Biological Journal of the Linnean Society, 2024, **143**, blae116
https://doi.org/10.1093/biolinnean/blae116
Advance access publication 17 December 2024
Original Article

OXFORD

Original Article

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

Didier Aurelle[1,2,*], Anne Haguenauer[3], Marc Bally[1], Frédéric Zuberer[4], Dorian Guillemain[4], Jean-Baptiste Ledoux[5], Stéphane Sartoretto[6], Cédric Cabau[7], Rachel Lapeyre[8], Lamya Chaoui[9], Hichem Kara[9], Sarah Samadi[2], Pierre Pontarotti[10,11,12]

[1]Aix Marseille Univ, Université de Toulon, CNRS, IRD, MIO, 13009, Marseille, France
[2]Institut Systématique Evolution Biodiversité (ISYEB), Muséum national d'Histoire naturelle, CNRS, Sorbonne Université, EPHE, Université des Antilles, CP 26, 75005 Paris, France
[3]CNRS - Délégation Provence et Corse, 13009, Marseille, France
[4]Aix Marseille Université, CNRS, IRD, INRAE, OSU Institut PYTHEAS, 13009, Marseille, France
[5]CIIMAR/CIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, 4450-208, Porto, Portugal
[6]IFREMER, COAST, 83500 La Seyne-sur-Mer, France
[7]Sigenae, GenPhySE, Université de Toulouse, INRAE, ENVT, 31326, Castanet Tolosan, France
[8]MGX-Montpellier GenomiX, Univ. Montpellier, CNRS, INSERM, 34094, Montpellier, France
[9]Laboratoire Bioressources marines, Université d'Annaba Badji Mokhtar, Annaba, 23000, Algérie
[10]Aix Marseille Université, MEPHI, 13005, Marseille, France
[11]IHU Méditerranée Infection, 13005, Marseille, France
[12]CNRS SNC5039, 13005, Marseille, France

*Corresponding author. Aix Marseille Université, Université de Toulon, CNRS, IRD, MIO, 13009 Marseille, France. E-mail: didier.aurelle@univ-amu.fr

ABSTRACT

Understanding how species can form and remain isolated in the marine environment still stimulates active research. Here we study the differentiation and the possibility of hybridization among three temperate octocorals: *Eunicella cavolini*, *Eunicella singularis*, and *Eunicella 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 F_1 and F_2 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

Received 3 September 2024; revised 8 November 2024; accepted 26 November 2024

concepts (Pante *et al.* 2015b). Sound species delimitations are useful in estimating a 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 in 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 with 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 progress in developing methodologies 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 Symbiodiniaceae (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 and 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.* 2022).

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), *Eunicella singularis* (Esper, 1791), and *Eunicella 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 devoid 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 individual identified as *E. singularis*, nor individuals that are morphologically difficult to attribute to a named species (which could be hybrids) have been analysed. Here, we will further investigate 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 then 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 in 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 to 40 m in 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 lattermost individuals displayed intermediate colours and branching patterns between *E. cavolini* and *E. singularis* (Supporting information, Fig. S1), and they were analysed to test their hybrid status (Aurelle *et al.* 2017). The specimens have been sampled by scuba diving at different times of the year in the area of Marseille, where the three species are present in sympatry (Supporting information, Fig. 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; Supporting information, Table S2; Fig. 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, and included 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 Supporting information, Figure S3 and Supporting information, Table S3.

RAD sequencing

DNA was extracted with a Macherey-Nagel NucleoSpin DNA RapidLyse kit. RAD library preparation (with the *Pst*I restriction enzyme) and sequencing (Illumina NovaSeq600 with 150 nucleotide paired-end sequencing) were performed at the MGX platform (CNRS). The MGX platform performed quality control, demultiplexing, and removal of PCR duplicates with unique molecule identifiers. Potential contaminants were removed with kraken2 (Wood *et al.* 2019, Lu *et al.* 2022). RAD loci were 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.* personal communication).

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 summarized in Supporting information, Table S4).

Transcriptome sequencing and SNP calling

Total RNA was extracted as in Haguenauer *et al.* (2013). RNAs were sent to the LIGAN genomic platform for sequencing (Lille, France) on four flow cells of an Illumina NextSeq 500 (2 × 75 bp). The transcriptomes were 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 Supporting information, 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 the 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 SNP calling was 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, is thereafter 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 were used for demographic inferences, and are referred to 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* (Supporting information, Table S4).

Presence of Symbiodiniaceae

We analysed the presence of Symbiodiniaceae in *Eunicella* gorgonians with transcriptome data. First, we counted the number

