

New epizootic symbioses between sponges of the genera *Plakortis* and *Xestospongia* in cryptic habitats of the Caribbean

Jan Vicente · Sven Zea · Ryan J. Powell ·
Joseph R. Pawlik · Russell T. Hill

Received: 19 February 2014 / Accepted: 17 September 2014 / Published online: 4 October 2014
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Abstract Three new cases of sponge symbiosis between species of *Plakortis* and *Xestospongia* were found in reef caves and mesophotic reef habitats of the Caribbean. *Plakortis* sp. 1 from the Bahamas associates exclusively with *Xestospongia deweerdtae* which was originally described living freely on the deep fore-reef and caves of Jamaica. In addition, we found *Plakortis* sp. 2 from Puerto Rico which associates with both *X. deweerdtae* and a different *Xestospongia* sp. Sponge specimens were identified using cytochrome oxidase subunit 1, 28S rRNA and 18S rRNA gene sequence fragments, spicule analysis, and histological sections with SEM. Unlike previous sponge pairs, *Xestospongia* spp. not only grew as a thin veneer of tissue over the *Plakortis* host sponge but through the mesohyl, forming inner channels (0.1–1 cm) that may provide a benefit by facilitating more efficient water transport through the

dense *Plakortis* tissue. Symbioses with both *Plakortis* spp. were documented from an early recruit stage through adulthood. Spicule measurements conducted on symbiotic versus free-living *X. deweerdtae* revealed significantly smaller spicule sizes for symbiotic individuals, suggesting a cost in terms of silicon availability, or a benefit in terms of a lower investment in skeleton synthesis for support. This study reveals new specialized symbiotic associations between distantly related sponge genera that likely represent an alternative strategy of adaptation for life in reef caves and mesophotic reefs.

Introduction

Cryptic habitats and mesophotic reefs (30–150 m depth) harbor a remarkable diversity of sponges with an increasing number of new species being discovered (Granek et al. 2009; Gerovasileiou and Voultsiadou 2012; Slattery and Lesser 2012). Like shallow coral reefs and mangrove habitats, reef cave sponges play an integral role in benthic–pelagic coupling processes (Southwell et al. 2008; Granek et al. 2009). For example, in reef caves of the Caribbean, sponges can remove from the flushing waters much of the dissolved organic matter returning nutrients and particulate matter to adjacent coral reefs (de Goeij et al. 2008; Slattery et al. 2013; de Goeij et al. 2013). On mesophotic reefs, sponge diversity exceeds that of coral and algae species making them biodiversity refuges for the more threatened shallower reef systems (Lesser et al. 2009; Kahng et al. 2010).

Reef caves can be crowded environments where sponges cope with space limitation by forming epizootic interactions with other sponge hosts without interfering with their host sponge pumping rates (Rützler 1970; Sarà 1970). In open

Communicated by M. G. Chapman.

Electronic supplementary material The online version of this article (doi:10.1007/s00227-014-2546-z) contains supplementary material, which is available to authorized users.

J. Vicente · R. J. Powell · R. T. Hill (✉)
Institute of Marine and Environmental Technology, University
of Maryland Center for Environmental Science, 701 E Pratt St
Suite 236, Baltimore, MD 21202, USA
e-mail: hill@umces.edu

S. Zea
Centro de Estudios en Ciencias del Mar-CECIMAR, Universidad
Nacional de Colombia, Sede Caribe, c/o INVEMAR, Calle 25
2-55, Rodadero Sur – Playa Salguero, Santa Marta, Colombia

J. R. Pawlik
Department of Biology and Marine Biology, Center for Marine
Science, University of North Carolina Wilmington, 5600 Marvin
K. Moss Lane, Wilmington, NC 28409, USA

coral reef communities, sponge epizoid interactions have been described as a strategy for surviving abiotic threats such as strong storm surges, but the cost of the interaction can also result in overgrowth and death of the host sponge (Wulff 1997, 2008b). The ability of opportunistic sponges to outgrow other sponge species may result from faster growth because of photosynthetic symbionts or by their ability to invest more energy in growing quickly rather than in producing chemical defenses (Rützler 1981; Walters and Pawlik 2005; Pawlik et al. 2008).

There have been several studies highlighting facilitation interactions between coral reef and sea grass bed sponges, where epizoidism does not result in the host being smothered (Wilcox et al. 2002; Ávila et al. 2007; Wulff 2008a). For example, to avoid predation, the coral reef sponge *Lissodendoryx colombiensis* forms assemblages with several sea grass sponge species (*Cliona caribbaea*, *Amphimedon erina*, *Clathria schoenus*, and *Tedania klausii*) that deter the sponge-eating seastar, *Oreaster reticulatus* (Wulff 2008a). In turn, the sea grass species use *L. colombiensis* as a substrate for stable settlement. Other relationships that have persisted for longer periods of time have resulted in more specialized associations as observed between the sponge pairs *A. erina* and *Geodia vosmaeri* (Engel and Pawlik 2000; Wilcox et al. 2002; Ramsby et al. 2012). Intriguingly, *A. erina* that associates with *L. colombiensis* commonly overgrows the palatable sponge *G. vosmaeri* protecting it from *O. reticulatus* (Ramsby et al. 2012).

In the present study, we report three novel specialized sponge associations between species of *Plakortis* and *Xestospongia* found living in reef caves and on the upper mesophotic reefs (30–36 m) of the Bahamas and Puerto Rico. Photographic images of the association from these sponges have been documented by Pomponi (Twilight Zone Expedition Team 2007, NOAA-OER www.photolib.noaa.gov/htmls/reef3932.htm) at a depth of 60 m in the Cayman Islands and by Zea et al. (2009). Initial morphological analysis of associated individuals showed a similar spicule complement among all the basibiont *Plakortis* samples but with smaller spicule size than nearby free-living specimens of *Plakortis halichondrioides*, the latter previously thought to be the species in the association (Zea et al. 2009). Also, owing to its strongyle spicules and its skeletal arrangement, associated epibiont specimens were initially thought to be *Xestospongia deweerdtiae* until free-living specimens of this species were found to have the same but larger spicules; this led Zea et al. (2009) to suggest that the epibiont *Xestospongia* was of a different species. In addition, while sampling the association, a clearly different species with oxea instead of strongyle spicules (hereafter named *Xestospongia* sp.) was found.

Frequent field observations of these interactions motivated the following three objectives of this study: (1) to

carry out the taxonomic identification and classification of all species involved in the association using the cytochrome oxidase I (COI), 28S rRNA and 18S rRNA genes for bar coding in combination with classical morphological characteristics; (2) to compare structural differences between free-living versus symbiotic individuals of the epibiotic *Xestospongia* species where both stages have been found, to look at possible benefits or disadvantages gained from the association, and (3) to conduct surveys to determine the frequency of associated individuals. We hypothesize that this is a mutualistic relationship in which the *Plakortis* and the *Xestospongia* are receiving reciprocal benefits based on the observation that the associated *Plakortis* spp. and *Xestospongia* spp. persist for long periods of time without the one species smothering the other.

Methods

Sample collection

Sponge individuals were collected from three geographic locations: Mexico in July 2012, Bahamas in July 2011 and Puerto Rico in October 2011, between a depth of 25–36 m (Supplementary Table 1). The number of individuals per species and the metadata for the samples collected are given in Table 1. Samples of free-living *P. halichondrioides* were also taken, as at the time it was believed that it was the basibiont species of the association (Zea et al. 2009). Sponges were cut at the base to reduce tissue damage and to avoid DNA cross-contamination between species. Whole individuals were placed in 15-l O₂/CO₂ permeable plastic bags Ken's Fish (Taunton, MA) for transportation to the surface where they were placed in 200-l tanks. Careful dissections and close examinations of the association were performed in the holding tanks. The associated sponge individuals were carefully dissected away from their sponge pair to retrieve clean samples for DNA from each of the species in the association. Sponge samples were cut into 1 cm³ pieces and were frozen or fixed and preserved using different methods depending on the objective (see below).

DNA extraction and sequencing

Fresh sponge tissue cubes were fixed in RNAlater and frozen at −80 °C. DNA was extracted using Qiagen's AllPrep DNA/RNA Mini Kit. Universal primers were used initially to amplify and sequence the cytochrome oxidase subunit I (Folmer et al. 1994), 18S rRNA gene (18SHOMOR, 18SHOMOF, from Gazave et al. 2010), and 28S rRNA gene (28SLSU5F, 28SLSU300F, 28SLSU300R, 28SLSU1200R, and 28SLSU1642R, from Redmond et al. 2011). Once initial sequences were retrieved, specific internal and pair-end

Table 1 Species and number of individuals used for phylogenetic and morphological analysis

Species or species pair	Status	N	Location	Location (acronym)	Latitude	Longitude	COI gene	28S rRNA gene	18S rRNA gene
<i>X. deweerdtae</i>	FL	1	Plana Key, Bahamas	PKBH	22°36.270'N	73°32.792'W	+	+	+
<i>X. deweerdtae</i>	FL	1	Little Inagua, Bahamas	LIBH	21°27.048'N	73°03.404'W	+	+	+
<i>X. deweerdtae</i>	FL	4	Cozumel, Mexico	COMX	20°22.937'N	87°01.746'W	+	+	+
<i>X. deweerdtae</i>	FL	1	Desecheo, Puerto Rico	DCPR	18°23.506'N	67°28.558'W	+	+	+
<i>X. deweerdtae</i>	A, PLsp2	2	Desecheo, Puerto Rico	DCPR	18°23.506'N	67°28.558'W	+	+	+
<i>X. deweerdtae</i>	A, PLsp2	1	La Parguera, Puerto Rico	LPPR	17°53.297'N	66°59.887'W	+	+	+
<i>Xestospongia</i> sp.	A, PLsp2	3	La Parguera, Puerto Rico	LPPR	17°53.297'N	66°59.887'W	+	—	+
<i>X. deweerdtae</i>	A, PLsp1	1	Mira Por Voz, Bahamas	MPVBH	22°06.052'N	73°32.444'W	+	+	+
<i>X. deweerdtae</i>	A, PLsp1	3	San Salvador, Bahamas	SSBH	24°02.433'N	74°31.882'W	+	+	+
<i>P. halichondrioides</i>	FL	3	La Parguera, Puerto Rico	LPPR	17°53.297'N	66°59.887'W	+	+	+
<i>P. halichondrioides</i>	FL	3	San Salvador, Bahamas	SSBH	24°02.433'N	74°31.882'W	+	+	+
<i>Plakortis</i> sp. 1	A, XD	3	San Salvador, Bahamas	SSBH	24°02.433'N	74°31.882'W	+	—	+
<i>Plakortis</i> sp. 2	A, Xsp	3	La Parguera, Puerto Rico	LPPR	17°53.297'N	66°59.887'W	+	+	+
<i>Plakortis</i> sp. 2	A, XD	2	La Parguera, Puerto Rico	LPPR	17°53.297'N	66°59.887'W	+	+	+
<i>Plakortis</i> sp. 2	A, XD	2	Desecheo, Puerto Rico	LPPR	18°23.506'N	67°28.558'W	+	+	+

