

Endolithic algae (*Ostreobium*) diversity in *Porites* corals at the Western Atlantic and Eastern Tropical Pacific

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Abstract

Ostreobium comprise endolithic algae commonly seen in conjunction with scleractinian corals. In the past, it was solely recognized as a coral skeleton bioeroder. Yet, their relationship with corals is critical because they give photosynthetic byproducts and help the coral when it loses its primary symbionts due to stress. The variety of these algae among coral species of the genus *Porites* in the tropical eastern Pacific and western Atlantic was investigated here. We extracted *Ostreobium* samples from seven *Porites* species including two from the Tropical Eastern Pacific-TEP (*P. panamensis*, *P. lobata*) and five from the Caribbean (*P. furcata*, *P. porites*, *P. colonensis*, *P. branneri*, and *P. astreoides*). We also compared the new *rbcL* sequences from algae found within various coral species from other parts of the world. A biogeographic analysis and two methodologies, PTP (Poisson tree process) and GMYC (General Mixed Yule-Coalescent), were used to delineate the different species. The findings revealed a significant degree of genetic diversity within *Ostreobium*, with more than 15 groups of no more than three individuals and 40 individual lineages. Its origins date back to the Ordovician, 500 Ma, and it does not appear to preserve host-specificity. The sampled locations include a wide variety of *Ostreobium* still, biogeographically, varied patterns were confirmed, making it impossible to pinpoint the precise origins of most clades. The ancestry analyses revealed convergent events for the emergence of *Ostreobium* in a few genera of local corals, but the phenomenon also occurred in genera from far-off places.

Introduction

A complex endosymbiotic and euendolithic microbial community that maintains symbiotic interactions in various ways, such as parasitism or mutualism, is found in association with reef-building corals (Peixoto et al. 2017; Sweet y Bulling 2017). Numerous indications suggest that the ecological link between this community and the coral, which together make up the holobiont (Rohwer et al. 2002), is essential for the growth of hosts and their adaptability to the environment (Kelly et al. 2014). Due of their ability to degrade the coral's calcium carbonate skeleton, certain coral microbes were occasionally regarded as parasites (Tribollet et al. 2009; Wisshak 2012). However, recent studies suggest that nutrients are transferred to the coral and pathogenic infections were prevented (Hambleton et al. 2019; Wijayanti et al. 2018). Actually, they have successfully colonized the coral skeleton as a result of providing their host with energy (del Campo et al. 2017; Sheppard et al. 2017; Weber y Medina 2012). The euendolithic alga *Ostreobium* is one most prevalent and widespread coral microeroder. More than 85% of the scleractinian coral species contain these algae, which has a wide geographic and bathymetric distribution (Chazottes et al. 2009). These algae belong to Bryopsidales, originating in the Ordovician approximately 500 million year ago-Ma (Del Cortona et al. 2020; Jackson et al. 2018; Verbruggen et al. 2009) and their relationship with scleractinian corals is at least 150 MA according to the fossil record in geological deposits of the Early Cretaceous (Kołodziej et al. 2012; Wisshak et al. 2017). The algae, which forms small channels linked to growth, can be seen as a green or brown ring inside the coral's calcareous skeleton (Gonzalez-Zapata, Gómez-Osorio, y Sánchez 2018). Up to one kilogram of reef carbonate per square meter per year is lost due to the disintegration of the coral's skeleton caused by this process (Grange, Rybarczyk, y Tribollet 2015). But given that a carbon transfer to

the host occurs when stressed, the interaction between coral and *Ostreobium* appears to be mutualistic (Fine, Steindler, y Loya 2004). Additionally, due to their photosynthetic pigments' capacity to collect wavelengths close to the infrared, it is able to live in conditions of low light intensity (Marcelino y Verbruggen 2016) and can change the concentration of chlorophyll a and chlorophyll b depending on the depth (Magnusson, Fine, y Kühl 2007).

Ostreobium is thought to be sister to the two main suborders of Bryopsidales, Bryopsidineae and Hali-medineae, according to the phylogeny provided by Verbruggen et al. (2009). *Ostreobium queketii* and *O. constrictum* were identified as the two species in the Ostreobiaceae based on taxonomic analyses and morphologic traits (Lukas 1974). Later, a study in the Red Sea used DNA analysis to identify seven different clades of the endolithic algae by taking samples from the coral skeletons of two species: *Goniastrea perisi* and *Porites lutea* (Gutner-Hoch y Fine 2011). However, each group may represent more than one species. For instance, the mesophotic coral species *Agaricia undata* provided *rbcL* gene sequences to distinguish 12 different types of cryptic *Ostreobium* species, mostly structured along a depth gradient (Gonzalez-Zapata et al. 2018). Understanding the origin and diversification of the biota requires the ability to recognize species (Wallace 2008). However, it can be difficult to identify and delineate species boundaries in taxa that have a uniform morphology and have existed on Earth for hundreds of millions of years.