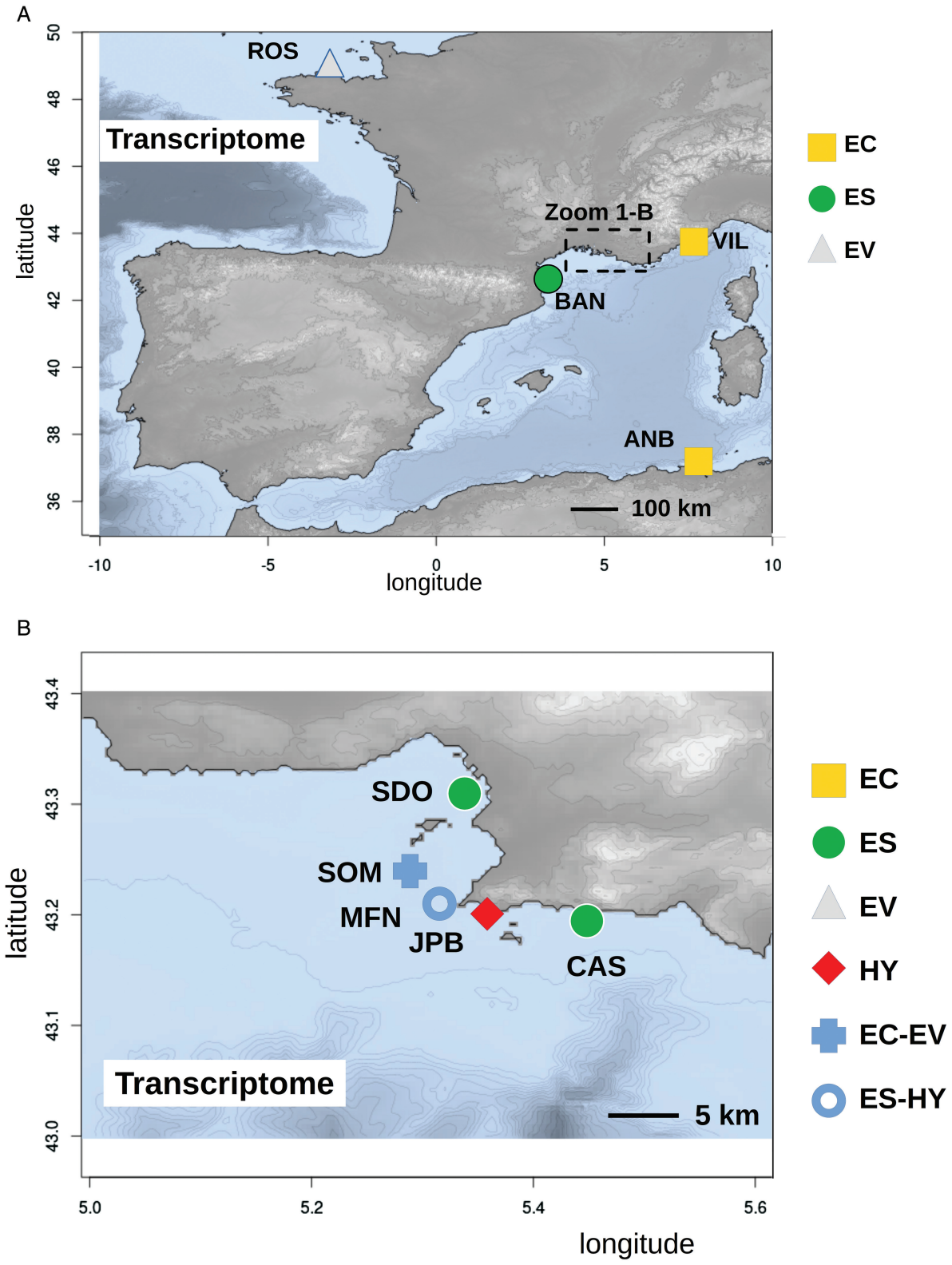

Figure 1. Map of sampling sites for transcriptomes: (A) general view, (B) close-up on the area of Marseille. The symbols correspond to different samples: EC *E. cavolini*, ES *E. singularis*, EV *E. verrucosa*, HY potential hybrids. The three letters correspond to the codes of the sampling. The latitude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

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 nuclear ribosomal large subunit (LSU), Internal Transcribed Spacers (ITS), and photosystem II protein D1 (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* (Supporting information, 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 and 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 the 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 when there is a too 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 10 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 10 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 Supporting information, Figure S4 (see Fraïsse *et al.* 2021 for details). Briefly, DILS allows testing a scenario with current migration (i.e. gene flow), such as isolation/migration or secondary contact, vs. 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 (Supporting information, 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* (Supporting information, Fig. S3). The closest species to this group was *Eunicella racemosa*. All other *Eunicella* MutS sequences (*Eunicella tricoronata* and *Eunicella 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 58 406 reads; Supporting information, Table S6). The proportion of contigs corresponding to Symbiodiniaceae with BLAT was also very low (between 0.00276 and 0.03686; Supporting information, Table S6). Significant differences were observed among species in both cases (Kruskal–Wallis test, $P = .047$ for reads counts, and $P = .002$ 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 (Supporting information, Table S7; Fig. 2). The mean values of reads counts and contigs for 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 (Supporting information, File S2). The same OTU was also observed in *E. cavolini* with a low abundance of reads, but still representing 99% of all