Status: A associated, FL free-living, XD *X. deweerdtae*, Xsp *Xestospongia* sp., (PLsp1, PLsp2) *Plakortis*

primers were designed for amplification of each species-specific gene (6 primer pairs for COI, 8 primer pairs for 18S rRNA, 11 primer pairs for 28S rRNA). Primer direction and sequence were determined using Sequencher, and primer sequences were evaluated using NetPrimer (<http://www.premierbiosoft.com/netprimer/index.html>). Species-specific primer sequences designed for each gene of the sponges involved in the association are listed in Supplementary Tables 2 and 3.

Preliminary optimization runs were performed using a 15 °C gradient, and specific annealing temperatures for each primer pair were selected for each species of the association. The highest annealing temperature showing a strong PCR product from the optimization gradient was selected for highest primer specificity. For each reaction, DNA from both sponges was used as a control to make sure that the PCR product was from the species of interest. Polymerase chain reactions were carried out in 50 µl total volume including the following: 39.3 µl of H₂O, 1 µl of each primer (10 mM), 1 µl dNTPs (10 mM), 5 µl 10× Buffer, 1.5 µl of MgCl₂ (50 mM), 0.2 µl of Platinum Taq polymerase, 1 µl of template DNA (30 µM). The PCR program consisted of an initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s; annealing at 50 °C (PLRV2/PLFWD3 *P. halichondrioides*), 63 °C (PLRV2/PLFWD3 other *Plakortis* spp.), and 68 °C (XDRV6/XDFWD6 associated *Xestospongia* spp.) for COI; 60.9 °C (PLRV1/PLFWD1 *P. halichondrioides*), 63 °C (PLRV2/PLFWD3 *Plakortis* spp.), and

63 °C (XDRV1/XDFWD1 *Xestospongia* spp.) for 18S rRNA gene; 54.6 °C (PLRV1/PLFWD1 *P. halichondrioides*), 63 °C (XDRV1/XDFWD1 *X. deweerdtae*) for 28S rRNA gene; and an extension at 72 °C for 5 min and a final extension of 72 °C for 5 min. PCR products were ran in 1 % agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Sequencing reactions were performed using the BigDye™ terminator v. 3.1 with species-specific PCR primers. Sequencing was done with an ABI Prism 2100 automated sequencer.

Phylogenetic analysis

Sequences were assembled and edited using Sequencher. Double coverage reads allowed for accurate editing of sequences. Complete consensus sequences for each gene fragment were exported into a single fasta file format from Sequencher and uploaded with MEGA 5 (Tamura et al. 2011). Sequences were then aligned using the ClustalW function with default parameters. The closest relative to our sequences was searched using the BLAST function from GenBank. These sequences were used as reference sequences for alignment and tree generating purposes. Aligned sequences for each gene (COI/18SrRNA/28SrRNA) were joined. MEGA 5 was used to construct a concatenated tree of the three genes using a neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood analysis. We ran the NJ analysis using a maximum composite likelihood model of

nucleotide substitution with substitutions to include Transitions + Transversions, and data were re-sampled using 1,000 bootstrap replicates. The MP analysis consisted of a heuristic search with a subtree pruning regrafting method using 10 random addition replicates. A Close-Neighbor-Interchange branch-swapping algorithm was used, and data resampled using 1,000 bootstrap replicates. The ML analysis was run using the Tamura Nei model with uniform rates among sites (Tamura and Nei 1993). A nearest-neighbor interchange ML heuristic method was used, and data were resampled using 1,000 bootstrap replicates. All sequences were deposited in GenBank.

Spicule morphology and size

Sponge spicules were prepared by immersing 1 mm³ pieces of previously frozen tissue into 1 ml of 65 % nitric acid. Samples were hand-stirred and incubated at room temperature for 3 h. Samples were centrifuged and washed with distilled water, and a few drops of spicules suspensions were mounted fresh on a microscope slide; spicules were characterized and measured under a light microscope. Fifty spicules were measured for each sample of free-living and associated *Xestospongia* spp. One hundred diod spicules and 10 triod spicules were measured for each sample of *Plakortis* spp. A nested analysis of variance (ANOVA) for *X. deweerdtiae* spicule dimensions for the Bahamas was carried out using association as a factor, and individuals as a nested factor (there being 50 spicule measurements per individual), separately for length and width. The same was carried out to compare associated *Plakortis* sp. 1 with *P. halichondroides* from the Bahamas with the factor being species, and individuals as nested factor. A third ANOVA was carried out to compare spicule sizes between the three *Plakortis* spp. in Puerto Rico with the factor being species, and individuals as nested factor. Tukey's post hoc test was run following significant ($P < 0.05$) ANOVA outcomes. Log₁₀ or square root transformations were employed when necessary to adjust for normality of residuals and homogeneity of variance among levels of the main factor. Statistical analyses were performed using the software program Statgraphics Plus 5.1.

Sectioning and scanning electron microscope imaging

To observe tissue arrangements between *Plakortis* and *Xestospongia* sponge pairs, sponge pieces (0.25 cm³) were prepared. Fresh sponge tissue cubes were fixed in 9 ml of 0.1 M sodium cacodylate prepared in artificial seawater (ASW) with 1 ml of 2.5 % glutaraldehyde at 4 °C for 24 h. Pieces were transferred to 10 ml of sodium cacodylate buffer in ASW and held at 4 °C until further processing. Fixed sponge tissues were then placed in 1 % (w/v) osmium

tetroxide solution (prepared in 0.2 M potassium phosphate buffer, pH 7.4) for 3.5 h and subsequently dehydrated in a graded ethanol (EtOH) series (15, 35, 55, 75, 85, and 95 % (v/v) ethanol). Gold-coated sponge pieces (0.25 cm³) were mounted on lead stubs and gold-coated in a SeeVac Auto conductavac IV sputter coater. Samples were visualized under a JEOL JCM-5700 Scanning Electron Microscope.

Hematoxylin and eosin staining

Sponge samples preserved in 4 % paraformaldehyde were embedded in paraffin. Tissue was cut in 7–60-μm-thick sections with a LKB 2128 Ultratome IV. Paraffin sections were placed on slides, immersed in xylene, and dehydrated in a graded EtOH series (70, 85, 95 %). Sections were immersed in PBS buffer for two minutes and then washed with deionized water (DI). Sections were stained with Harris hematoxylin solution for 20 min followed by a 5-min rinse with running DI. Sections were then destained in 1 % acid EtOH for 30 s and subsequently washed with DI for 1 min. Sections were exposed to bluing in 0.2 % ammonia water for 1 min and washed in DI for 5 min. Sections were dehydrated in a graded EtOH series (25, 50, 70, 95 %), counterstained in eosin–phloxine solution for 1 min, and washed in 95 % EtOH before mounting with xylene-based mounting medium.

Reef surveys

Candidate survey sites were scouted and selected based on the presence of steep drop-offs, overhangs, and caves, all habitats likely to host associated individuals. GPS coordinates and full survey site names can be found in Supplementary Table 1. Surveys using 100-m transect tape between 20 and 30 m depth were conducted along the Yucatan coast ($N = 4$) in July 2012, in Puerto Rico in June 2011 ($N = 6$), and in the Bahamas ($N = 9$) in July 2011 and July 2013. The tape was loosely laid or hanged on the reef wall, and all caves and crevices found in about 1 m on each side of the tape were examined. Since *Plakortis* sp. 1 and *Plakortis* sp. 2 individuals are difficult to distinguish in the field, we refrained from separating them as two different species in our analysis. There are obvious morphological differences between *X. deweerdtiae* and *Xestospongia* sp. that made it easy for us to indicate which species of *Xestospongia* was spotted. For our surveys, we recorded the presence of *Plakortis/Xestospongia* associated pairs and of free-living *X. deweerdtiae* and observations on their association status were noted (associated/free-living). Underwater digital photographs of different sponge morphologies were taken for associated and free-living individuals. In Puerto Rico, five sponge associates were tagged (*Plakortis* sp. 2/*Xestospongia* sp. ($N = 4$) and *Plakortis* sp. 2/*X.*

deweerdtae ($N = 1$)), photographed, and monitored for growth for 8 months.

Results

Phylogenetic analysis

The concatenated phylogenetic tree generated from partial sequences of the COI (463 bp), the 18S rRNA (1,300 bp), and the 28S rRNA (750 bp) gene is shown in Fig. 1. The closest concatenated sequences to the COI/18S rRNA/28S rRNA genes of *Plakortis* sp. 2 were those from *Plakortis simplex* (91.9 % identity, 100 % coverage), *P. halichondrioides* (91.2 % identity, 100 % coverage), *Plakina jani* (91.1 % identity, 100 % coverage), and *Plakinastrella* cf. *onkodes* (90.9 % identity, 100 % coverage). All *Plakortis* sp. 2 individuals shared identical sequence irrespective of whether they were associated with *X. deweerdtae* or *Xestospongia* sp. and were supported by bootstrap values of 89/100/100. *P. halichondrioides* from Puerto Rico and the Bahamas were 100 % identical and showed 0.48 % divergence (5 bp) with *P. halichondrioides* (GenBank accession no. HM118543). Identical COI/18S rRNA/28S rRNA gene sequences for *P. halichondrioides* samples were obtained from Puerto Rico and the Bahamas. Samples of *P. halichondrioides* collected in this study showed 1.29 % (29 bp)

divergence to *P. halichondrioides* with 100 % coverage supported by bootstrap values of 88/100/100.