One of the few genera of coral that may be found in the entire tropical region of the globe is *Porites* (Link 1807) (Paz-García et al. 2016), which is one of the most numerous groups of scleractinian corals that have existed from the Miocene to the present (Baker, Correa, y Cuning 2017; Grizzle et al. 2016; Klaus et al. 2008; Zhao et al. 2016). In the Triassic, it started to colonize many environments, and in the Jurassic, it displayed a rapid pace of diversification (Flügel y Senowbari-Daryan 2001). Due to its extensive distribution range and intricate patterns of morphological diversity, this type of coral is a great illustration of the "species problem" (Zlatarski 2010). A consensus about their phylogeny is not established, and the limits of some species are still debated (Prada et al. 2014; Serrano et al. 2016). *Porites* holobionts have been identified as ocean acidification-tolerant corals (Connell et al. 2013). Also, some studies have evaluated the permanence of symbiont groups corals showing that most of the associations did not experience seasonal or annual variation in the dominant symbiont, indicating strong mutualistic relationships among species (Thornhill et al. 2014). And also, relationships between corals and their holobiont varied geographically, leading to the theory that coral might adapt to various ecological niches by "changing" its microbial relationships (Buddemeier et al. 2004), suggesting a co-evolving geographic mosaic with widely distributed host populations and more isolated symbiont populations.

Numerous data are available to compare the diversity of *Ostreobium* around the world (del Campo et al. 2017; Gonzalez-Zapata et al. 2018; Gutner-Hoch y Fine 2011; Massé et al. 2018), allowing us for a comparison of samples from various geographic locations, including those that were divided by the Panama Isthmus, and providing insight into the impact of the closure of the Isthmus on the evolutionary process. Since this geological formation created different oceanographic conditions on either side of Panama, which resulted in processes of diversification and extinction (Bowen et al. 2013; O'Dea et al. 2016), it is a natural experiment that enables us to determine what effects it had on the evolution of the endolithic algae. Run 50 km from coast to coast, certain geological studies claim it closed 15 Ma (Montes et al. 2012) whereas accepted dogma suggests that they stopped existing at 3 Ma (O'Dea et al. 2016). The biological data yield conflicting conclusions because, according to molecular analyses of 68% of the marine species examined (Marko, Eytan, y Knowlton 2015), the ages of separation occurred more recently than 12 Myr, and, according to the paleontological record using the neodymium isotope, the deep-water connection was severed between 12 and 9.2 Ma (Osborne et al. 2014).

We must comprehend the evolutionary biology of coral symbiosis in order to predict how the ecosystem will react to environmental changes like ocean acidification and climate change. The degree of host specificity between different coral species and their symbionts is not entirely understood (Silverstein, Correa, y Baker 2012). The diversity of coral and algae pairings may be a sign of the association's plasticity or distinctiveness, the partners' capacity to adapt to their environmental surroundings, and even the possibility of coevolution

(Apprill et al. 2012; LaJeunesse et al. 2010). Because these connections form the basis of many ecosystems, host-symbiont system research is essential for comprehending ecological patterns (Baker et al. 2017). Sadly, we still don't fully grasp these associations, the impact of holobionts on host development and diversity, and vice versa. Here, we start to fill in this knowledge gap by describing the genetic diversity of *Ostreobium* spp. using molecular data. The findings deepen our understanding of this symbiont's biology and the ecology of their relationships, prompting us to assess the biodiversity and molecular ecology of the endolithic algae associated with *Porites* and consider how different species of *Porites* ' reef-building corals from the Atlantic and Pacific differ in their *Ostreobium* clades. We also inquired about other geographical areas or hosts. We attempted to determine the patterns of *Ostreobium* 's diversification and how it relates to *Porites* species in the Atlantic and Pacific oceans. As shown by data from other marine organisms (O'Dea et al., 2016), it is predicted that the populations of encrusting algae would be significantly impacted by the closing of the Isthmus, dividing into groups according to their geographic locations shortly after the geological event. However, *Ostreobium* diversification could happen prior to the closure of the Panama Isthmus (Del Cortona et al. 2020; Verbruggen et al. 2009). To resolve these issues, a phylogeny of *Ostreobium* spp. was inferred for samples collected from within corals of the genus *Porites* , and comparing with sequences reported previously in NCBI, to make inferences about the genotypic diversity of *Ostreobium* spp. in the Atlantic and Pacific oceans and compare with studies from the Red Sea (Gutner-Hoch y Fine 2011), Indo-Pacific (O'Kelly et al, 2015), and Atlantic oceans (Gonzalez-Zapata et al. 2018).