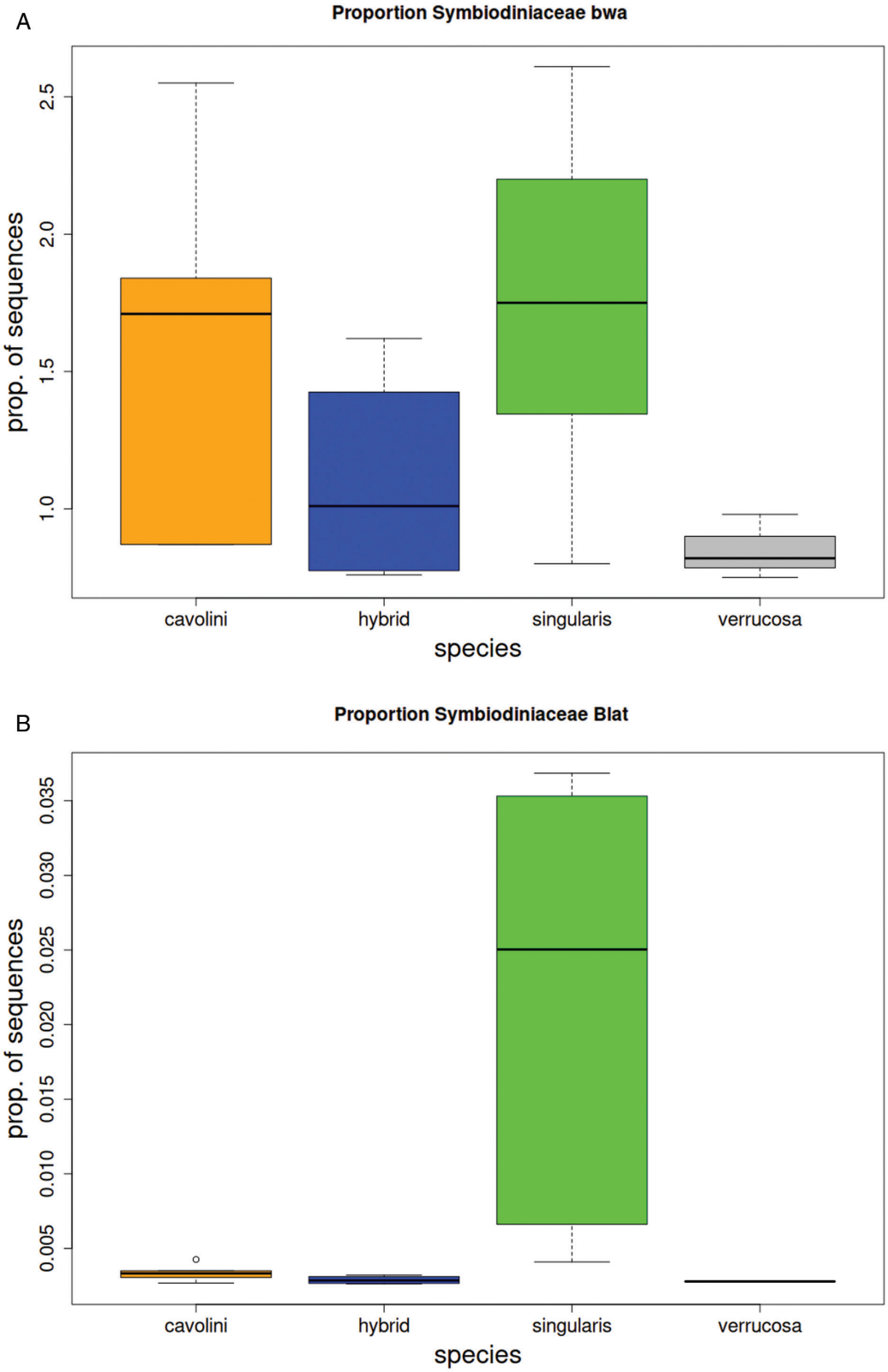

Figure 2. Distribution of the frequency of Symbiodiniaceae sequences in the individual transcriptomes according to the species based (2A) on the number of reads estimated with Salmon, and (2B) on the proportion of assembled sequences (contigs) with the BLAT analyses. 2A, Read counts with Salmon; mean values per group: *E. cavolini*: 16 508; hybrids: 10 238; *E. singularis*: 26 023; *E. verrucosa*: 4285. Kruskal–Wallis test of the differences among groups: chi-squared = 7.9467, d.f. = 3, *P*-value = .047. 2B, 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, d.f. = 3, *P*-value = .002.

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 the *E. cavolini* and *E. verrucosa* genomes, respectively (Supporting information, 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; Supporting information, 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 the comparisons between hybrids and *E. cavolini* or *E. singularis* (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 (Supporting information, Fig. 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 (Supporting information, Fig. S6). The second axis separated *E. cavolini* and *E. singularis*, with the potential hybrids in an 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; Supporting information, 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 (Supporting information, Fig. 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 (Supporting information, Fig. S8). At K = 2, the first distinction was observed between *E. verrucosa* and all other samples (Fig. 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 (Fig. 4). The second axis separated *E. cavolini* and *E. singularis*, with the potential hybrids in an intermediate position between them. The third axis separated the *E. cavolini* samples from Algeria (ANB site) from all other samples (Supporting information, Fig. S6).

The NewHybrids analysis on RAD sequencing indicated that all morphologically intermediate individuals, except one, appeared as hybrids: first generation (F_1), second generation (F_2), or backcrosses with *E. singularis* or *E. cavolini* (Table 1). These samples also appeared admixed on the basis of LEA (Supporting information, Fig. 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: F_2 or backcross with *E. cavolini* in two cases, F_1 or F_2 in two cases. Potential parental individuals not included in the priors were 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 10 iterations of the analysis. One individual was an F_1 hybrid, another one was an F_2 hybrid, and the two other ones corresponded to backcrosses with *E. singularis* (Fig. 3; Table 1). In the same analysis, the *E. cavolini* and *E. singularis* individuals not included as priors for parental species (see Fig. 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* (Supporting information, Table S9, Aurelle 2024). 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 = .87 and .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 $P \geq .87$: 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 ($P \geq .99$) for all analyses. In the case of current gene flow (between *E. cavolini* and *E. singularis*), a genomic heterogeneity in migration rates was inferred ($P \geq .82$). 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 parameter inferences, we used the complete datasets, with all *E. cavolini* samples. The inferred parameters for the different scenarios are presented in Supporting information, 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