All *X. deweerdtae* individuals formed a monophyletic group supported by bootstrap values of 99/100/90. However, there were some subtle base pair differences between individuals. For example, *X. deweerdtae* individuals (PKBHFL, LPPRA, DCPRA) were all 100 % identical, but were 0.13 % (3 bp) divergent to *X. deweerdtae* (DCPRFL, COMXFL). In addition, *X. deweerdtae* individuals (LSBHA, MPVBHA) were 0.42 % (10 bp) divergent to *X. deweerdtae* individuals (PKBHFL, LPPRA, DCPRA). Amplification of the 28S rRNA gene of *Xestospongia* sp. and *Plakortis* sp. 1 was attempted with eight different primer combinations listed in Supplementary Table 2 without successful amplification. This failure suggests the possible presence of mutations in the primer annealing sites.

A second concatenated tree from partial sequences (463 bp) of the COI and (1,300 bp) of the 18S rRNA was generated to include sponges *Xestospongia* sp. and *Plakortis* sp. 1 (Fig. 2). The *Xestospongia* sp. and *Plakortis* sp. could not be included in the concatenated tree shown in Fig. 1 because we were unable to amplify the 28S rRNA genes from these sponges. The closest concatenated sequence to the COI/18S rRNA genes of *Plakortis* sp. 1 is that of *Plakortis* sp. 2 (96.6 % identity, 100 % coverage) and together formed a clade supported by bootstrap values of 97/97/99. *Xestospongia* sp. shared 89.9 % identity

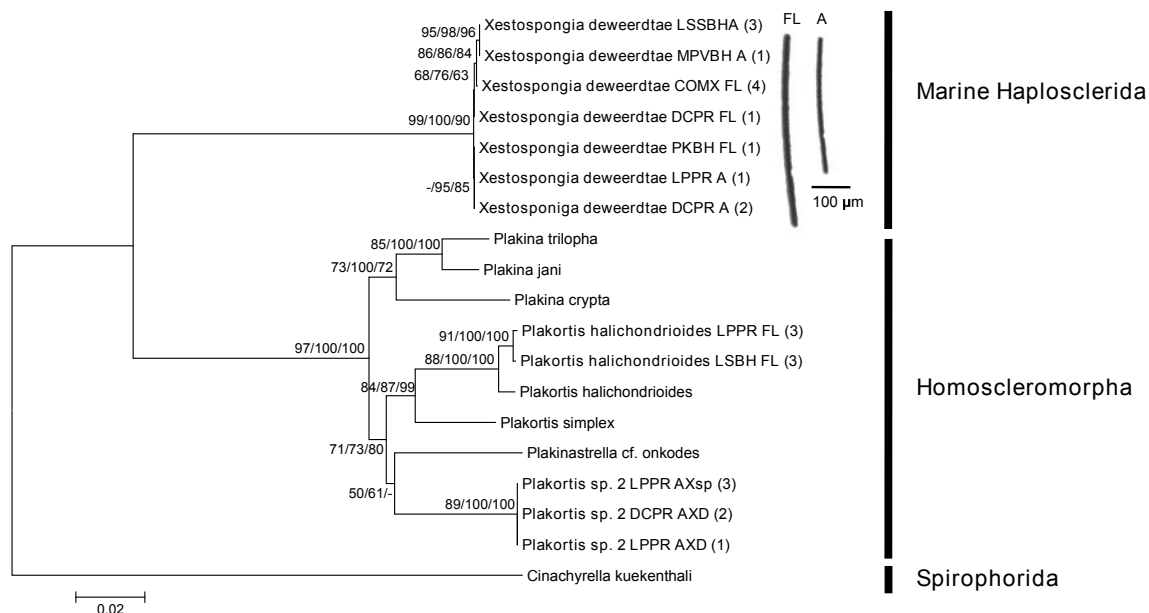


Fig. 1 Concatenated phylogenetic tree generated from an alignment of 463 bp of the cytochrome subunit 1, 1300 bp of the 18S rRNA gene, and 750 bp of the D1-D2 regions of the 28S rRNA gene of sponges sequenced in this study (**bold**). Reference sequences downloaded from GenBank are indicated by species name in *italics*. Coding preceding sample name refers to collection location as referenced

in Table 1. Following the location is the association status of the individual (A—associated; FL—free-living). The tree topology was obtained from neighbor-joining (NJ) analysis. The bootstrap values at each node were generated from (NJ), maximum parsimony (MP), and maximum likelihood (ML) analysis, respectively

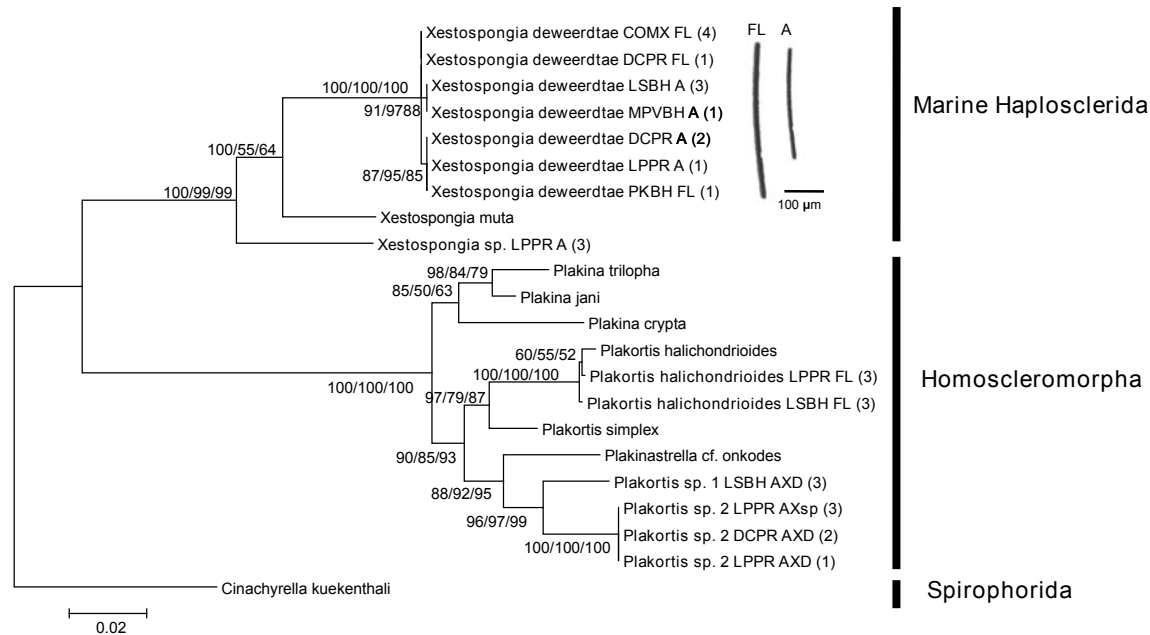


Fig. 2 Concatenated phylogenetic tree generated from an alignment of 463 bp of the cytochrome subunit 1 and 1300 bp of the 18S rRNA gene of sponges sequenced in this study (**bold**). Reference sequences downloaded from GenBank are indicated by species name in *italics*. Coding preceding sample name refers to collection location as refer-

enced in Table 1. Following the location is the association status of the individual (A—associated; FL—free-living). The tree topology was obtained from neighbor-joining (NJ) analysis. The bootstrap values at each node were generated from (NJ), maximum parsimony (MP), and maximum likelihood (ML) analysis, respectively

(100 % coverage) with *X. deweerdtiae* and 91.5 % identity (97 % coverage) with *Xestospongia muta*. The closest 18S rRNA gene sequence to *Xestospongia* sp. was *Oceanapia* sp. (GenBank accession no. DQ927317), *Gelliodes callista* (GenBank accession no. KC902338), and *Dasychalina fragilis* (GenBank accession no. DQ927316) all showing 91 % identity (100 % coverage) (Supplementary Fig. 1). However, the closest COI gene sequence to *Xestospongia* sp. LPPRA was *Petrosia* sp. (GenBank accession no. JN242216) showing 94 % identity (100 % coverage) (Supplementary Fig. 2).

Tree topologies supported by bootstrap values from NJ, ML, and MP analysis were consistent in separating haplosclerid from homoscleromorph sponges for the three genes; *X. deweerdtiae* individuals and associated *Plakortis* spp. were each also monophyletic for the three genes used in this study (Figs. 1 and 2). Basibiont *Plakortis* spp. was definitely not *P. halichondrioides* or any of the other included species of Homoscleromorpha. Regardless of origin (free-living vs. associated), all samples of strongyle-bearing *Xestospongia* were >99 % identical for the three genes. This led us to identify them as *X. deweerdtiae* and to hypothesize that the association is responsible for the differences in spicule size (see below). Oxea-bearing epibionts were genetically very far from *X. deweerdtiae* in the two genes that could be amplified, confirming them to be a different species. We have tentatively named this species

as *Xestospongia* sp. owing to its spicule size and skeletal architecture; however, judging from the polyphyletic nature of this genus (Supplementary Figs. 1 and 2), and the genetic proximity to some *Haliclona*-like species, further analyses are needed to clarify its taxonomic status.

