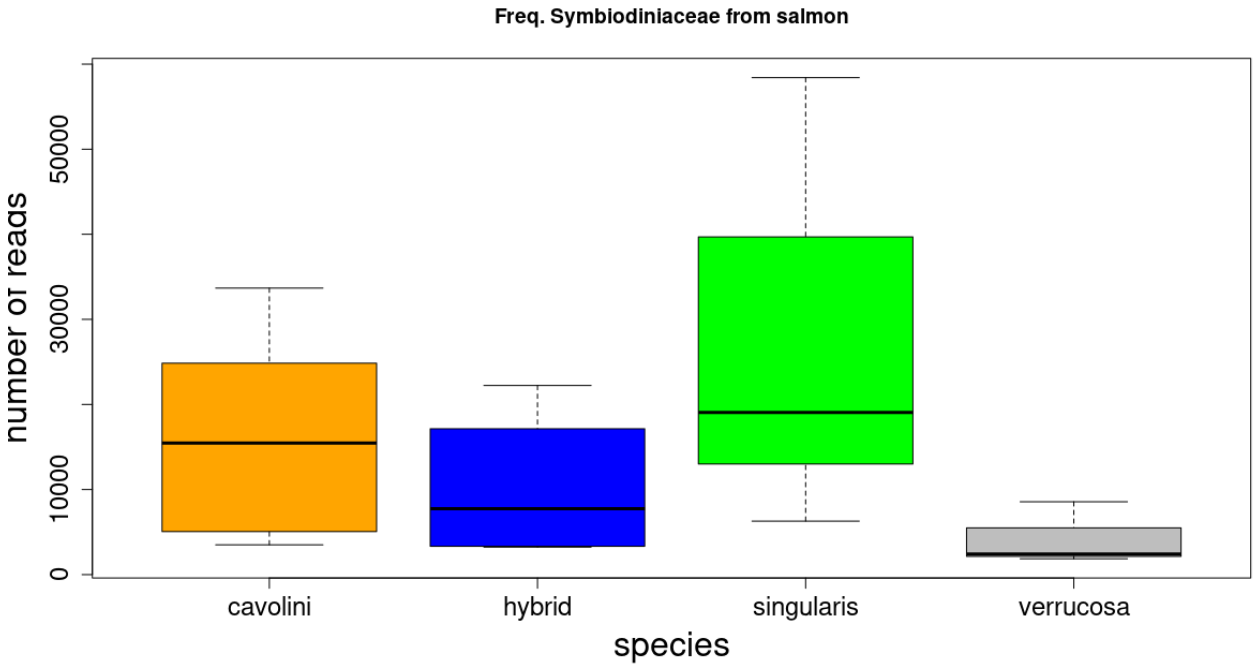


Figure 2: distribution of the frequency of Symbiodiniaceae sequences in the individual transcriptomes according to the species based A) on the number of reads estimated with Salmon, and B) on the proportion of assembled sequences (contigs) with the BLAT analyses.

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.