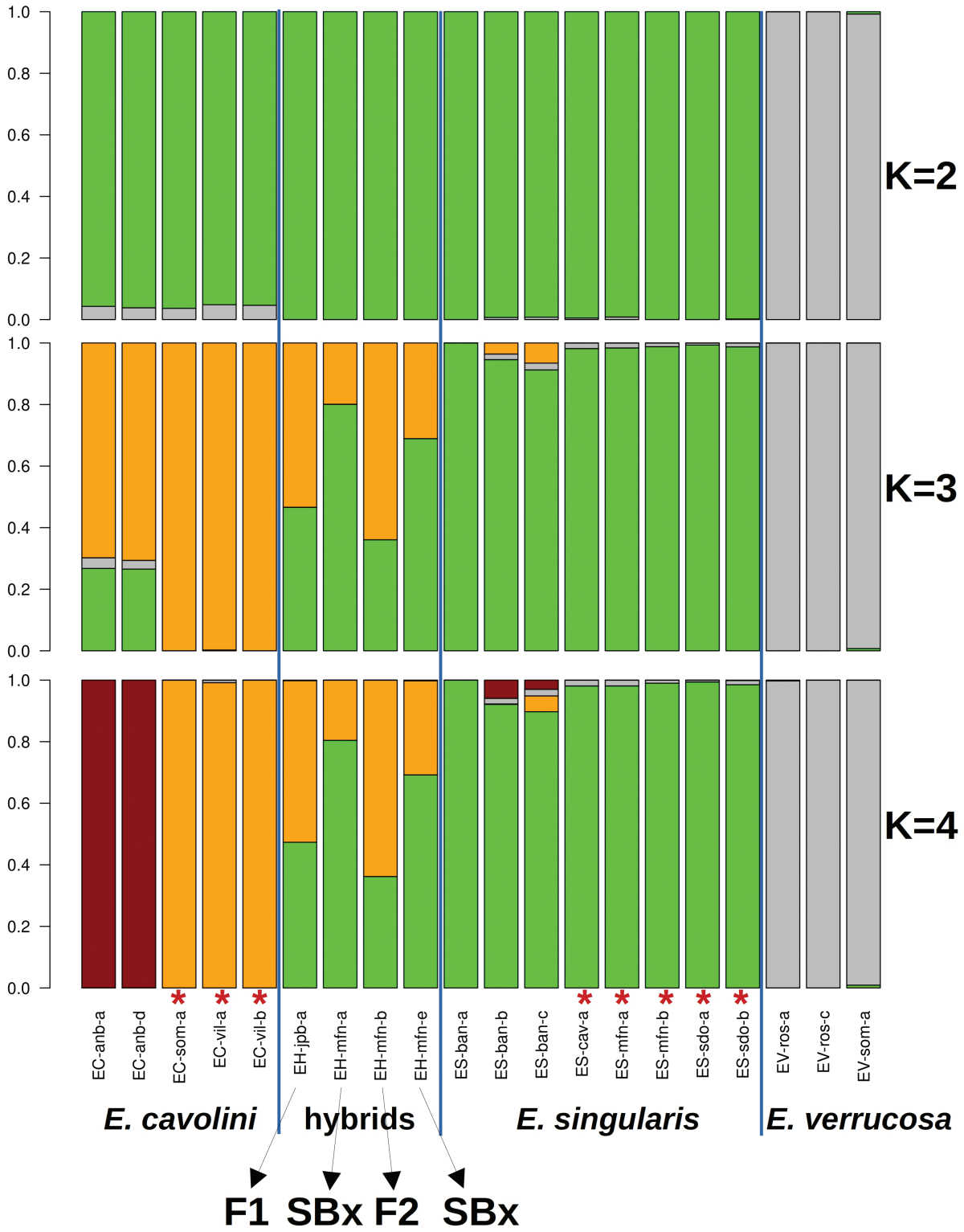

Figure 3. Barplots of coancestry coefficients inferred with the LEA R package. The analysis is based on the 'polymorphic sites' transcriptome dataset. The asterisks indicate the individuals used as priors for parental status in the NewHybrids analysis. The results of the NewHybrids analysis are indicated below the hybrid individuals: F_1, 1st generation; F_2, 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*.

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

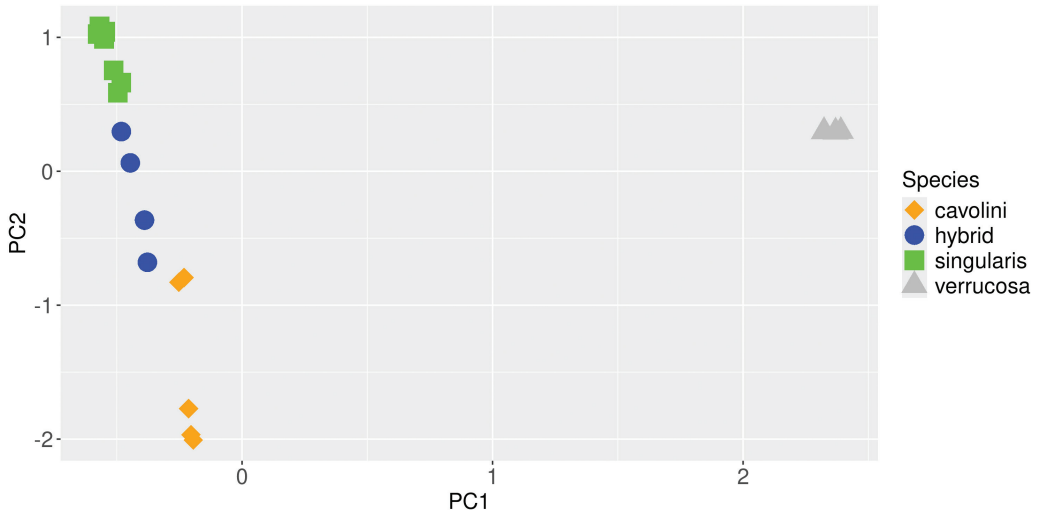

Figure 4. Principal Component Analysis based on the 'polymorphic SNPs' transcriptome dataset; axis 1 represents 33.2% of the variance, axis 2 represents 13% of the variance

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 10 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 10 replicates. In the other cases, the numbers indicate the minimal probability threshold over the 10 replicates for this status (and the status was coherent over the 10 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 was 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	F_2	F_2	F_2	F_2
EC-X-MFNC	F_2	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	$F_2 > 0.95$	Bx-EC > 0.92	F_2
EC-X-MFNG	F_2	F_2	F_2	F_2
EC-X-MFNH	Bx-ES	Bx-ES	Bx-ES > 0.67	Bx-ES > 0.98
EC-X-MFNI	F_1	F_1	F_1	F_2
EC-X-MFNL	F_1	$F_1 > 0.99$	F_1	F_2
ES-X-MFNA	Bx-ES	Bx-ES	Bx-ES	Bx-ES
ES-X-MFNJ	F_2	$F_2 > 0.96$	F_2	F_2
ES-X-MFNK	ES	ES	ES	ES
Individual—transcriptome				
EH-JPB-a	F_1			
EH-MFN-a	Bx-ES			
EH-MFN-b	F_2			
EH-MFN-e	Bx-ES			

E. singularis and *E. verrucosa*, respectively. For these last two cases, the gene flow (forwards 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, sclerite 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 characterized by a reduced frequency of Symbiodiniaceae compared to *E. singularis*. On the other hand, *E. verrucosa* appears

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	-

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 discriminate the three species due to the 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 (Pante *et al.* 2015a, Erickson *et al.* 2021). The slow rate of evolution of mitochondrial DNA in octocorals has been linked to the presence of the mitochondrial locus MutS, a homologue 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 F_1, F_2, 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, personal communication) 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 semi-permeable 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 survival under elevated temperature conditions (Chan *et al.* 2018).

Regarding *E. verrucosa*, the more ancient divergence corresponded to many 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 vs. isolation between *E. cavolini* and *E. singularis*. The posterior probabilities for ancestral migration (for *E. verrucosa* vs. 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 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 frequently in the Mediterranean Sea, whereas both other species (*E. cavolini* and *E. singularis*) 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 ago, 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 ranges 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 at greater depths 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 either *E. cavolini* or *E. verrucosa*. Interestingly, the hybrids showed a lower frequency of Symbiodiniaceae than *E. singularis*, and *E. cavolini*, though the difference between hybrids and *E. cavolini* 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). Neither transmission modes restored 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 water 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 hybrid 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 may 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 prey 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 may 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 showed 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 adaptations 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*.

CONCLUSION

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 these correlate with particular environments with a coupling between endogenous and exogenous barriers to gene flow (Bierne *et al.* 2011)? For example, characterizing the genomic landscape of introgression would help to identify effects of introgression on adaptation or symbiosis. 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.

SUPPORTING INFORMATION

Supplementary data is available at *Biological Journal of the Linnean Society* online.