Distribution and morphological characters of sponge pairs

Plakortis sp. 1 was found in the Bahamas, and individuals were thickly encrusting to massive with lobate morphology. Individuals of *Plakortis* sp. 1 measured 3 × 30 cm by 1–8 cm thick with oscules 0.2–0.9 cm in diameter. *Plakortis* sp. 1 individuals were dark brown in vivo and exuded a brown pigment when preserved in ethanol. Individuals had a soft compressible consistency and exhibited an irregular shape of the outer surface with grooves along the surface of the sponge body. The ectosome (40–60 μm) in transversal sections was poorly differentiated but distinguishable, being denser than the choanosome. A confused choanosomal skeleton with spherical and irregular canals (100–300 μm) was visible. Both adults and sponge recruits of *Plakortis* sp. 1 were exclusively associated with *X. deweerdtiae* in cryptic habitats but were also found settling on the bedrock at a depth of 36 m. Since we initially assumed free-living individuals of this association were *P. halichondrioides*, we did not purposely search for other possible species. We observed the association from

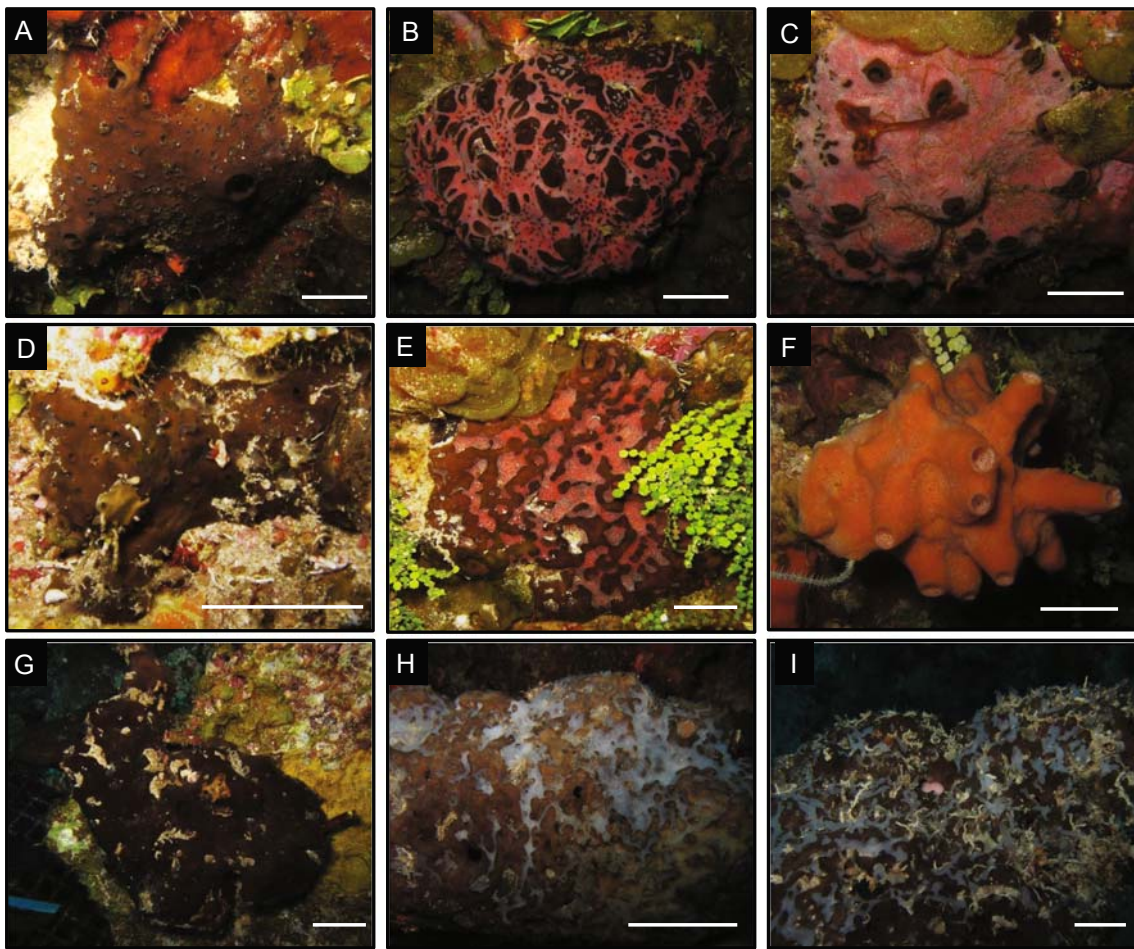


Fig. 3 Growth stages of *Plakortis/Xestospongia* associations. **a** *Plakortis* sp. 1 (brown) associated with *X. deweerdtiae* (translucent spots) at a recruit stage. **b, c** *Plakortis* sp. 1 adult growing massively with *X. deweerdtiae* (pink). **d** *Plakortis* sp. 2 recruit associated with *X. deweerdtiae* (translucent spots). **e** *Plakortis* sp. 2 adult associated

with *X. deweerdtiae*. **f** Free-living morphotype of *X. deweerdtiae*. **g–i** *Plakortis* sp. 2 associated with *Xestospongia* sp. (blue) growing with encrusting and papillate morphology. Scale bar (**a**) 1 cm, (**b–d**), (**g–i**) 5 cm, (**e–f**) 2 cm

an early recruitment stage (Fig. 3a) where *X. deweerdtiae* is growing as small translucent colonies through the *Plakortis* sp. 1 tissue. In adult individuals, *X. deweerdtiae* can grow as patches over the *Plakortis* sp. 1 body (Fig. 3b) or completely cover *Plakortis* sp. 1 as a pink encrusting tissue without overgrowing the oscula (Fig. 3c). Diod spicules were observed for *Plakortis* sp. 1 where diod lengths ranged between 21 and 223 μm , individual means ranging from 103 to 124 μm (Table 2). Although rare, small diods (21–40 μm) were observed in all individuals, large diods in *Plakortis* sp. 1 had mixed morphologies between straight and irregular with acerate endings. Diod centers were thick and occasionally S-bent (Supplementary Fig. 3A).

In the Puerto Rican archipelago, *Plakortis* sp. 2 can be found in reefs off the south and west continental shelf (30–36 m) as well as shallow reef caves (12 m) in Mona and Desecheo Island (personal observations of JV). Only associated individuals of *Plakortis* sp. 2 were found.

Plakortis sp. 2 individuals were more commonly found thinly encrusting, measuring between 3 and 10 cm of maximum diameter by 1–3 cm thick, with oscules 0.3–1.7 cm in diameter. *Plakortis* sp. 2 individuals were dark brown to dark green in vivo and exuded a brown pigment when preserved in ethanol. Individuals had a soft compressible consistency and exhibited a smooth outer surface with occasional grooves along the surface of the sponge body. Like *Plakortis* sp. 1, the ectosome (30–50 μm) in transversal sections was poorly differentiated. Irregular shaped canals (100–300 μm) were surrounded by a confused choanosomal skeleton. Diod length in *Plakortis* sp. 2 ranged between 43 and 160 μm , individual means ranging from 115 and 121 μm when associated with *X. deweerdtiae* and 107 to 114 μm when associated with *Xestospongia* sp. (Table 2). Like *Plakortis* sp. 1, the *Plakortis* sp. 2/*X. deweerdtiae* association was found at a recruit stage and an adult phase (Fig. 3d, e). However, overgrowth by *X.*

deweerdtae throughout the *Plakortis* sp. 2 body is limited to grooves along the sponge surface as well as the mesohyl, but never overgrows individuals completely, as observed in *Plakortis* sp. 1 (Fig. 3e).

Although rare, both *Plakortis* sp. 1 and *Plakortis* sp. 2 had Y-shaped triods with sharp endings (Supplementary Fig. 3A, B). Triods in *Plakortis* sp. 1 measured between 40 and 107 μm (longest span); triods in *Plakortis* sp. 2 measured between 38 and 108 μm averaging 67 to 78 μm when associated with *X. deweerdtae* and 57 to 64 μm when associated with *Xestospongia* sp. (Table 2).

Following up on the original idea that associated *Plakortis* were *P. halichondrioides*, spicule size of *Plakortis* sp. 1 and 2 was compared with those of *P. halichondrioides* individuals from the Bahamas and Puerto Rico (Supplementary Fig. 3C). Diod length and width of *P. halichondrioides* from the Bahamas were significantly larger than *Plakortis* sp. 1 (ANOVA, between species $P < 0.001$), but widths were not ($P > 0.05$). Mean sizes (± 1 standard deviation, length \times width) for *P. halichondrioides* individuals from the Bahamas were 166 ± 20 by 4.0 ± 1.0 μm in width ($N_{\text{individuals}} = 3$), while those of *Plakortis* sp. 1 were 113 ± 29.6 by 3.4 ± 0.9 μm ($N_{\text{individuals}} = 3$). A subsequent ANOVA and Tukey's post hoc analyses comparing diod mean length and width (± 1 standard deviation, length \times width) between *Plakortis* sp. 1, *Plakortis* sp. 2, and *P. halichondrioides* from Puerto Rico revealed significantly larger diod lengths ($P < 0.001$) but not widths ($P > 0.05$) for *P. halichondrioides* than for *Plakortis* sp. 1, which were in turn longer than for *Plakortis* sp. 2 (Fig. 4). Mean sizes (± 1 standard deviation, length \times width) for *P. halichondrioides* individuals from Puerto Rico were higher in length [162 ± 14 by

4 ± 0.8 μm in width ($N_{\text{individuals}} = 3$)], followed by *Plakortis* sp. 2 associated with *X. deweerdtae* [117 ± 16 by 3.7 ± 0.9 μm ($N_{\text{individuals}} = 3$)], *Plakortis* sp. 1 [113 ± 30 by 3.4 ± 0.9 μm ($N_{\text{individuals}} = 3$)], and with smallest diod size *Plakortis* sp. 2 associated with *Xestospongia* sp. [110 ± 16 by 3.6 ± 0.9 μm ($N_{\text{individuals}} = 3$)].

Free-living individuals of *X. deweerdtae* grew as thickly (1–2 cm) encrusting circular patches (6–12 cm) with 5–10 oscules per individual measuring up to 5 cm in height and 1 cm in diameter (Fig. 3f). Color between *X. deweerdtae* individuals varied between purple, bright pink, and white. Sponges had a smooth surface and were slightly compressible, although dried specimens were hard. Associated individuals grew as a thin veneer of tissue through the mesohyl and surface of *Plakortis* spp.