Methods

Porites samples were taken in three sites, two in the Colombian Caribbean and one in the Pacific between November 2018 and October 2019, where seven species of *Porites* were collected, two of them, *P. panamensis* and *P. lobata*, in the Pacific, and the other five, *P. astreoides*, *P. furcata*, *P. colonensis*, *P. branneri*, and *P. porites*, in the Atlantic (Figs. 1A and 2B-G). One of the places in the Caribbean was in San Andres island (12 ° 31'15.45 "N, 81 ° 43'48.60" W) at the SeaFlower Biosphere reserve. The other sampling point in the Caribbean was in Cartagena specifically in Barú island in a National Natural Park (PNN Corales del Rosario y San Bernardo, 10 ° 10'30 " N 75 deg 45'00 " W). The sampling site in the Easter Tropical Pacific corresponded to PNN Gorgona Island (2 deg 58'17 " N 78 deg 11'04 " W) (Fig. 1A).

Sixty samples were taken from each site, to complete in all 180 samples. The process used was SCUBA diving in a bathymetric range of 5 m to 40 m taking 10 m to avoid the collection of clones. The fragments of each coral were extracted mechanically with the use of a chisel, where part of the sample was used for taxonomic identification purposes and the other was preserved in individual tubes with DMSO (0.5 M EDTA; 20% DMSO (v / v), saturated with NaCl (Dawson, 1998) and stored at -20 degC until DNA extraction. In the same way, a dry voucher was kept for each sample obtained and deposited in the Museo de Historia Natural C.J. Marinkelle of the Universidad de los Andes, Bogota, Colombia. The identification of the corals of the genus *Porites* was carried out based on morphological characters described for each species in different geographic locations supported by different investigation (Humann y DeLoach 2002; Prada et al. 2014; Venera-Ponton, Reyes, y Diaz-Pulido 2008; Veron 2000; Von Prahl, Guhl, y Grogl 1979). Research and collection of specimens were approved by the National Environmental Licensing Authority (ANLA, Spanish acronym): Collection Framework Agreement granted to Universidad de los Andes through resolution 1177 of October 9, 2014 - IBID 0359.

Sample processing

To evaluate the biodiversity and molecular identity of the endolithic algae associated with the collected *Porites* , we used the CTAB DNA extraction protocol (Coffroth et al. 1992), adopting modifications to increase the quality of the DNA associated with *Ostreobium* spp., for example, the coral tissue was removed using a WaterPik, leaving the bare skeleton to avoid contamination of the host's DNA. A piece of coral skeleton was crushed to become a powder and we continued with extraction process. The resulting product was quantified and pass to a concentration of 30 ng/ul so that it reacted adequately in the amplification process. Next, the *rbcL* gene was amplified with primers *rbcLF250* [5'GATATTGARCCTGTTGTTG GTGAAGA 3 '], and *rbcLR670* [5' CCAGTTTCAGCTTGWGCCTTATAAA 3 '], based on the sequence of amino acids

(Masse et al. 2020). The mixture to carry out the PCR had a total of 15 μ l distributed as follows, 6.35 μ l of water, 2 μ l of $MgCl_2$, 3 μ l of buffer, 0.75 μ l BSA (Bovine Serum Albumin, 20ng / μ l), 0.6/ μ l of each primer, 1 μ l Taq DNA polymerase, and 1 μ l of genomic DNA. Subsequently, the following conditions were established for amplification, starting at a temperature of 94 degC for 2 minutes, followed by 40 cycles of 94 degC for 90 s, 54 degC for 90 s, and 72 degC for 60 seconds. When the cycles ending, proceed with a temperature of 72 degC for 5 minutes. The amplified fragments were visualized in a 1% agarose gel using Gelgreen (Invitrogen) to finally be sequenced by the Sanger method in the GenCore laboratory of the Universidad de los Andes.