ACKNOWLEDGEMENTS

We thank the ECCOREV Research Federation (FR 3098) for the financial support of part of this study (https://www.eccorev.fr/). The project leading to this publication has received funding from European FEDER Fund under project 1166-39417 and the Excellence Initiative of Aix-Marseille University - A*MIDEX, a French 'Investissements d'Avenir' programme. The authors are grateful for the use of the UMR 8199 LIGAN-PM Genomics platform (Lille, France, with special thanks to Véronique Dhennin) of the 'Federation de Recherche' 3508 Labex EGID (European Genomics Institute for Diabetes; ANR-10-LABX-46) supported by the ANR Equipex 2010 session (ANR-10-EQPX-07-01; 'LIGAN-PM'). The LIGAN-PM Genomics platform (Lille, France) was also supported by the FEDER and the Region Nord-Pas-de-Calais-Picardie. J-.B.L. was supported by the strategic funding grants UIDB/04423/2020, UIDP/04423/2020, and 2021.00855.CEECIND through national funds provided by FCT -Fundação para a Ciência e a Tecnologia. Reference genomes were obtained with the support from EASI-genomics funded from the European Union's Horizon 2020 research and innovation programme under grant agreement No 824110 to J-.B.L. Camille Roux, Jonathan Romiguier, and Christelle Fraïsse were of a great help for the analysis of scenarios of speciation. We thank the diving service of INSU/OSU Pytheas for fieldwork, and the Calanques National Park for sampling authorisations. We acknowledge the staff of the 'Cluster de calcul intensif HPC' Platform of the OSU Institut Pythéas (Aix-Marseille Université, INSU-CNRS) for providing the computing facilities. We are grateful to the Genotoul bioinformatics platform Toulouse Occitanie (Bioinfo Genotoul, https://doi.org/10.15454/1.5572369328961167E12) for providing help, and computing and storage resources. We thank Christophe Klopp and Marie-Stéphane Trotard for their help. We acknowledge the use of the computing cluster of MNHN (Plateforme de Calcul Intensif et Algorithmique PCIA, Muséum National d'Histoire Naturelle, CNRS). Part of the bioinformatics analyses were performed on the Core Cluster of the Institut Français de Bioinformatique (IFB) (ANR-11-INBS-0013). Montpellier GenomiX is financially supported by the France Génomique National infrastructure, funded as part of 'Investissement d'Avenir' programme managed by Agence Nationale pour la Recherche (contract ANR-10-INBS-09). Part of this work was performed during a CNRS detachment position of D.A. at the ISYEB laboratory.

CONFLICT OF INTEREST

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

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 (Aurelle 2024).

REFERENCES

Ålund M, Cenzer M, Bierne N *et al.* Anthropogenic change and the process of speciation. *Cold Spring Harbor Perspectives in Biology* 2023;**15**:a041455. https://doi.org/10.1101/cshperspect.a041455.

Anderson E, Thompson EA. A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* 2002;**160**:1217–29. https://academic.oup.com/genetics/article-abstract/160/3/1217/6052497

Arnold ML, Bulger MR, Burke JM *et al.* Natural hybridization: how low can you go and still be important? *Ecology* 1999;**80**:371–81. https://doi.org/10.2307/176618.

Aurelle D. Analysis of speciation and hybridization in Mediterranean octocorals. *Zenodo*. https://doi.org/10.5281/zenodo.14007931. 1 November 2024.

Aurelle D, Pivotto ID, Malfant M *et al.* Fuzzy species limits in Mediterranean gorgonians (Cnidaria, Octocorallia): inferences on speciation processes. *Zoologica Scripta* 2017;**46**:767–78. https://doi.org/10.1111/zsc.12245.

Baird NA, Etter PD, Atwood TS *et al.* Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS One* 2008;**3**:e3376. https://doi.org/10.1371/journal.pone.0003376.

Baumgarten S, Bayer T, Aranda M *et al.* Integrating microRNA and mRNA expression profiling in *Symbiodinium microadriaticum*, a dinoflagellate symbiont of reef-building corals. *BMC Genomics* 2013;**14**:704. https://doi.org/10.1186/1471-2164-14-704.

Berkelmans R, van Oppen MJH. The role of zooxanthellae in the thermal tolerance of corals: a 'nugget of hope' for coral reefs in an era of climate change. *Proceedings Biological Sciences* 2006;**273**:2305–12. https://doi.org/10.1098/rspb.2006.3567.

Bierne N, Borsa P, Daguin C *et al.* Introgression patterns in the mosaic hybrid zone between *Mytilus edulis* and *M. galloprovincialis*. *Molecular Ecology* 2003;**12**:447–61. https://doi.org/10.1046/j.1365-294x.2003.01730.x.

Bierne N, Welch J, Loire E *et al.* The coupling hypothesis: why genome scans may fail to map local adaptation genes. *Molecular Ecology* 2011;**20**:2044–72. https://doi.org/10.1111/j.1365-294X.2011.05080.x.

Bonacolta AM, Miravall J, Gómez-Gras D *et al.* Differential apicomplexan presence predicts thermal stress mortality in the Mediterranean coral *Paramuricea clavata*. *Environmental Microbiology* 2024;**26**:e16548.

Bordenstein S. Symbiosis and the origin of species. In: Bourtzis K, Miller TA (ed.), *Insect Symbiosis*. Boca Raton: CRC Press, 2003, 283–303.

Brener-Raffalli K, Vidal-Dupiol J, Adjeroud M *et al.* Gene expression plasticity and frontloading promote thermotolerance in *Pocillopora* corals. *Peer Community Journal* 2022;**2**:e13. https://peercommunityjournal.org/articles/10.24072/pcjournal.79/

Brucker RM, Bordenstein SR. Speciation by symbiosis. *Trends in Ecology & Evolution* 2012;**27**:443–51. https://doi.org/10.1016/j.tree.2012.03.011.

Cabau C, Escudié F, Djari A *et al.* Compacting and correcting trinity and Oases RNA-Seq *de novo* assemblies. *PeerJ* 2017;**5**:e2988. https://doi.org/10.7717/peerj.2988.

Cahill AE, Meglécz E, Chenuil A. Scientific history, biogeography, and biological traits predict presence of cryptic or overlooked species. *Biological Reviews of the Cambridge Philosophical Society* 2024;**99**:546–61. https://doi.org/10.1111/brv.13034.

Cairns SD. Deep-water corals: an overview with special reference to diversity and distribution of deep-water scleractinian corals. *Bulletin of Marine Science* 2007;**81**:311–22.