Associated and free-living forms of *X. deweerdtae* share a unispicular isotropic reticulation of strongyle spicules. Strongyle length and width of free-living individuals of *X. deweerdtae* from the Bahamas were significantly larger than associated individuals of *X. deweerdtae* (ANOVA, between associations $P < 0.001$) (Supplementary Fig. 3D, E). Indeed, mean sizes (± 1 standard deviation, length \times width) for associated individuals were lower [218 ± 17 μm in length by 8.2 ± 1.7 μm in width ($N_{\text{individuals}} = 4$)] than those of free-living individuals [340 ± 22 by 11.2 ± 2.2 μm ($N_{\text{individuals}} = 2$)]. The same situation was found in Puerto Rico, although statistical comparisons could not be carried out owing to lack of replication, and associated *X. deweerdtae* individuals averaged 236 ± 21 by 9.7 ± 1.9 μm ($N_{\text{individuals}} = 4$) compared to a single free-living individual which averaged 327 ± 15 by 13.3 ± 2.1 μm . In Mexico, only free-living individuals were observed and

Table 2 Spicule types and lengths of *P. halichondrioides*, *Plakortis* sp. 1, and *Plakortis* sp. 2

Sponge	Location	Association	Diod length range (μm)	Average diod length (μm)	Triod length range (μm)	Average triod length (μm)
<i>Plakortis</i> sp. 1 (1)	BH	XD	24–223	124 ± 37	40–107	82 ± 20
<i>Plakortis</i> sp. 1 (2)	BH	XD	21–119	103 ± 23	48–83	62 ± 9
<i>Plakortis</i> sp. 1 (3)	BH	XD	29–140	111 ± 22	60–79	67 ± 6
<i>Plakortis</i> sp. 2 (1)	PR	XD	72–146	115 ± 16	38–108	78 ± 18
<i>Plakortis</i> sp. 2 (2)	PR	XD	106–150	121 ± 15	51–85	67 ± 12
<i>Plakortis</i> sp. 2 (3)	PR	XD	43–129	115 ± 17	51–94	70 ± 14
<i>Plakortis</i> sp. 2 (1)	PR	Xsp	89–140	107 ± 18	42–89	64 ± 16
<i>Plakortis</i> sp. 2 (2)	PR	Xsp	83–127	109 ± 12	49–101	74 ± 14
<i>Plakortis</i> sp. 2 (3)	PR	Xsp	85–160	114 ± 17	51–74	57 ± 10
<i>P. halichondrioides</i> (1)	BH	FL	113–204	160 ± 21	–	–
<i>P. halichondrioides</i> (2)	BH	FL	101–175	143 ± 17	–	–
<i>P. halichondrioides</i> (3)	BH	FL	135–206	173 ± 17	–	–
<i>P. halichondrioides</i> (2)	PR	FL	99–200	163 ± 15	57–108	82 ± 18
<i>P. halichondrioides</i> (3)	PR	FL	125–194	165 ± 13	76–114	88 ± 12
<i>P. halichondrioides</i> (4)	PR	FL	112–202	159 ± 15	75–115	92 ± 13

Association: FL free-living, XD *X. deweerdtae*, Xsp *Xestospongia* sp.

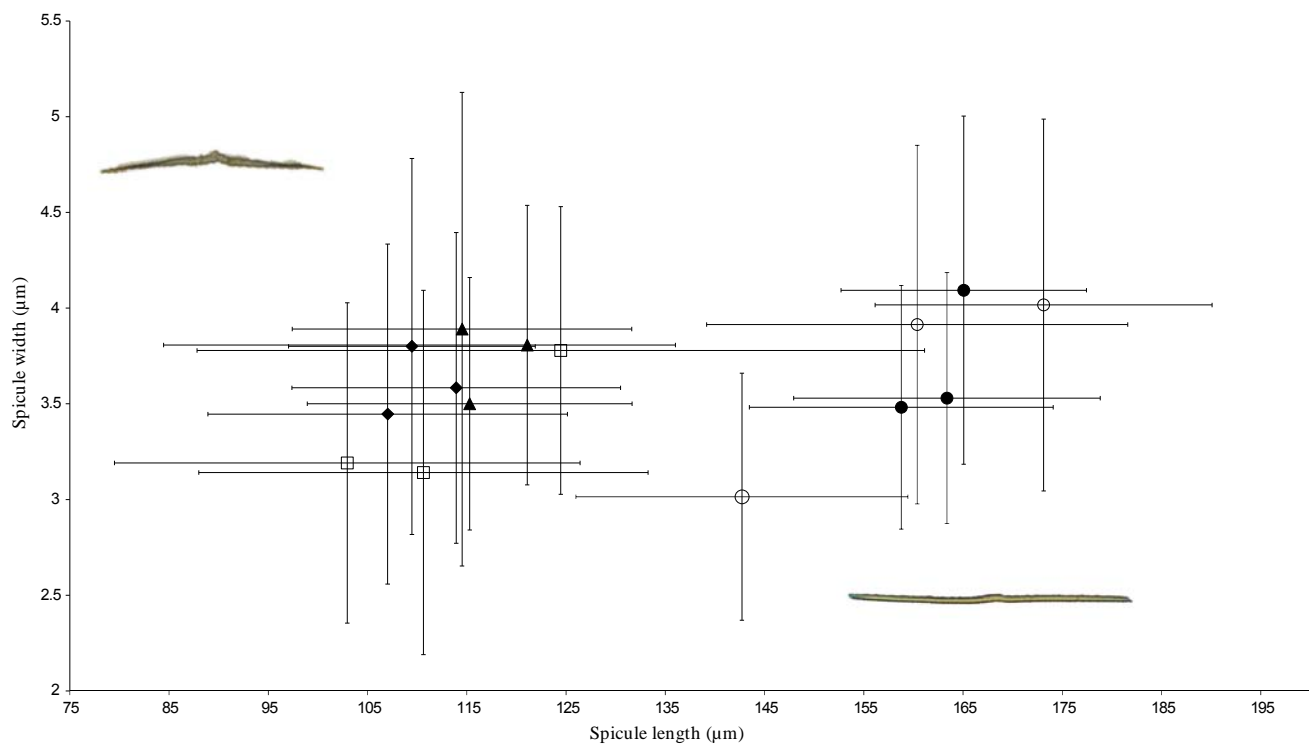


Fig. 4 Diod spicule length and width (mean \pm 1 standard deviation) of diods from *P. halichondrioides* (circles), *Plakortis* sp. 1 associated with *X. deweerdtiae* (squares), *Plakortis* sp. 2 associated with *X. deweerdtiae* (triangles) and with *Xestospongia* sp. (diamonds). Open

shapes represent samples from the Bahamas and closed shapes from Puerto Rico. Light microscope photographs of the spicules of each species at relative size are shown for comparisons

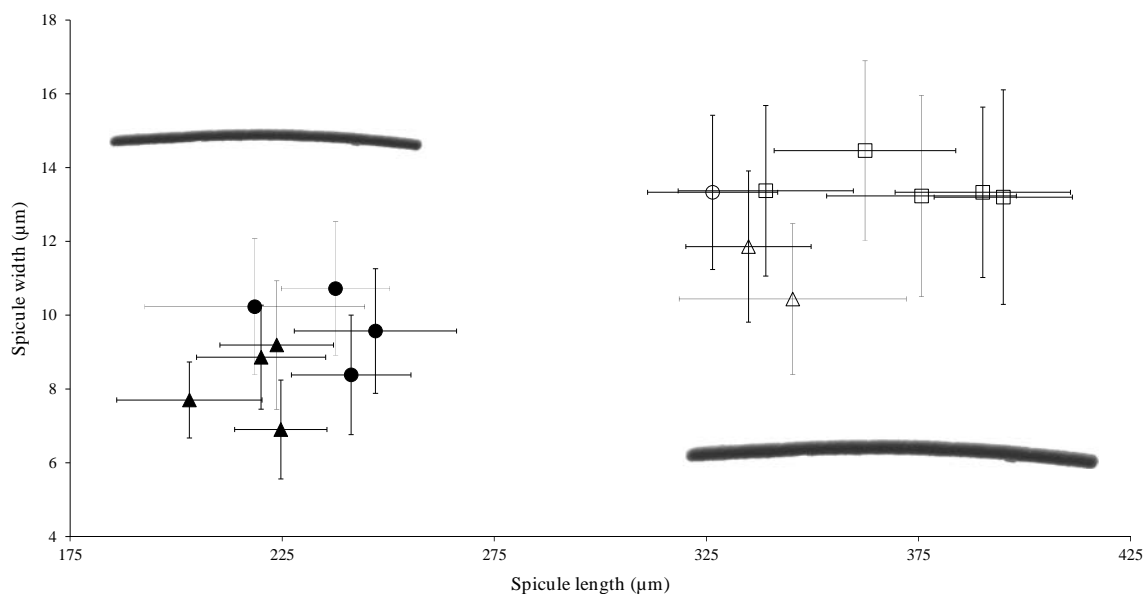


Fig. 5 Strongyle spicule length (mean \pm 1 standard deviation) of associated (closed shapes) and free-living (open shapes) individuals of *X. deweerdtiae* from the Bahamas (triangles) and Puerto Rico (cir-

cles). SEM photographs of the spicules of each species at relative size are given for comparisons

measured 373 ± 27.9 by $13.5 \pm 2.6 \mu\text{m}$ ($N_{\text{individuals}} = 5$) (Fig. 5).

Xestospongia sp. grew as encrusting or papillate morphology with a light blue to white veneer of tissue that also penetrated the *Plakortis* sp. 2 mesohyl (Fig. 3g–i). The association between *Plakortis* sp. 2 and *Xestospongia* sp. has currently been observed only on the south continental shelf reef habitats of La Parguera, Puerto Rico. The skeleton arrangement of *Xestospongia* sp. consisted of a unispicular isodictyal reticulation of oxeas (Supplementary Fig. 3F). Oxea size ranged between 199 and 277 μm individual means ranging from 229 to 230 μm .