Data analysis

The F and R sequences were assembled in Geneious Prime. Sequences of *Ostreobium* obtained from NCBI were included in the comparison and analyses (Table 1). The alignment was performed in the MEGA X program using the MuscleG algorithm, considering identical sequences when performing the alignment were combined into a representative one. The result obtained was the basis for making the selection of evolutionary models in the jModelTest 2.1.2 software (Darriba et al. 2012), where the result was the GTR model with a Gamma distribution of rates, according to the Bayesian Information criterion. The phylogenetic relationships among the individuals were inferred using a Bayesian inference (BI) method (Rambaut et al. 2018) and maximum likelihood (ML) (Hoang et al. 2018). The outgroups were *Codium fragile*(AB103021), *Caulerpa taxifolia* (KF419327) and *Caulerpa webbiana* (KY062946); in both cases the selection was based on papers that showed the nearness of the lineage (Jackson et al. 2018; Verbruggen et al. 2009) and authors like Gonzalez-Zapata (2018) and Gutner-Hotch (2011) uses as base of their analyses. The ML tree topology was estimated in IQ-Tree v.2.0 (Minh et al., 2020). Subsequently, the Bayesian tree was useful as support to estimate divergence times. To complain this, use BEAST v. 2.0, the substitution rate parameter assumed a normal prior distribution whit mean= 0.00056 , SD=0.0001 (Villarreal y Renner 2014) substitutions/site/lineage/million years. The molecular clock model for the dataset was selected based on the likelihood ratio test in MEGA 6.0 (Tamura et al. 2013) and the result implies the use of an uncorrelated relaxed clock, set the number of generations for the MCMC (Markov chain Monte Carlo) analysis at 50 million and sampling every 1000 generations. Finally we used tracer to verify the quality of the analysis and obtain parameters with an effective sample size, ESS >200 (Rambaut et al. 2018). The substitution model corresponded to GTR together with a gamma distribution of 4 rate categories and the maximum credibility tree was computed in TREEANNOTATOR (Drummond et al. 2012) removing 10% of the trees as burn-in. The nodes indicate the posterior probability of the Bayesian analysis, accepting a suitable grouping at probabilities [?] 0.95. Data on the origin of *Ostreobium* were based on prior studies (Del Cortona et al. 2020; Jackson et al. 2018; Marcelino y Verbruggen 2016) with a range of between 470 Ma and 500 Ma for its origin. To point ideas about the grouping of the species, the GMYC (General Model Yule Coalescent) and mPTP (multi-rate Poisson Tree Process) analyses were employed. In this case the setting of GMYC analysis was 100000000 in MCMC, sampling every 1000 steps and discarded 10% as burn-in. The lineages that were congruent in both methods were considered as potential species.

The last analysis included a biogeographical history hypothesis of *Ostreobium* inferred using parsimony-based statistical dispersal vicariance (S-DIVA), Bayesian binary MCMC analysis (BBM), and dispersal-extinction-cladogenesis analysis (DEC). These three models were implemented in RASP v. 3.0 (Yu et al. 2015). The initial parameter was setting as default, based on the minimum result for AIC number was made selection of analysis DEC+J, and the maximum clade credibility (MCC) tree produced in BEAST analysis was used as the input tree. To evaluate the degree to which *Ostreobium* and its hosts were co-evolving, ML analyses of a Markov k-state parameter performed in Mesquite v. 2.75 (Maddison and Maddison 2011) were used. Parsimony analysis weights the contribution of each character state to a node equally, while ML uses the information provided by the lengths of the branches to estimate the probability that a given character state exists at each node in the tree (Cunningham, Zhu, y Hillis 1998). The ancestral patterns of change in the hosts were compared, paying special attention to the lineages that showed passage between more than two hosts and the genera that belonged to different geographic regions.

Table 1. NCBI accession codes for the sequences of *rbcL* gene of *Ostreobium* spp. present in different

substrates along locations in the world. The number of sequences was obtained after filtering the repeat sequences and the unidentified host. The average length was obtained after alignment.

NCBI Sequence	Host coral	Geographic region	Source/Voucher	Number of unique sequences	Mean sequence length (pb)
MG570021- MG569988	<i>Pocillopora damicornis</i>	Indo-Pacific	(Massé et al. 2018)	9	420
JF801728- JF801734	<i>Porites lutea</i> , <i>Goniastrea perisi</i>	Red Sea	(Gutner-Hochy Fine 2011)	7	396
KC685553- KC685526	Briozoo, spyrorbis tube, bivalve shell	Pacific	(O’Kelly et al, 2015)	6	400
MF135622- MF135643.	<i>Agaricia undata</i> <i>A. lamarcki</i> <i>Hellioseris cuculata</i>	Atlantic	(Gonzalez-Zapata et al. 2018)	15	395
MK095220- MK095212	<i>Pocillopora acuta</i>	Indo-Pacific	(Massé et al. 2020)	5	400
OQ983515- OQ983530	<i>Porites furcata</i> <i>P. colonensis</i> <i>P. astreoides</i> <i>P. branneri</i>	Atlantic	Present study	15	380
OQ983500- OQ983514	<i>Porites panamensis</i> <i>P. lobata</i>	Pacific	Present study	15	391