Calderón I, Garrabou J, Aurelle D. Evaluation of the utility of *COI* and ITS markers as tools for population genetic studies of temperate gorgonians. *Journal of Experimental Marine Biology and Ecology* 2006;**336**:184–97. https://doi.org/10.1016/j.jembe.2006.05.006.

Campoy AN, Addamo AM, Machordom A *et al.* The origin and correlated evolution of symbiosis and coloniality in scleractinian corals. *Frontiers in Marine Science* 2020;**7**:1–13. https://www.frontiersin.org/articles/10.3389/fmars.2020.00461/full

Canessa M, Bavestrello G, Bo M *et al.* Filling a gap: A population of Eunicella verrucosa (pallas, 1766) (Anthozoa, Alcyonacea) in the Tavolara-punta Coda Cavallo marine protected area (Ne Sardinia, Italy). *Diversity* 2022;**14**:405. https://doi.org/10.3390/d14050405

Carpine C, Grasshoff M. Les gorgonaires de la Méditerranée. *Bulletin de l'Institut Océanographique de Monaco* 1975;**71**:140.

Carugati L, Moccia D, Bramanti L *et al.* Deep-dwelling populations of Mediterranean *Corallium rubrum* and *Eunicella cavolini*: distribution, demography, and co-occurrence. *Biology* 2022;**11**:333. https://doi.org/10.3390/biology11020333.

Casado-Amezúa P, Terrón-Sigler A, Pinzón JH *et al.* General ecological aspects of anthozoan-*Symbiodinium* interactions in the Mediterranean Sea. In: Goffredo S, Dubinsky Z (eds), *The Cnidaria, Past, Present and Future: The World of Medusa and Her Sisters*. Switzerland: Springer, 2016, 375–86.

Chan WY, Peplow LM, Menéndez P *et al.* Interspecific hybridization may provide novel opportunities for coral reef restoration. *Frontiers in Marine Science* 2018;**5**:160.

Chimienti G. Vulnerable forests of the pink sea fan *Eunicella verrucosa* in the Mediterranean Sea. *Diversity* 2020;**12**:176. https://doi.org/10.3390/d12050176

Coelho M, Pearson G, Boavida J *et al.* Not out of the Mediterranean: Atlantic populations of the gorgonian *Paramuricea clavata* are a separate sister species under further lineage diversification. *Ecology and Evolution* 2023;**13**:e9740. https://onlinelibrary.wiley.com/doi/abs/10.1002/ece3.9740

Coma R, Llorente-Llurba E, Serrano E *et al.* Natural heterotrophic feeding by a temperate octocoral with symbiotic zooxanthellae: a contribution to understanding the mechanisms of die-off events. *Coral Reefs* 2015;**34**:549–60. https://doi.org/10.1007/s00338-015-1281-3.

Combosch DJ, Vollmer SV. Trans-Pacific RAD-Seq population genomics confirms introgressive hybridization in Eastern Pacific *Pocillopora* corals. *Molecular Phylogenetics and Evolution* 2015;**88**:154–62. https://doi.org/10.1016/j.ympev.2015.03.022.

Csilléry K, François O, Blum MGB. abc: an R package for approximate Bayesian computation (ABC). *Methods in Ecology and Evolution* 2012;**3**:475–9. https://doi.org/10.1111/j.2041-210x.2011.00179.x.

Danecek P, Auton A, Abecasis G *et al.*; 1000 Genomes Project Analysis Group. The variant call format and VCFtools. *Bioinformatics* 2011;**27**:2156–8. https://doi.org/10.1093/bioinformatics/btr330.

De Jode A, Le Moan A, Johannesson K *et al.* Ten years of demographic modelling of divergence and speciation in the sea. *Evolutionary Applications* 2023;**16**:542–59. https://doi.org/10.1111/eva.13428.

De Queiroz K. Species concepts and species delimitation. *Systematic Biology* 2007;**56**:879–86. https://doi.org/10.1080/10635150701701083.

Eaton DA, Overcast I. Interactive assembly and analysis of RADseq datasets. *Bioinformatics* 2020;**36**:2592–4.

Erickson KL, Pentico A, Quattrini AM *et al.* New approaches to species delimitation and population structure of anthozoans: two case studies of octocorals using ultraconserved elements and exons. *Molecular Ecology Resources* 2021;**21**:78–92. https://doi.org/10.1111/1755-0998.13241.

Estaque T, Richaume J, Bianchimani O *et al.* Marine heatwaves on the rise: one of the strongest ever observed mass mortality event in temperate gorgonians. *Global Change Biology* 2023;**29**:6159–62. https://doi.org/10.1111/gcb.16931.

Ezzat L, Merle PL, Furla P *et al.* The response of the Mediterranean gorgonian *Eunicella singularis* to thermal stress is independent of its nutritional regime. *PLoS One* 2013;**8**:e64370. https://doi.org/10.1371/journal.pone.0064370.

Faria R, Johannesson K, Stankowski S. Speciation in marine environments: diving under the surface. *Journal of Evolutionary Biology* 2021;**34**:4–15. https://doi.org/10.1111/jeb.13756.

Forcioli D, Merle PL, Caligara C *et al.* Symbiont diversity is not involved in depth acclimation in the Mediterranean sea whip *Eunicella singularis*. *Marine Ecology Progress Series* 2011;**439**:57–71. https://doi.org/10.3354/meps09314.

Fourt M, Goujard A. Rapport final de la campagne MEDSEACAN (Têtes des canyons méditerranéens continentaux) novembre 2008–avril 2010. *Partenariat Agence des aires marines protégées–GIS Posidonie*. Marseille: GIS Posidonie, 2012, 1–218. http://paleopolis.rediris.es/benthos/TaP/Rapport_Final_MEDSEACAN.pdf

Fraïsse C, Popovic I, Mazoyer C *et al.* DILS: demographic inferences with linked selection by using ABC. *Molecular Ecology Resources* 2021;**21**:2629–44. https://doi.org/10.1111/1755-0998.13323.

Frichot E, François O. LEA: an R package for landscape and ecological association studies. *Methods in Ecology and Evolution* 2015;**6**:925–9. https://doi.org/10.1111/2041-210x.12382.