Morphology of the association

Images from dissected specimens revealed that the epibiont *X. deweerdtiae* penetrated through the *Plakortis* body forming inner channels that measured 0.1–1 cm in diameter (Fig. 6a–c). SEM images of the association between *Plakortis* sp. 1, 2 and *X. deweerdtiae* sponge pairs showed

detailed spicule arrangement in the mesohyl (Fig. 6a, b). Despite boring into the *Plakortis* body, a well-defined barrier between the two species was observed in the mesohyl (Fig. 7). The choanosome of *Plakortis* spp. and *Xestospongia* spp. is separated in the sponge mesohyl (Fig. 7a, c, e). Diodes and triodes from *Plakortis* spp. never crossed the surface of the interface, and it was the strongyles from *X. deweerdtiae* and oxeas from *Xestospongia* sp. that slightly penetrated the *Plakortis* sp. 1 and 2 tissue instead (Fig. 7b, d, f).

Reef surveys

The highest number of free-living individuals of *X. deweerdtiae* was found in the Bahamas with an average of 4.1 individuals per site (LIBH, $N = 4$; AIBH, $N = 3$; PKBH, $N = 1$; GIBH, $N = 1$; MIBH, $N = 1$) from nine sites that were surveyed, followed by Mexico with 0.75 individuals per site (CB2COMX, $N = 1$; CBCOMX, $N = 1$, CACOMX, $N = 1$) from five sites that were

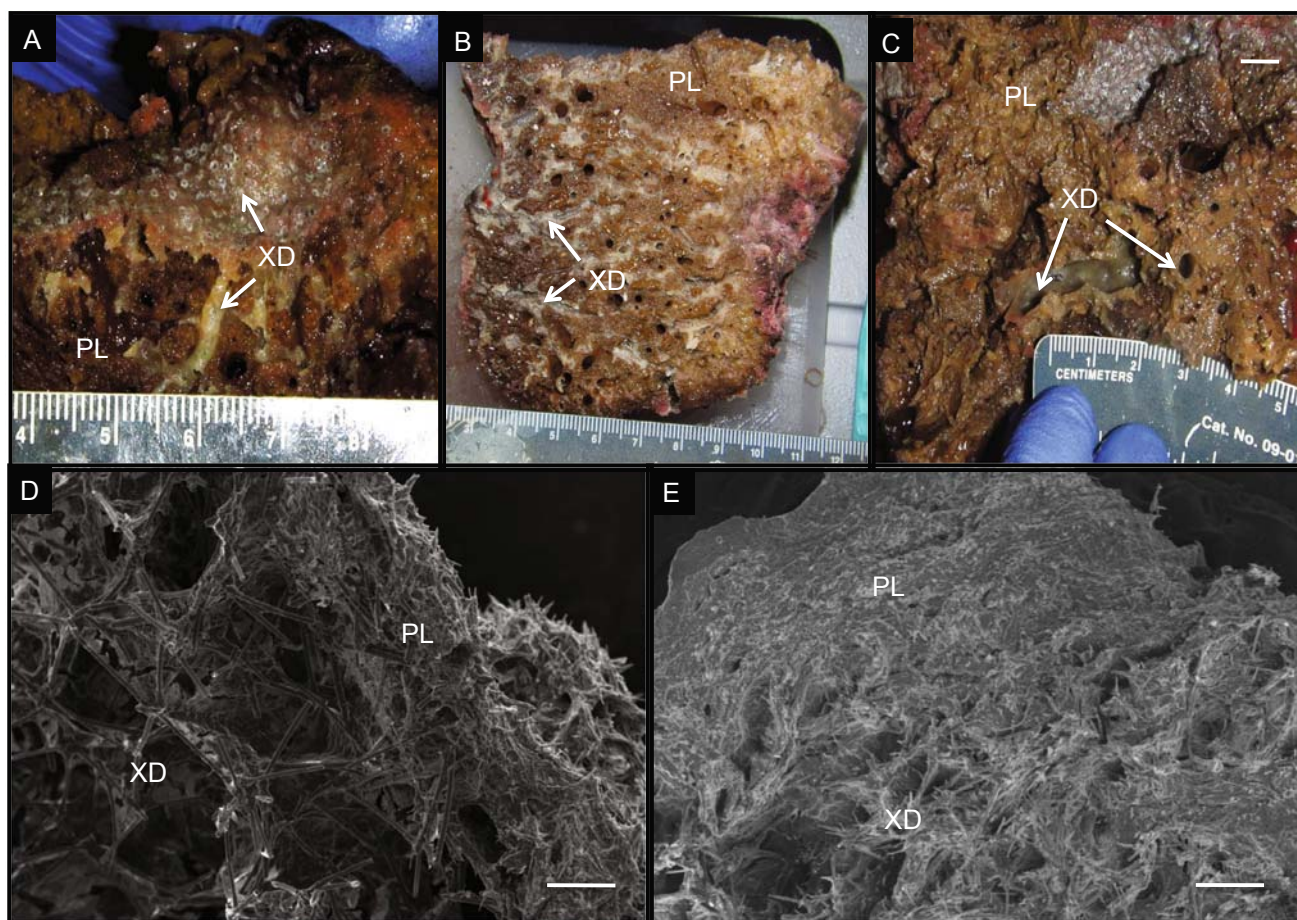


Fig. 6 Transversal dissection of **a** *Plakortis* sp. 1 adult with *X. deweerdtiae* (epibiont) boring from the *Plakortis* outer surface into the mesohyl. **b, c** Inner channels formed by *X. deweerdtiae* measuring

from 1 mm up to 1 cm in diameter. **d, e** Handmade transverse section of the mesohyl of *Plakortis* sp. 1 and 2, respectively, associated with *X. deweerdtiae* (SEM). Scale bar (**d, e**) 200 μm

surveyed and Puerto Rico with 0.33 individuals per site (DCPR, $N = 2$) from four sites that were surveyed. Associated individuals of *X. deweerdtiae* were confined to the Bahamas sites (LIBH, $N = 8$; MIBH, $N = 7$; SSBH, $N = 7$; HRBH, $N = 4$; GIBH, $N = 3$; PKBH, $N = 2$; MVPBH, $N = 1$) and in Puerto Rico (IMMOPR, $N = 13$; PCMOPR, $N = 11$; DCPR, $N = 9$; CNMOPR, $N = 6$; CECMOPR,

$N = 5$; LPPR, $N = 2$) (Fig. 8; Supplementary Table 1). Individuals of *Xestospongia* sp. were only found associated with *Plakortis* in the southern continental shelf reef habitats of Puerto Rico (LPPR, $N = 6$) with an average of 1.2 individuals. Associated individuals of *Plakortis* spp. were absent in all transects done in Mexico. From the six associated individuals that were tagged and photographically

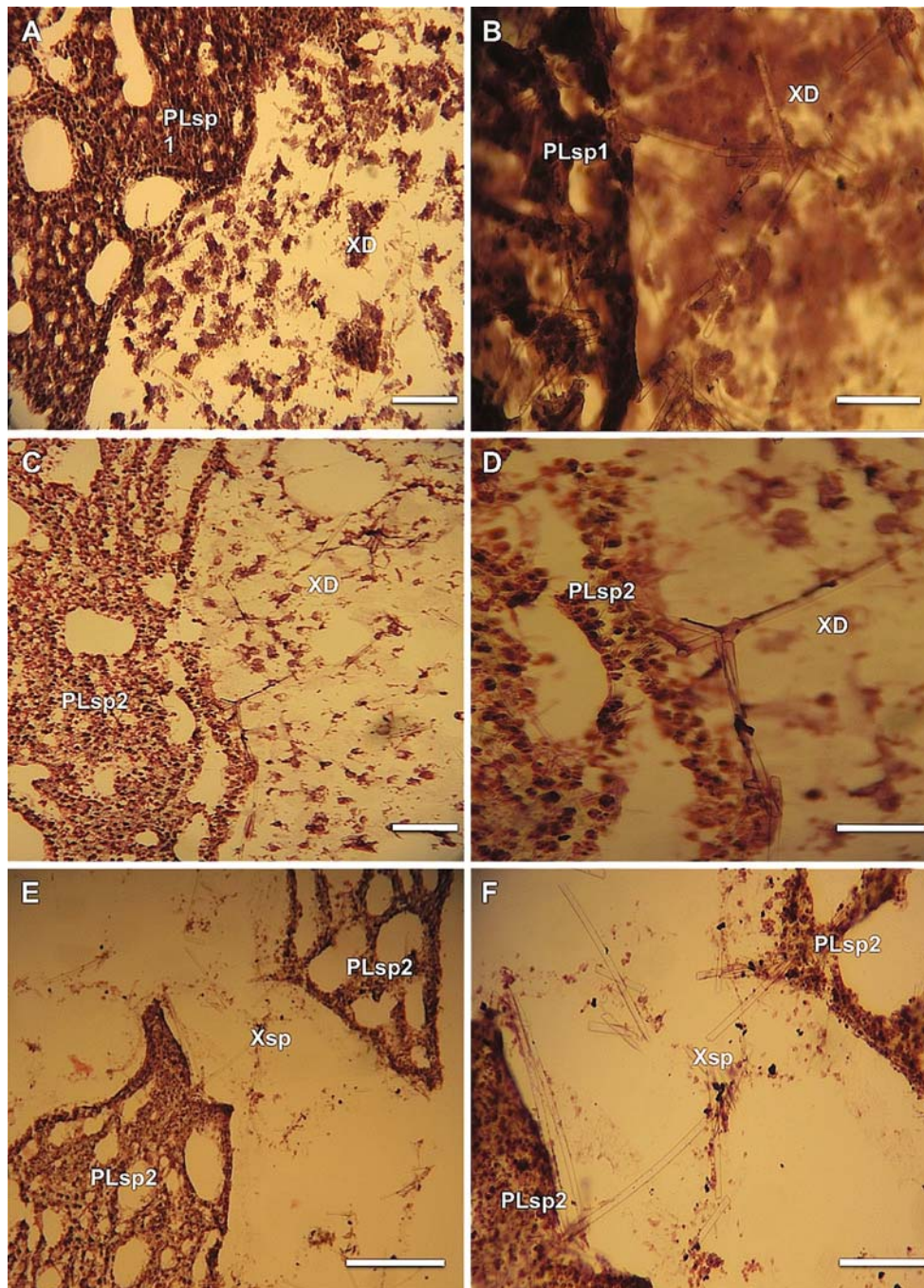


Fig. 7 Hemocytoclylin and eosin-stained transverse section of the mesohyl at the interface of *Plakortis* sp. 1 and *X. deweerdtiae* (a, b), *Plakortis* sp. 2 and *X. deweerdtiae* (c, d), *Plakortis* sp. 2 and *Xesto-*

spongia sp. (e, f). Scale bar (a, c, e) 200 μm (B, D, F) 100 μm. Section thickness (a, b) 30 μm, (c–f) 10 μm

monitored for an 8-month period, none of the sponge pairs' growth morphologies showed signs of smothering over the other (Supplementary Fig. 4). Zooanthids were observed growing exclusively on individuals of *X. deweerdtiae* associated with both *Plakortis* spp. No free-living individuals of *X. deweerdtiae* intercepted in our transects were observed with zooanthids.