Results

Sequences were obtained from the 30 samples (total of 180 samples in the three sites), 15 of them belonging to the Atlantic Ocean and 15 of them to the Pacific Ocean, these sequences were compared with the data available in NCBI from different geographical regions as described in table 1, for a total of 78 sequences to perform the analyses. The time-calibrated Bayesian phylogeny (Fig. 1B) placed the common ancestor of *Ostreobium* spp. in the Ordovician, around 500 Ma (95% HPD: 550– 450 Ma). The initial divergence within the genus were predicted to have occurred about 410 Ma (95% HPD: 420–350 Ma) during the Silurian. During the Mesozoic and Cenozoic occurred the most of the divergence events in *Ostreobium* s pp. Biogeographical results, shows many vicariant events that coincide whit the closure of the Panamanian Isthmus, nevertheless, this kind of result should be interpreted carefully, because the range of times that appear in error may show the event in different ages. Figure 2A shows a dominant event in the pacific that seems separate in two lineages towards Atlantic and pacific, but many of the groups at lower taxonomic level shows sequences founded in Pacific, Atlantic, Indo-Pacific and Red Sea.

The result of the analysis of species delimitation were similar, GMYC generated 21 groups with maximum 5 sequences and 25 individual categories, the mPTP produced 18 groups with maximum 3 sequences and 38 individual sequences, both analyses generated in common 15 groups (Fig. 2). Many of the individuals in the groups belonging to different geographic regions and different hosts. The results of the RASP analysis showed interesting patterns associated with the region of origin and the possible events of *Ostreobium* vicariance, out of a total of 146 nodes, 26 correspond to vicariousness (Fig. 2A) and 7 of them occurred in the proposed time interval. for the closure of the isthmus of Panama (15-3 Mya), 5 occur between individuals belonging to the Atlantic and the Pacific (nodes 32,94,101,133,139). Despite the difficulty of confirming it with certainty through our analyzes, the substantial oscillation in temperature and CO₂ reduction known to have occurred

in the late Miocene / early Pliocene, 3 Ma before the closure of the Central American seaway, could have initiated an expansion / bottleneck of the symbiont (LaJeunesse 2005). The rest of the vicariance events point to events that occurred during the Mesozoic, mainly in the Cretaceous and Triassic, at node 147 and 116 of figure 2 we observe the oldest events that are 293 Ma and 229 Ma ago respectively, although it is difficult to make inferences due to the temporal variation that the geographic regions have suffered, there is a slight tendency towards the separation of individuals from the Atlantic and the Pacific oceans.

Figure 3B shows the ancestry of the hosts of *Ostreobium*, so that it allows to give an idea about which hosts passed the symbiont and what was its main origin. It is observed how two convergence events occur on the individuals of the genus *Hellioseris* that acquired *Ostreobium* spp. from *Agaricia*, these individuals collected in the Atlántico locality are a perfect example of the low host specificity that *Ostreobium* presents, because although in this case the host passes between the same locality, this convergence phenomenon is repeated between *Agaricia* and other genera such as *Pocillopora* or as hosts of the Red Sea. Ancestral reconstruction (Fig. 3B) result of the analysis in mesquite V 2.75, in the first node of the tree present uncertainty about the first possible host, however, the final groups of the tree defined the ancestor with more confidence and showed pattern associates a host genus belonging to the same region, on the other hand, many of the transferences were acquired from genera from apart regions. Surprisingly, some branches evidence a pass from a genus and after a return at the same genus from the new host. The Atlantic individuals of the genus *Agaricia* in all cases passed *Ostreobium* spp. to the genus *Porites*, from then on *Porites* managed to make this step between most of the genera, except *Hellioseris* and the substrates that did not correspond to the coral. In a study developed by Massé in 2018, an experiment is carried out at the level of coral development to know when it acquires *Ostreobium* spp., showing that are less than 5 days between the time of settlement of the larva of coral, until the moment in which the presence of the endolytic algae is confirmed. Given the speed of colonization and the lack of host choice in *Ostreobium* spp., it is predicted that the figure depicts ancestral host patterns in which Red Sea species acquire their symbiont from species belonging to the same genus from different geographic locations. In the group with the most individuals gathered by the GMYC analysis (Fig. 2A) we include individuals belonging to the Atlantic and Pacific locality of the genus *Porites* together with an individual of the genus *Pocillopora*, this case is particularly interesting because there is a return to a previous host. Originally, it was observed that the ancestor is *Agaricia* (Atlantic) and that from this it makes a step towards *Porites*, however, a few nodes later it is observed how the passage from *Porites* towards *Agaricia* is made again confirming the generalist role that the endolithic algae has and the little importance that the genetic differentiation of the coral genera represents for their survival.