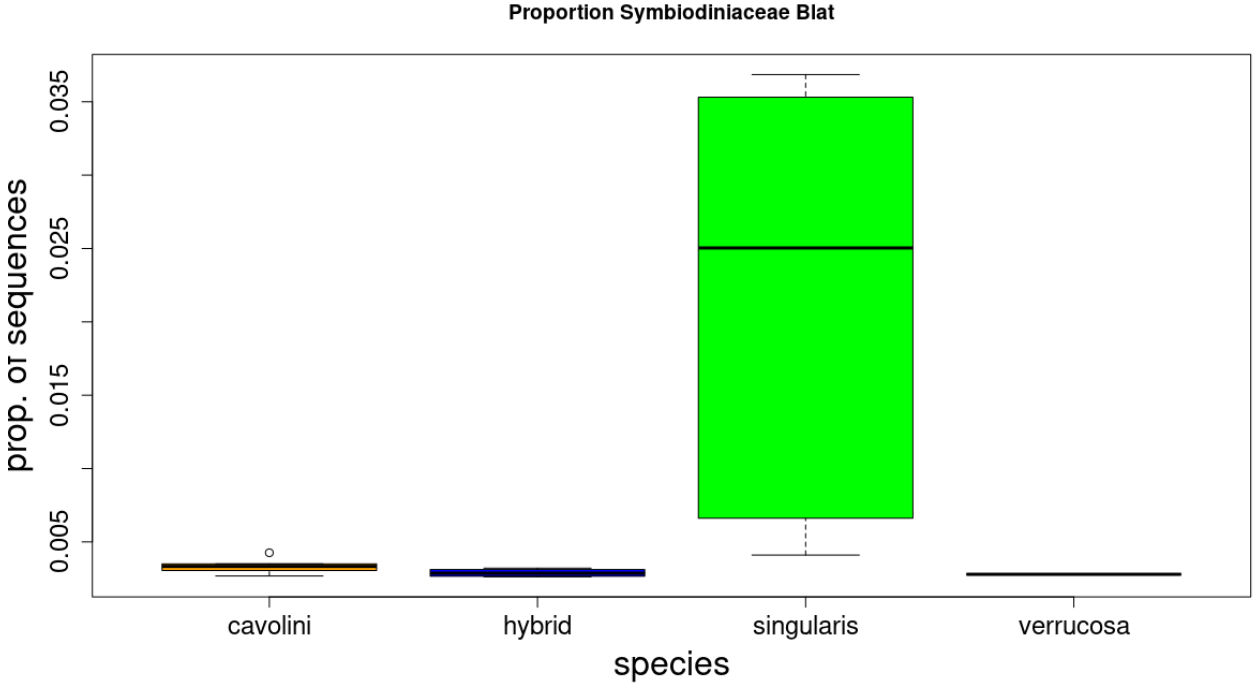


Figure 3: barplots of coancestry coefficients inferred with the LEA R package. The analysis is based on the “polymorphic sites” transcriptome dataset. The red asterisks indicate the individuals used as prior for parental status in the NewHybrids analysis. The results of the NewHybrids analysis are indicated below the hybrid individuals: F1, 1st generation; F2, 2nd generation; Sbx, backcross with *E. singularis*. The coancestry analysis is based on 31 369 SNPs, whereas the NewHybrids analysis is based on 326 SNPs showing high differentiation between *E. cavolini* and *E. singularis*.