Discussion

Mutualistic epizoic interactions have long been documented as a facilitation strategy by sponges to deal with competition in highly diverse and densely packed sponge communities of reef cave habitats (Rützler 1970). Likely to represent a facilitation strategy, three new specialized associations between Homoscleromorph and Haplosclerid sponges are reported in this study. We found *Plakortis* sp. 1 and 2 individuals always living beneath the thin veneer of tissue of *X. deweerdtiae* which was originally described living freely on the deep fore-reef (76–82 m) of Jamaica (Lehnert and van Soest 1999), and later in shallow reef caves of Curaçao (10–12 m) (Soest and Weerd 2001) and in the Bahamas (Zea et al. 2009). We also found a second species of *Xestospongia* sp. associated with *Plakortis* sp. 2 in the southern continental shelf of Puerto Rico. The tissues of both *Xestospongia* spp. grow within the mesohyl and part of the surface of *Plakortis* spp., unlike the basibiont–epibiont associations documented for other sponge pairs (Figs. 6 and 7). For example, in previously reported sponge pairs, the epibiont is found living on top of the basibiont where

there is a clear barrier between both sponge tissues and mixed sponge tissues are never found in the mesohyl (Wulff 2008a, Ramsby et al. 2012). Mixed tissues in the mesohyl are still differentiated, where the choanosome of *Plakortis* spp. is enclosed from *Xestospongia* spp. (Figs. 6b, 7a, d, f). A similar system was observed by Rützler (1970) in the Adriatic Sea where *Aplysilla sulfurea* was fully overgrown by the basobiont *Gellius fibulatus* but remained living in its mesohyl. Through extensive survey data, we observed the association from an early recruitment stage for both *Plakortis* spp. (Fig. 6a, d) where *X. deweerdtiae* is observed growing as small translucent colonies within the *Plakortis* spp. tissues. These are the first observations of sponge pairs ever reported from a recruiting stage that could be representative of a highly specialized mutualistic relationship.

Associated specimens of *X. deweerdtiae* were previously thought by Zea et al. (2009) to be a different species, to be compared with *Haliclona strongylophora* (Lehnert and Van Soest 1996), the only other sponge species with a unispicular skeleton of 150- to 200- μ m strongyles. However, our work with partial sequences of the 18S rRNA, 28S rRNA, and COI genes of associated individuals compared with free-living individuals of *X. deweerdtiae* collected from three different geographic locations revealed that associated individuals were all >99 % similar to free-living *X. deweerdtiae* (Figs. 1, 2). All *X. deweerdtiae* individuals were monophyletic and supported by a single clade across the three genes. However, the sequences most closely related to the *X. deweerdtiae* clade were those of *Geliodes gallista* and *Dasychalina fragilis*. The sponges *G. gallista* and *D. fragilis* both belong to sponges of the family Niphatidae but in our analysis are grouped with *X. deweerdtiae* which is in the family Petrosidae (Supplementary Figs. 1, 2). Similar groupings were observed by Redmond et al. (2011) and are characteristic of the polyphyletic nature of the Haplosclerida.

Molecular confirmation of the conspecificity of associated and free-living specimens of *X. deweerdtiae* suggests that differences in spicule size are due to its association with *Plakortis* spp. and not due to differences in silica concentrations from different geographic locations (Bavestrello et al. 1993). This would imply a cost in terms of silicon availability, or a benefit in terms of a lower investment in skeleton for support and defense (Hill et al. 2005). Assuming that sponges can be severely limited for silica (Maldonado et al. 2012a) and that there is a need for larger spicules for support or defense, these results suggest that the association could be beneficial for *X. deweerdtiae*, allowing it to invest less energy in spicule synthesis. A similar observation was made for the association between *G. vosmaeri* and *A. erina*, in which the cortex of *G. vosmaeri* was significantly reduced when associated with *A. erina* in comparison with the free-living form (Ramsby et al. 2012).

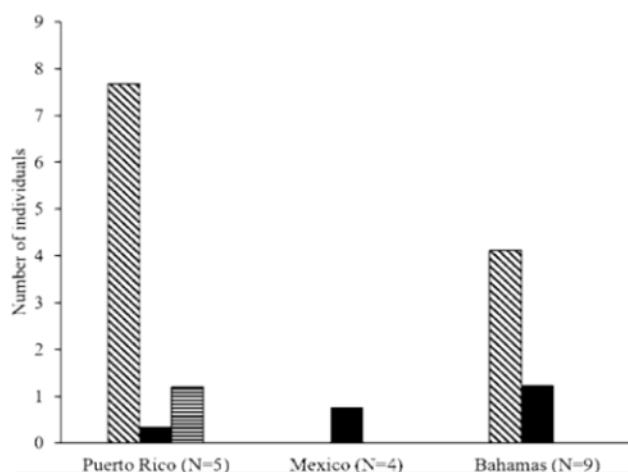


Fig. 8 Average number of free-living (solid bars) and associated (diagonal striped bars) individuals of *X. deweerdtiae* in Mexico ($N_{\text{sites}} = 4$), Puerto Rico ($N_{\text{sites}} = 5$), and the Bahamas ($N_{\text{sites}} = 9$) along 100-m transects; the number of associated *Xestospongia* sp. (horizontal striped bars); location names for each acronym and number of individuals spotted for each species are listed in Supplementary Table 1

Spicule composition of *Plakortis* sp. 1 and 2 consisted of diods and triods, but lacked microrhabds or quasiamphiblasters, which drew our attention to consider *Plakortis simplex* and *P. halichondrioides* as closely related species. The concatenated COI/18SrRNA gene sequences of *Plakortis* sp. 1 and 2 showed only 91.9 % identity, 100 % coverage with *Plakortis simplex* and 91.2 % identity, 100 % coverage with *P. halichondrioides* (Fig. 2), making it unlikely that *Plakortis* sp. 1 and 2 are closely related to either *P. simplex* or *P. halichondrioides*. *Plakortis* sp. 1 showed microscleer diods which has only been observed in *Plakortis berquistae* (Muricy 2011), *Plakortis galapagensis* (Desqueyroux-Faundez 1981), and the recently described *Plakortis dariae* and *Plakortis edwardsi* (Ereskovsky et al. 2013). Unfortunately, we did not have access to individuals of *P. dariae* and *P. edwardsi* to compare molecular sequences. However, morphological differences consisted of *Plakortis* sp. 1 only having Y-shaped triods and individual measuring up 3–30 cm by 1–8 cm thick with oscules 0.2–0.9 cm in diameter which are significantly larger than those of *P. dariae* and *P. edwardsi* individuals. Spicule arrangements in the ectosome of both *Plakortis* sp. 1 and 2 never crossed the surface, whereas spicules of *Plakortis dariae* do. *Plakortis* sp. 1 and 2 also exhibit an irregular shape of the outer surface resulting in grooves along the surface of the sponge body where *X. deweerdtiae* tissue occurs (Fig. 3b, e), whereas the surface of *P. edwardsi* and of *Plakortis dariae* is smooth (Ereskovsky et al. 2013). In addition, *P. dariae* and *P. edwardsi* are found in the same deep reef habitats (25–28 m) of Jamaica where *X. deweerdtiae* was originally documented (Lehnert and van Soest 1999), but neither of these species has been reported to be associated with *X. deweerdtiae*. If, in fact, *P. dariae* and *P. edwardsi* were to be either of the *Plakortis* species reported here, they would likely be found associated with *X. deweerdtiae* which at this point remains an exclusive characteristic of *Plakortis* sp. 1 and 2. We therefore conclude that *Plakortis* sp. 1 and 2 are likely undescribed species.

Although smaller diod size classes are characteristic of *P. dariae*, and *P. edwardsi*, we believe that the smaller spicule size for the associated *Plakortis* individuals is a benefit for *Plakortis* spp. By producing smaller spicules, these sponges are possibly investing less energy in skeleton synthesis. One additional potential benefit for *Plakortis* spp. is that the inner channels formed from the less dense *Xestospongia* spp. tissue would facilitate more efficient water transport through the denser *Plakortis* spp. tissue. This builds on the hypothesis from Vogel (1978) proposing that denser sponge tissues tend to have a greater abundance of small water channels that require more energy to pump water than less dense sponge tissues. Images of dissected *Plakortis* spp. associated with *Xestospongia* spp. have been observed to have a less dense arrangement of spicules

forming channels (0.1–1 cm) with wider meshes (200–500 μ m) throughout the *Plakortis* spp. body (Figs. 6, 7). Since both associated cases of *Xestospongia* spp. are thin walled sponges in comparison with the *Plakortis* spp., the *Xestospongia* spp. are also more likely to display current induced flow than their basibiont (Leys et al. 2011).