Frichot E, Mathieu F, Trouillon T *et al.* Fast and efficient estimation of individual ancestry coefficients. *Genetics* 2014;**196**:973–83. https://doi.org/10.1534/genetics.113.160572.

Furla P, Allemand D, Shick JM *et al.* The symbiotic anthozoan: a physiological chimera between alga and animal. *Integrative and Comparative Biology* 2005;**45**:595–604. https://doi.org/10.1093/icb/45.4.595.

Gagnaire P, Broquet T, Aurelle D *et al.* Using neutral, selected, and hitchhiker loci to assess connectivity of marine populations in the genomic era. *Evolutionary Applications* 2015;**8**:769–86. https://doi.org/10.1111/eva.12288.

Gayral P, Melo-Ferreira J, Glemin S *et al.* Reference-free population genomics from next-generation transcriptome data and the vertebrate–invertebrate gap. *PLoS Genetics* 2013;**9**:e1003457. https://doi.org/10.1371/journal.pgen.1003457.

Gori A, Bramanti L, López-González P *et al.* Characterization of the zooxanthellate and azooxanthellate morphotypes of the Mediterranean gorgonian *Eunicella singularis*. *Marine Biology* 2012;**159**:1485–96. https://doi.org/10.1007/s00227-012-1928-3.

Grasshoff M. Die Flachwasser-Gorgonarien von Europa und Westafrika (Cnidaria, Anthozoa). *Courier Forschunginstitut Senckenberg*, Vol. **149**. Frankfurt am Main: Senckenbergische Naturforschende Gesellschaft, 1992.

Guzman C, Shinzato C, Lu TM *et al.* Transcriptome analysis of the reef-building octocoral, *Heliopora coerulea*. *Scientific Reports* 2018;**8**:8397. https://doi.org/10.1038/s41598-018-26718-5.

Haguenauer A, Zuberer F, Ledoux JB *et al.* Adaptive abilities of the Mediterranean red coral *Corallium rubrum* in a heterogeneous and changing environment: from population to functional genetics. *Journal of Experimental Marine Biology and Ecology* 2013;**449**:349–57. https://doi.org/10.1016/j.jembe.2013.10.010

Jombart T. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 2008;**24**:1403–5. https://doi.org/10.1093/bioinformatics/btn129.

Kent WJ. BLAT—the BLAST-like alignment tool. *Genome Research* 2002;**12**:656–64. https://doi.org/10.1101/gr.229202.

Krueger-Hadfield S. *marmap.* https://www.molecularecologist.com/2015/07/03/marmap/. 2015.

LaJeunesse TC, Parkinson JE, Gabrielson PW *et al.* Systematic revision of Symbiodiniaceae highlights the antiquity and diversity of coral endosymbionts. *Current Biology* 2018;**28**:2570–80.e6. https://doi.org/10.1016/j.cub.2018.07.008.

LaJeunesse TC, Wiedenmann J, Casado-Amezúa P *et al.* Revival of Philozoon Geddes for host-specialized dinoflagellates, 'zooxanthellae', in animals from coastal temperate zones of Northern and Southern hemispheres. *European Journal of Phycology* 2022;**57**:166–80.

Leroy T, Louvet JM, Lalanne C *et al.* Adaptive introgression as a driver of local adaptation to climate in European white oaks. *The New Phytologist* 2020;**226**:1171–82. https://doi.org/10.1111/nph.16095.

Lesser MP, Stat M, Gates RD. The endosymbiotic dinoflagellates (*Symbiodinium* sp.) of corals are parasites and mutualists. *Coral Reefs* 2013;**32**:603–11. https://doi.org/10.1007/s00338-013-1051-z.

Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* 2009;**25**:1754–60. https://doi.org/10.1093/bioinformatics/btp324.

Li H, Handsaker B, Wysoker A *et al.*; 1000 Genome Project Data Processing Subgroup. The sequence alignment/map format and SAMtools. *Bioinformatics* 2009;**25**:2078–9. https://doi.org/10.1093/bioinformatics/btp352.

Lischer HE, Excoffier L. PGDSpider: an automated data conversion tool for connecting population genetics and genomics programs. *Bioinformatics* 2012;**28**:298–9. https://doi.org/10.1093/bioinformatics/btr642.

Lu J, Rincon N, Wood DE *et al.* Metagenome analysis using the Kraken software suite. *Nature Protocols* 2022;**17**:2815–39. https://doi.org/10.1038/s41596-022-00738-y.

Macleod KL, Jenkins TL, Witt MJ *et al.* Rare, long-distance dispersal underpins genetic connectivity in the pink sea fan, *Eunicella verrucosa*. *Evolutionary Applications* 2024;**17**:e13649. https://doi.org/10.1111/eva.13649.

Matz MV. Not-so-mutually beneficial coral symbiosis. *Current Biology* 2024;**34**:R798–801. https://doi.org/10.1016/j.cub.2024.07.047

Mayr E. Wu's genic view of speciation. *Journal of Evolutionary Biology* 2001;**14**:866–7. https://doi.org/10.1046/j.1420-9101.2001.00336.x.

McFadden CS, Benayahu Y, Pante E *et al.* Limitations of mitochondrial gene barcoding in Octocorallia. *Molecular Ecology Resources* 2011;**11**:19–31. https://doi.org/10.1111/j.1755-0998.2010.02875.x.

McFadden CS, Quattrini AM, Brugler MR *et al.* Phylogenomics, origin, and diversification of anthozoans (phylum Cnidaria). *Systematic Biology* 2021;**70**:635–47. https://doi.org/10.1093/sysbio/syaa103.

Muir PR, Obura DO, Hoeksema BW *et al.* Conclusions of low extinction risk for most species of reef-building corals are premature. *Nature Ecology & Evolution* 2022;**6**:357–8. https://doi.org/10.1038/s41559-022-01659-5.

Munro L. Determining the reproductive cycle of *Eunicella verrucosa*. Gosport, UK: *Reef Research*. 2004, 1–28. https://www.marine-bio-images.com/RR_Eunicella_PDFS/Report_RR12Jul2004reproductive%20cycle%20pdf.pdf

Muthye V, Mackereth CD, Stewart JB *et al.* Large dataset of octocoral mitochondrial genomes provides new insights into mt-mutS evolution and function. *DNA Repair* 2022;**110**:103273. https://doi.org/10.1016/j.dnarep.2022.103273.