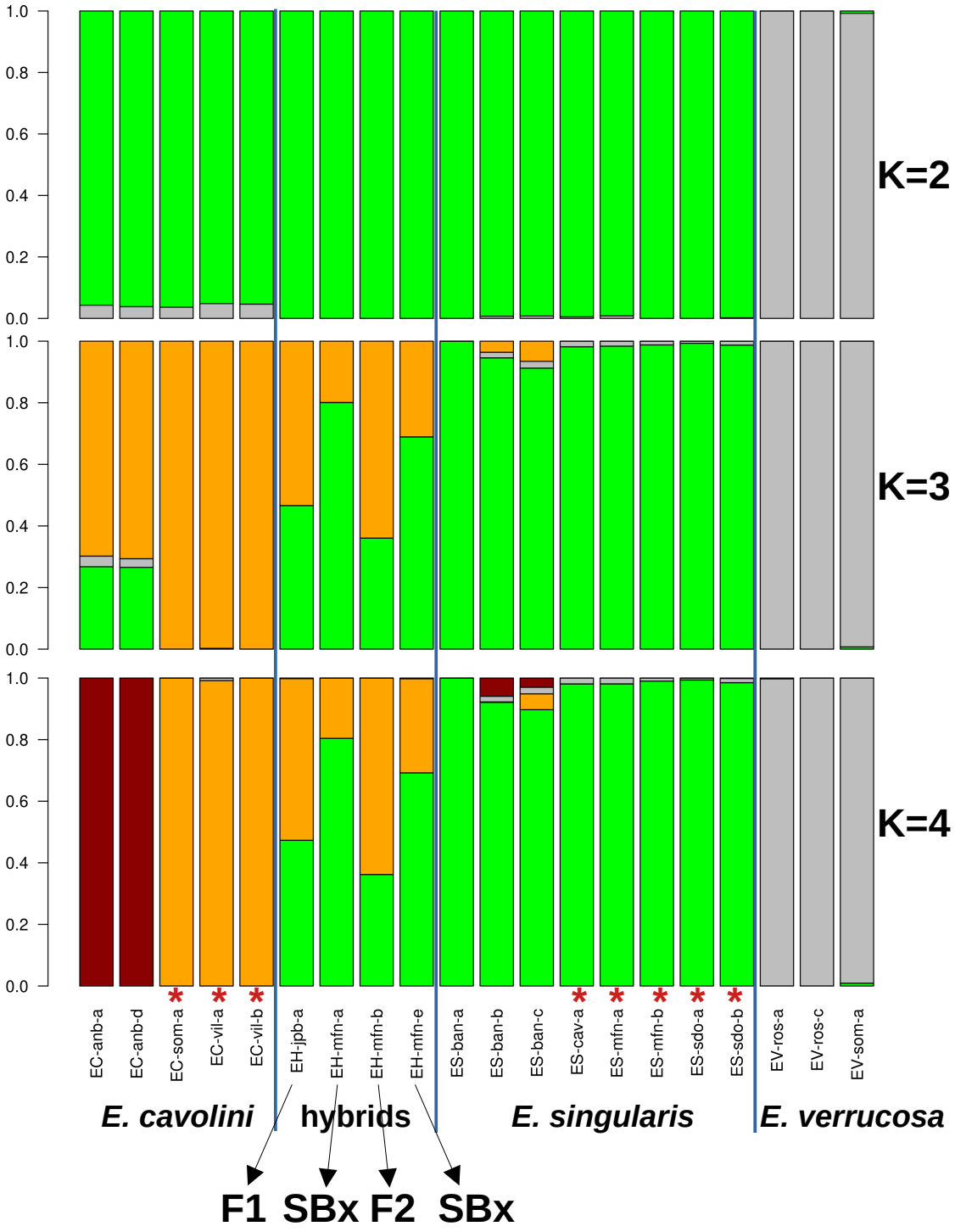

1.0
0.8
0.6
0.4
0.2
0.0
K=2
K=3
K=4
EC-anb-a
EC-anb-d
EC-som-a
EC-vil-a
EC-vil-b
EH-jpb-a
EH-mfn-a
EH-mfn-b
EH-mfn-e
ES-ban-a
ES-ban-b
ES-ban-c
ES-cav-a
ES-mfn-a
ES-mfn-b
ES-sdo-a
ES-sdo-b
EV-ros-a
EV-ros-c
EV-som-a
E. cavolini
hybrids
E. singularis
E. verrucosa
F1
SBx
F2
SBx

Figure 4: principal Component Analysis based on the “polymorphic SNPs” transcriptome dataset; axis 1 represents 33.2% of the variance, axis 2 represents 13% of the variance