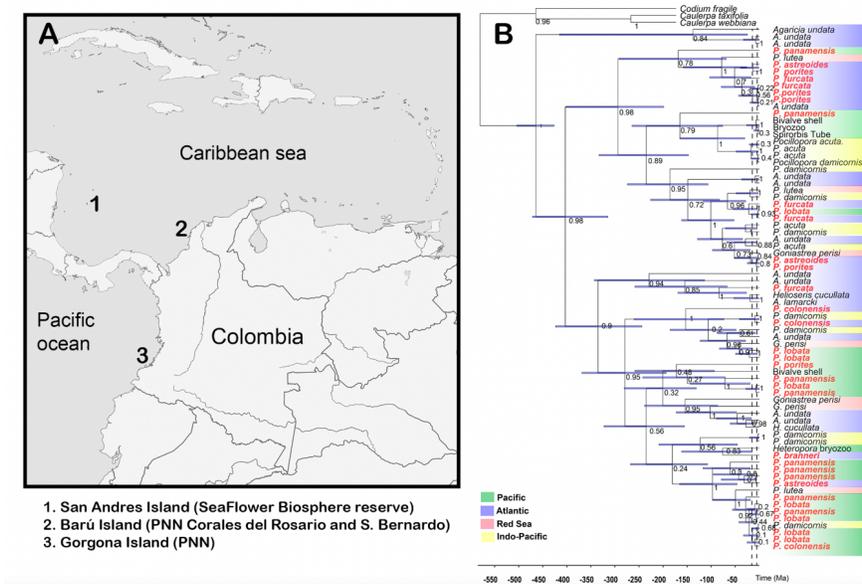


Figure 1. A. Location of new collection from *Porites* spp. Included in this study. **B.** Bayesian posterior consensus tree of *Ostreobium* spp. based on chloroplast gene *rbcL*, with red labels indicating new sequence data resulting from this study. Numbers to the right of each branch represent the posterior probability of the indicated clade. The two dotted lines refer to possible closing date of the Isthmus of Panama according to Montes et al. (2012) (older date, 12-15 Ma) and O’Dea et al. (2016) (the younger date of 3 Ma). Localities are indicated by the four colors, where green corresponds to samples from the Atlantic ocean, purple indicates Pacific, red is the Red Sea, and samples highlighted in Yellow are from the Indo-Pacific. The purple horizontal bars represent the 95% Highest Posterior Density for the node divergence time.

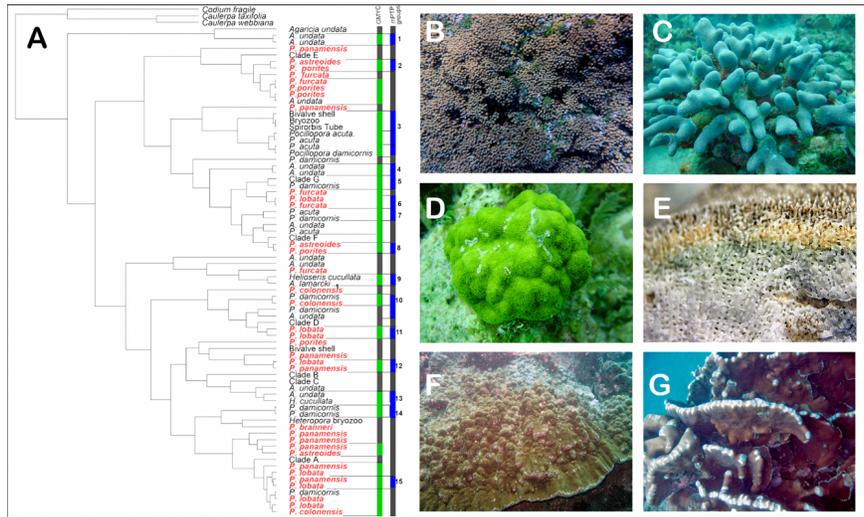


Figure 2. A. Bayesian MCMC consensus tree of algae samples in the genus, *Ostreobium*, based on the chloroplast gene *rbcL* and species delimitation results, the names correspond with host or source that inhabit the algae. Colored bars to the right of the phylogeny represent grouping based on GMYC and mPTP and the origin of the numbers represent the groups that coincide between the two analyses. Grey bars represent the sequences that are not grouping by the analysis. Red labels represent sequences obtaining in this study. Images of some *Porites* spp. Included in this study **B.** *Porites furcata* (Rosario islands, 3 m), **C.** *P. porites* (San Andrés, 22 m) **D.** *Porites astreoides* (San Andrés island, 5 m), **E.** *P. furcata* broken skeleton showing the green *Ostreobium* band, **F.** *P. lobata* (Gorgona island, 7 m), **G.** *P. panamensis* (Gorgona island, 10 m) (Photo credits: J.A. Sánchez).