Palumbi SR. Marine speciation on a small planet. *Trends in Ecology & Evolution* 1992;**7**:114–8. https://doi.org/10.1016/0169-5347(92)90144-Z.

Pante E, Simon-Bouhet B. marmap: a package for importing, plotting and analyzing bathymetric and topographic data in R. *PLoS One* 2013;**8**:e73051.

Pante E, Puillandre N, Viricel A *et al.* Species are hypotheses: avoid connectivity assessments based on pillars of sand. *Molecular Ecology* 2015a;**24**:525–44. https://doi.org/10.1111/mec.13048.

Pante E, Abdelkrim J, Viricel A *et al.* Use of RAD sequencing for delimiting species. *Heredity* 2015b;**114**:450–9. https://doi.org/10.1038/hdy.2014.105.

Patro R, Duggal G, Love MI *et al.* Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods* 2017;**14**:417–9. https://doi.org/10.1038/nmeth.4197.

Pelosi JA, Bernal MA, Krabbenhoft TJ *et al.* Fine-scale morphological, genomic, reproductive, and symbiont differences delimit the Caribbean octocorals *Plexaura homomalla* and *P. kükenthali*. *Coral Reefs* 2022;**41**:635–53. https://doi.org/10.1007/s00338-021-02175-x.

Peñalba JV, Runemark A, Meier JI *et al.* The role of hybridization in species formation and persistence. *Cold Spring Harbor Perspectives in Biology* 2024;**a041445**:a041445. https://doi.org/10.1101/cshperspect.a041445.

Picard Toolkit. *Broad Institute. GitHub Repository.* https://broadinstitute.github.io/picard/. 2019.

Porro B. Diversités génétiques chez l'holobiote *Anemonia viridis*: des morphotypes de l'hôte à la différenciation symbiotique. Doctoral dissertation, COMUE Université Côte d'Azur, 2019.

Pudlo P, Marin JM, Estoup A *et al.* Reliable ABC model choice via random forests. *Bioinformatics* 2016;**32**:859–66. https://doi.org/10.1093/bioinformatics/btv684.

Readman J, Hiscock K. *Eunicella verrucosa. Pink sea fan*. 2017. https://www.marlin.ac.uk/species/detail/1121.

Reynes L, Aurelle D, Chevalier C *et al.* Population genomics and Lagrangian modeling shed light on dispersal events in the Mediterranean endemic *Ericaria zosteroides* (= *Cystoseira zosteroides*) (Fucales). *Frontiers in Marine Science* 2021;**8**:683528.

Rivera-García L, Rivera-Vicéns RE, Veglia AJ *et al. De novo* transcriptome assembly of the digitate morphotype of *Briareum asbestinum* (Octocorallia: Alcyonacea) from the southwest shelf of Puerto Rico. *Marine Genomics* 2019;**47**:100676. https://doi.org/10.1016/j.margen.2019.04.001.

Rosenberg E, Zilber-Rosenberg I. The hologenome concept of evolution after 10 years. *Microbiome* 2018;**6**:78. https://doi.org/10.1186/s40168-018-0457-9.

Rousset F. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* 2008;**8**:103–6. https://doi.org/10.1111/j.1471-8286.2007.01931.x.

Rousset F, Lopez J, Belkhir K. Package 'genepop'. R package version 1. 2020. https://cran.r-project.org/web/packages/genepop/index.html

Roux C, Fraïsse C, Romiguier J *et al*. Shedding light on the grey zone of speciation along a continuum of genomic divergence. *PLoS Biology* 2016;**14**:e2000234. https://doi.org/10.1371/journal.pbio.2000234.

Sachs JL, Wilcox TP. A shift to parasitism in the jellyfish symbiont *Symbiodinium microadriaticum*. *Proceedings Biological Sciences* 2006;**273**:425–9. https://doi.org/10.1098/rspb.2005.3346.

Sartoretto S, Francour P. Bathymetric distribution and growth rates of *Eunicella verrucosa* (Cnidaria: Gorgoniidae) populations along the Marseilles coast (France). *Scientia Marina* 2011;**76**:349–55.

Schulz MH, Zerbino DR, Vingron M *et al*. Oases: robust *de novo* RNA-seq assembly across the dynamic range of expression levels. *Bioinformatics* 2012;**28**:1086–92. https://doi.org/10.1093/bioinformatics/bts094.

Sini M, Kipson S, Linares C *et al*. The yellow gorgonian *Eunicella cavolini*: demography and disturbance levels across the Mediterranean Sea. *PLoS One* 2015;**10**:e0126253. https://doi.org/10.1371/journal.pone.0126253.

Stanley RRE, Jeffery NW, Wringe BF *et al*. GENEPOPEDIT: a simple and flexible tool for manipulating multilocus molecular data in R. *Molecular Ecology Resources* 2017;**17**:12–8. https://doi.org/10.1111/1755-0998.12569.

Tricou T, Tannier E, de Vienne DM. Ghost lineages can invalidate or even reverse findings regarding gene flow. *PLoS Biology* 2022;**20**:e3001776. https://doi.org/10.1371/journal.pbio.3001776.

Tsagkogeorga G, Cahais V, Galtier N. The population genomics of a fast evolver: high levels of diversity, functional constraint, and molecular adaptation in the tunicate *Ciona intestinalis*. *Genome Biology and Evolution* 2012;**4**:852–61. https://doi.org/10.1093/gbe/evs054.

van Oppen MJH, Medina M. Coral evolutionary responses to microbial symbioses. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* 2020;**375**:20190591. https://doi.org/10.1098/rstb.2019.0591.

Van Oppen M, Mieog JC, Sanchez C *et al*. Diversity of algal endosymbionts (zooxanthellae) in octocorals: the roles of geography and host relationships. *Molecular Ecology* 2005;**14**:2403–17.

Wagner D, Pochon X, Irwin L *et al*. Azooxanthellate? Most Hawaiian black corals contain *Symbiodinium*. *Proceedings Biological Sciences* 2011;**278**:1323–8. https://doi.org/10.1098/rspb.2010.1681.

Weir BS, Cockerham CC. Estimating F-statistics for the analysis of population structure. *Evolution* 1984;**38**:1358–70. https://doi.org/10.1111/j.1558-5646.1984.tb05657.x.

Wood DE, Lu J, Langmead B. Improved metagenomic analysis with Kraken 2. *Genome Biology* 2019;**20**:257. https://doi.org/10.1186/s13059-019-1891-0.