Survey data also revealed a higher abundance of associated *X. deweerdtiae* individuals over free-living individuals only when either *Plakortis* sp. 1 or 2 were present indicating that recruitment might be enhanced in the presence of either *Plakortis* sp. (Fig. 8). However, the apparent obligate symbiosis between *Plakortis* spp. and *Xestospongia* spp. could also be due to our inability to exhaustively survey all possible habitats where these sponges may be found in free-living form. Interestingly, *Plakortis* sp. 2 was found associated with *Xestospongia* sp. only in La Parguera, Puerto Rico (Fig. 8), suggesting possible host competition between different Haplosclerid species for *Plakortis* sp. 2. Associated individuals of *X. deweerdtiae* were sometimes exposed on the wall of the deep fore-reef or on the bedrock at >30 m. Free-living *X. deweerdtiae* were only found in entirely shaded areas and never exposed to light. Perhaps, *Plakortis* spp. allows *X. deweerdtiae* to survive in more exposed habitats with higher predator pressure, either by providing the sponge with chemical defenses that it lacks or by providing UV protecting pigments to its tissue (Makarchenko and Utkina 2006). The chemical defense hypothesis is a likely possibility as sponges belonging to the genus *Plakortis* are well known to produce a wide range variety of biologically active cyclic peroxides (del Sol Jiménez et al. 2003). More specifically, bioactive polyketide endoperoxides and peroxide lactones have been isolated from *Plakortis* sp. 1 and 2 (Dalisay et al. 2010; Jiménez-Romero et al. 2010). Functional roles of cyclic peroxides from *P. halichondrioides* have been speculated to be involved in causing bleaching and death of 14 different coral species (Porter and Targett 1988). Chemical extracts from tissue of several Homoscleromorph species have also proven to provide chemical defense in feeding assays with the bluehead (*Thalassoma bifasciatum*) (Pawlik et al. 1995). Despite the overwhelming evidence of *Plakortis* spp. being heavily defended species, it could be that the *Xestospongia* spp. sponge pairs are chemically defended as well, therefore invalidating the chemical defense hypothesis as an ecological trade-off. Regardless, we are uncertain of possible predation pressures from spongivorous organisms in low-light habitats like reef caves and mesophotic reefs, and it is not known whether spongivory is similar to that found in shallow coral reef habitats or sea grass beds. We suspect that cave habitats have few spongivorous fish species, but cryptic mollusks, echinoderms, and crustaceans could be a threat to undefended sponges. Potential spongivores in these habitats

may include cryptic starfish, sea urchins, nudibranchs, limpets, or cowries (Faulkner and Ghiselin 1983; McLean and Harasewych 1995; Wilson and Clarkson 2004). Unfortunately, there are limited studies on spongivory in reef cave habitats of the Caribbean.

Despite the evidence suggesting that the symbioses between *Plakortis* and *Xestospongia* are mutualistic, it is important to discuss any potential negative effects of the association. For example, individuals of *Plakortis* sp. 1 can be completely overgrown by *X. deweerdtiae* leaving only the oscula of *Plakortis* sp. 1 free of tissue (Fig. 3c). We believe that when completely overgrown by *X. deweerdtiae*, water is pumped through the *X. deweerdtiae* ostia and enters the *Plakortis* spp. mesohyl through the inner channels (Fig. 6b) that interact with the *Plakortis* spp. body. *X. deweerdtiae* could be at an advantage over *Plakortis* sp. 1 as it is the first to access any particulates in the water column. Although unlikely, we are uncertain whether *X. deweerdtiae* could completely outgrow and eliminate *Plakortis* sp. 1. We have photographically monitored several individuals of *Plakortis* sp. 2 with *X. deweerdtiae* and *Xestospongia* sp. which showed even distribution and no evidence of smothering by either species throughout an 8-month period (Supplementary Fig. 4). Stable growth morphologies of both species support a mutualistic relationship. In addition, individuals of both *Plakortis* sp. 1 and 2 are associated with *X. deweerdtiae* from a recruiting stage (5 × 2 cm) (Fig. 6a, d), suggesting that these associations begin early in their life cycle. We also noticed that associated *X. deweerdtiae* individuals were commonly colonized by zoanthids (personal observations by JV) (Fig. 3b, c) which are known to reduce pumping rates of host sponges (Lewis 1982). Intriguingly, zoanthids only colonized the *X. deweerdtiae* tissue but not the *Plakortis* sp. 1 or 2 tissue. None of the free-living individuals of *X. deweerdtiae* encountered in our surveys showed the presence of zoanthids although they have been observed in individuals collected in Curacao (Soest and Weerdt 2001). The presence of zoanthids could therefore affect the pumping rates and be a disadvantage for the *Plakortis* host that, without the presence of *X. deweerdtiae*, would possibly avoid fouling as it is uncommon for Homoscleromorphs to host zoanthids (Swain and Wulff 2007).

We have discussed both mutualistic and commensalism scenarios for the associations between *Plakortis* and *Xestospongia*, and we conclude that these are likely of mutualistic nature. There are several hypothetical possibilities on how these associations originated. Based on biomass proportions of each associated sponge species, it seems as though at one point *X. deweerdtiae* larvae likely settled on the *Plakortis* spp. becoming the epibiont. *X. deweerdtiae* could have been resistant to antifouling allelochemicals from *Plakortis* species and overtime managed

to grow from the *Plakortis* pinacoderm through the mesohyl. Persistence of these associations overtime presumably allowed for reproductive synchronization between both species. A reef cave habitat rather than the bedrock of the upper mesophotic reef is the best hypothetical setting where these associations first occurred, where larval dispersal from different sponge species is confined and more susceptible to interact with each other (Harmelin et al. 1985). Similar to the genetic isolation observed in Mediterranean caves that allowed for speciation of four different cryptic species of *Plakina trilopha* (Muricy et al. 1996), a similar process may have resulted in speciation of *Plakortis* sp. 1 and 2. These are all hypothetical scenarios that need to be tested in future studies. Long-term monitoring studies of tagged *Plakortis* sp. 1 and 2 recruits in combination with histological sections highlighting reproductive strategies of both sponges would help determine if in fact reproduction between both sponges is synchronous.

Another interesting topic for future study is to determine the allrecognition mechanism between free-living individuals of *X. deweerdtiae* and *Plakortis* spp., to determine whether the interaction results in recognition or rejection of the heterospecific tissues (Connes et al. 1974; Mukai and Shimoda 1986). Parabiosis experiments using free-living individuals of *X. deweerdtiae* with *Plakortis* sp. 1 and 2 would help determine how these sponges interact with one another at the interphase (Gaino et al. 1999). Characterization of the aggregation factors involved in cellular adhesion of both heterospecifics could also aid in understanding self- to non-self-recognition mechanisms of these sponges (Haseley et al. 2001).

Resolving the genetic structure of associated *X. deweerdtiae* in cave habitats where free-living individuals co-occur would reveal whether there is genetic isolation between associated and the free-living *X. deweerdtiae* individuals (López-Legentil and Pawlik 2009). *Plakortis* species are known to be a “high microbial abundance” (HMA) sponge, while *Xestospongia* species belong to the order *Haplosclerida* which have many species that are “low microbial abundance” (LMA) sponges (Maldonado et al. 2012b). It would be interesting to test the hypothesis that tissue densities between both species correlate with bacterial abundance and whether the more bacteria-rich *Plakortis* tissue serves as a source for key symbionts with important metabolic pathways that *Xestospongia* spp. would not be able to otherwise access. In this regard, it would also be interesting to look at the proportions of choanocyte chambers for both species and see how these are distributed and fit with the HMA/LMA hypothesis suggested by Poppell et al. (2013) where HMA sponges have a lower amount of choanocyte chambers and rely more on a heterotrophic lifestyle than LMA sponges.

Conclusions

Our study reveals two potentially new *Plakortis* species from reef caves and mesophotic reefs of the Caribbean that are found exclusively in association with either *X. deweerdtiae* or *Xestospongia* sp. resulting in three new sponge–sponge symbiotic associations. Phylogenetic analysis using partial 28S rRNA, 18S rRNA, and COI gene sequences of associated and free-living individuals of *X. deweerdtiae* revealed that these were all >99 % identical, thus being of the same species. Spicule sizes for associated *X. deweerdtiae* individuals were significantly smaller than those of free-living individuals, suggesting a possible benefit in terms of costs by investing less energy in synthesizing smaller spicules. Symbiotic individuals of *Plakortis* spp. are thought to benefit from the association by receiving more efficient water flow through its mesohyl by a complex system of inner channels created by *Xestospongia* spp. Other possible roles of these associations are under investigation. This study reflects the unusual adaptations of marine sponges in light-limited habitats where they are likely subjected to different space limitations and a different suite of predators than sponges in open reef environments.

Acknowledgments Funding for this project was supported by NOAA's Living Marine Resource Cooperative Science Center award number NA11SEC4810002. JV received additional support by the American Museum of Natural History Lerner-Gray Fund for Marine Research for the collection of sponges in Puerto Rico. JV's stipend was provided by NOAA's Nancy Foster Scholarship award NA12NOS4290142. Support for sequencing of sponge samples was provided by the NSF BIO/IOS Program (IOS-0919728) Grant to RTH. We would like to thank the government of the Bahamas for letting us collect samples provided by an unnumbered scientific permit for the operation of the R/V Walton Smith in their territorial waters; the government of Mexico for providing the CONAPESCA permit DAPA/2/06504110612/1608; and the government of Puerto Rico for providing permit 2010-IC-043 (R-VS-PVS15-SJ-00190-08062010). All sponge collections from the Bahamas and Mexico were supported by NSF award OCE-1029515 to JRP. Scanning electron microscope images were provided by the University of Maryland Baltimore County's NanoImaging facility with the assistance of Dr. Laszlo Takacs. Paul Jensen and Laura Grice are thanked for providing key points to the discussion of this manuscript. Micah Marty is thanked for his assistance in performing dissections and photographing sponge individuals during cruises to the Bahamas. JV is indebted to Milton Carlo at the University of Puerto Rico at Mayaguez for his dive assistance with the collection of sponge individuals from Puerto Rico. Fred Lentz and Jose A. Rivera are also thanked for providing boat access for the collection of sponges in Desecheo, Puerto Rico. Niamh E. Redmond is thanked for her advice in suggesting primers for this study. We thank Leah Blasiak for her assistance in troubleshooting phylogenetic tree analyses. Nilli Zmora is thanked for providing histological equipment to perform hematoxylin and eosin staining for light microscopy images. SZ's work is contribution of the Centro de Estudios en Ciencias del Mar – CECIMAR, Universidad Nacional de Colombia, Sede Caribe. We thank two anonymous reviewers for very helpful comments. This is IMET contribution no. 14–136 and UMCES contribution no. 4960.

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