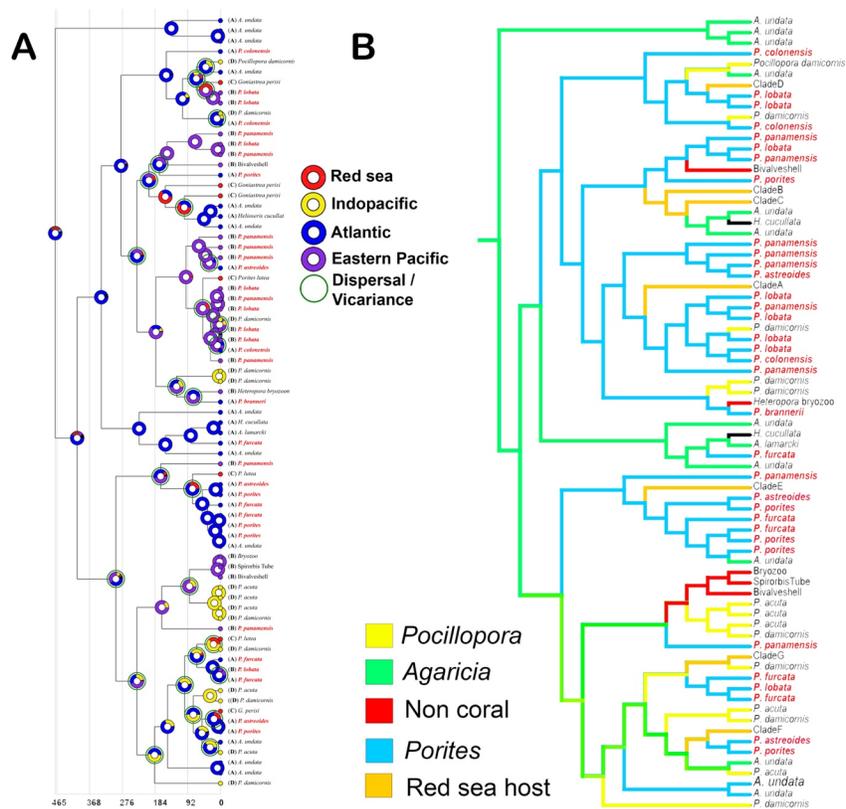


Figure 3. Biogeographic and ancestral analyses. **A.** Inference of the historical biogeography of *Ostreobium* spp. using Bayesian binary MCMC analysis (BBM) in RASP v. 3.0. Colors of pie wedges at each node represent geographical areas inferred to have been occupied by ancestral taxa. **B.** Mesquite Parsimony ancestral state mapping suggesting the host for ancestors of *Ostreobium* spp. Samples. The color of the branches represents the genera of the ancestral host, some branches have two colors that represent the uncertainty about the inferred host. The red labels in both cases represent the new sequences of this study.

Discussion

We found a complex of haplotypes belonging to the endolytic algae *Ostreobium* dispersed across hosts and geographical regions. The results showed an organism that has been on earth for 500 Mya and that is distributed in more than 50 taxonomic units, agreeing with what was proposed by (Marcelino y Verbruggen 2016) who had similar unexpected results in other green algae phylogenies. *Ostreobium* for some years was thought to be green algae with little species diversity and whose main function was to erode corals. An intriguing result of the study was the diversity of *Ostreobium* lineages among the several coral host species. Previous findings (del Campo et al., 2017; Gonzalez-Zapata et al., 2018; Gutner-Hoch & Fine, 2011) predicted that the analysis would produce a result with a number less than or equal to 12 lineages. However, the results of GMYC grouped 46 categories and those of mPTP 56, including 18 groups in common; many of the categories were represented by individual items. In fact, the group with the highest number of sequences was number 3 in figure 2A, which is where the non-coral substrates are grouped. The research based on the sequences of three genes (16s, rbcL, and 18S: del Campo et al. 2017) indicated the greatest variety of *Ostreobium* in the Atlantic but three main clades worldwide. Our findings corroborate these findings, which depicts polyphyletic groups of *Ostreobium* that originate from various hosts and geographical locations. The sequences from the Atlantic locality are consistent with sequences from the Red Sea, the Pacific, and the Indo-Pacific.