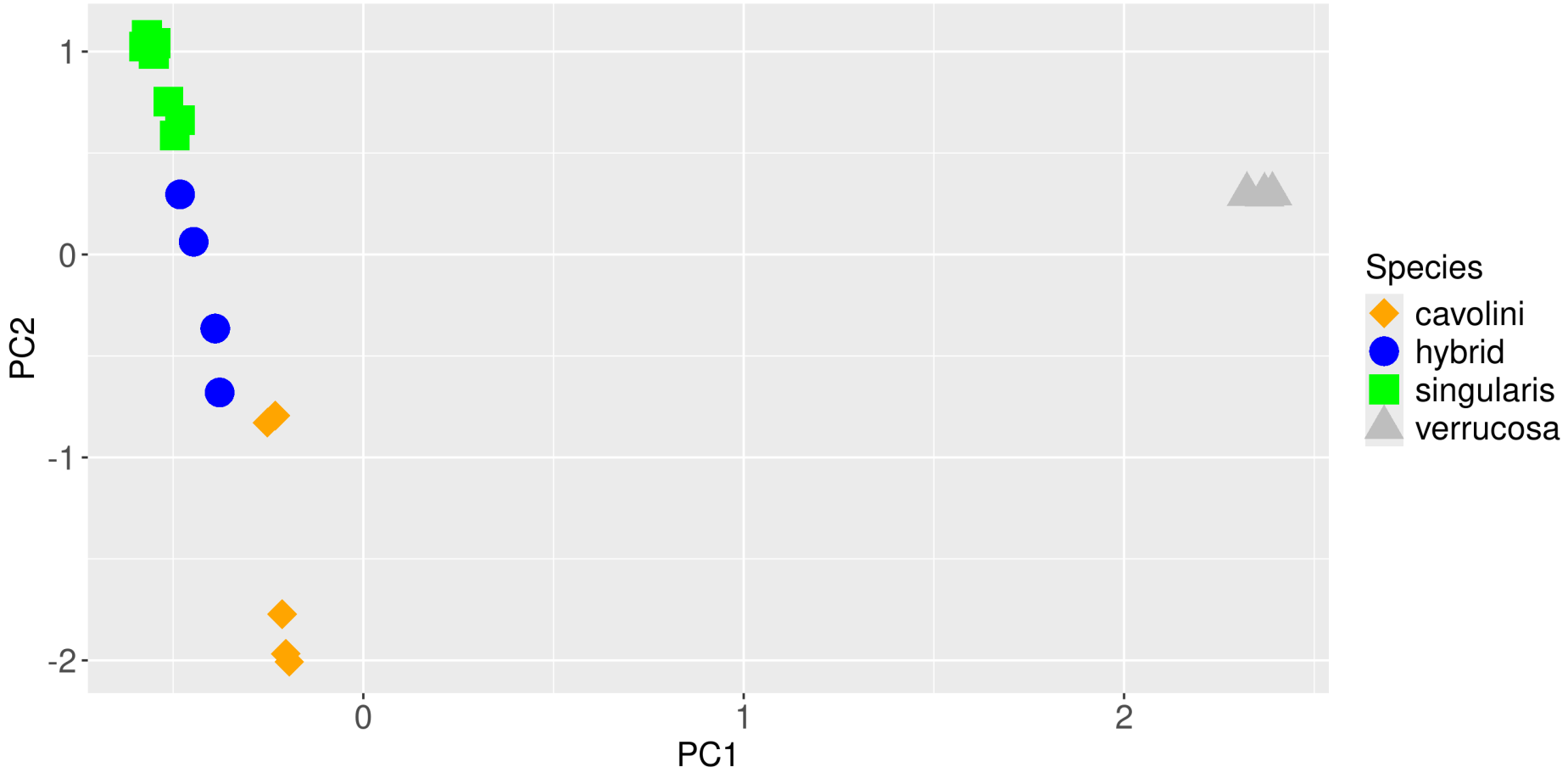


Table 1 : inference of hybrid status with NewHybrids for transcriptome and RAD sequencing. For transcriptomes, all probabilities were at 1 for the inferred status and for the ten replicates. For RAD sequencing, the results are given for the four datasets (different assembly strategies). If no probability is mentioned for RAD sequencing, the hybrid status was supported by a probability higher than 0.999 over the ten replicates. In the other cases, the numbers indicate the minimal probability threshold over the ten replicates for this status (and the status was coherent over the ten replicates as well, with slight variations in probability). NA indicates an individual which was removed during the filtering of SNPs because of too many missing data. The lines highlighted in grey indicate the cases where different status were inferred depending on the dataset. Bx-ES and Bx-EC indicate backcrosses with *E. singularis* and *E. cavolini* respectively; ES indicates parental *E. singularis*.

Individual - RAD sequencing	***de novo***	**ref. *E. cavolini***	**ref. *E. singularis***	**ref. *E. verrucosa***
EC-X-MFNB	F2	F2	F2	F2
EC-X-MFNC	F2	NA	Bx-EC	NA
EC-X-MFND	Bx-ES	Bx-ES	Bx-ES	Bx-ES
EC-X-MFNE	Bx-EC	Bx-EC	Bx-EC	Bx-EC
EC-X-MFNF	Bx-EC	F2 > 0.95	Bx-EC > 0.92	F2
EC-X-MFNG	F2	F2	F2	F2
EC-X-MFNH	Bx-ES	Bx-ES	Bx-ES > 0.67	Bx-ES > 0.98
EC-X-MFNI	F1	F1	F1	F2
EC-X-MFNL	F1	F1 > 0.99	F1	F2
ES-X-MFNA	Bx-ES	Bx-ES	Bx-ES	Bx-ES
ES-X-MFNJ	F2	F2 > 0.96	F2	F2
ES-X-MFNK	ES	ES	ES	ES
Individual - transcriptome				
EH-JPB-a	F1			
EH-MFN-a	Bx-ES			
EH-MFN-b	F2			
EH-MFN-e	Bx-ES			

Table 2: results of demographic inferences with DILS with transcriptome data. The columns indicate the species comparison, the model choice for population size (constant vs. variable), and the results of inferences: current (on-going) gene flow (migration vs isolation); if current migration is inferred DILS compares isolation / migration (IM) with secondary contact (SC); if no current migration is inferred, the comparison is between strict isolation (SI) and ancestral migration (AM); the last columns give the results of the tests of homogeneity or heterogeneity among loci for inferences in effective size (N-homo vs N-hetero), and gene flow (M-homo vs M-hetero). The probability of each scenario is given in the same case. Homogeneity and heterogeneity indicate no variation or variation among loci respectively.

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