It is not surprising that haplotype grouping analyses show a pattern of groups that include various regions and *Ostreobium* hosts because of the widespread distribution of endolytic algae and the basal origin (480Ma) in the Bryopsidales phylogeny previously proposed by Jackson et al. (2018) and Verbruggen et al. (2009). In fact, it is long enough to go through thousands of biological pressures, making host species not a fundamental factor for their diversification. Microendolytic species employ every calcareous substrate accessible for colonization, including calcite crystals, foraminifera, bivalve and brachiopod shells, and coral skeletons, at least in recent geological times. Only its abundance varies in different types of substrates, as observed and measured in modern samples (Vogel y Brett 2009). On the other hand, the algae went through an invertebrate diversification event that happened in the Ordovician (Aberhan, Nürnberg, y Kiessling 2012) and that according to the fossil record shows how the amount of bioeroders increased considerably, at least considering the number of tunnels per substrate area, there is an increase through the Ordovician (reaching a peak in the Late Ordovician to the Early Silurian (Wilson y Palmer 2006), which favors the diversification of the species by expanding the number of available niches. This radiation event also promoted a greater intensity of symbiotic interactions, both skeletal carbonate and life within the host could have been advantageous, since the new life sites also offered protection against herbivores, against environmental variations such as temperature, salinity, etc. physical stress or desiccation during intertidal periods. Despite this, it seems that endolytic algae only occupy those environments in which they could best survive and gradually their way of life within almost any element with calcium carbonate made them fundamental in the ecosystem for hundreds of millions of years.

The results also provide evidence of multiple *Ostreobium* lineages that survived the Permian mass extinction and diversified after the Triassic (Fig. 2), along with the emergence of scleractinian reefs (Stanley 2003). Later it also diversified in the Mesozoic, especially among the sequenced species of the Atlantic, probably associated with phenomena such as temperature changes associated with CO₂ concentrations that considerably changed sea level. We can speculate about the *Ostreobium* endurance at current temperature regimes and its function as a support for coral when its main symbiont, *Symbiodinium*, during bleaching events. Finally, we see during the Cretaceous (66Mya) a diversification of lineages associated with the Pacific, which contrasts with what is found in corals where there is a diversification of Atlantic species during the Cenozoic (Johnson y Kauffman 2001). Despite the significance of the symbiotic relationship between the algae and coral for both organisms, *Ostreobium* spp. and some coral species have no particular relationship, so it is not surprising that their evolutionary events—radiation or extinction—do not exactly coincide. *Ostreobium* encountered adaptation challenges because of the coral skeleton's low light exposure, daily pH and oxygen level variations brought on by photosynthesis, and holobiont respiration (Tribollet et al. 2009). The niche inside the coral also varies greatly from species to species, which suggests that the groups identified by the analyses were not evenly distributed among their hosts or areas. In fact, as suggested in the study of the Red Sea developed by Gutner-Hotch (2011), there is greater specificity at the depth level than at the species level. During the last 500 Mya, endolithic algae have surely been exposed to depth variations that exceed 1000 meters in some geographical regions (Parsons y Sclater 1977), prompting it to develop adaptations associated with the lack of light rather than the search for a host.

To summarize, it is important to remember that future research must strengthen all of the inferences proposed, including a larger number of genes and developing higher resolution sequencing methods; however, this research provides interesting results to the biology of a fundamental, but little known, symbiont. Both the species delimitation and the phylogeny show that, in addition to the wide diversity of clades found in *Ostreobium*, there is no clear pattern of differentiation between geographic region or coral hosts. This low specificity may be a response to an opportunistic lineage that has been on Earth for more than 500 Mya and has, of course, undergone many evolutionary pressures. In fact, the presence of endolithic algae in non-coral samples and the relationship between some groups provides yet another hint concerning a generalist endolytic algae at the host level. It should be highlighted that the lack of a specificity relationship does not diminish the significance of the symbiotic interaction between endolithic algae and corals. Finally, *Ostreobium* spp. appears to be traveling through a dispersion of niches by depth, thus genotypes on diverse substrates (because there is no specificity) across large depth gradients would be worthwhile to study.

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Ethics.

All samples used in the study were endorsed with the collection permits granted by the national environmental licensing authority (ANLA) and by the Ministry of the Environment, whose resolution corresponds to 204 of November 13, 2009, for the Pacific, and 1177 of October 9, 2014, for the Atlantic.

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