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Sponges and their prokaryotic communities sampled from a remote karst ecosystem

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Abstract
Marine karst ecosystems exist at the land-sea interface and are characterised by underwater formations sculpted over time by the action of seawater. Submerged caves and crevices of these ecosystems host a rich array of marine life of which sponges are among the most abundant and diverse components. In the present study, we describe elements of the sponge fauna sampled from a unique karst ecosystem at a remote island, Orchid Island, off the southeastern coast of Taiwan. The present study includes several understudied sponge taxa, including sclerosponges (*Acanthochaetetes wellsi*, and *Astrosclera willeyana*) and several lithistid species from dark, shallow-water caves. Prokaryotic communities were obtained from a total of 22 demosponge species, of which 11 are potentially new to science. The tetracladinid, lithistids harboured prokaryotic communities, which clustered separately from all other sponge species, contrasting with the non-tetracladinid, lithistid *Vetulina incrustans*. The tetracladinid, lithistids, furthermore, formed two distinct clusters with species of the Spirophorina suborder clustering apart from those of the Astrophorina suborder. The sclerosponge *A. wellsi* also harboured a distinct prokaryotic community in terms of composition including five unique, abundant OTUs with relatively low sequence similarities to organisms in GenBank. All cave sponges were enriched with SAR202 members, a group of bacteria known for their role in the degradation of recalcitrant compounds. The highest relative abundance of SAR202 was found in *A. wellsi*. We propose that the cave sponges of Orchid Island may play an as-yet uncharted role in nutrient dynamics at the land-sea interface.

Keywords 16S · Composition · Porifera · Prokaryotes · Taiwan

Introduction

Marine karst areas are unique ecosystems of spellbinding beauty and considerable potential scientific importance. They are often renowned for their striking geological formations, adorned with intricate rock structures. Clinging to the coast, they consist of intricate networks of pools, crevices, and caves with a plethora of life forms inhabiting these varying habitats. Situated along rocky coastlines, they are subject to ebb and flow such that the flora and fauna have adapted to fluctuations in temperature, light intensity, and salinity (Meroz-Fine et al. 2005; Sket 1996). Sought-after destinations for diving and ecotourism, among other activities, they have come under increasing scientific scrutiny although much of this focus has been heavily skewed to the Mediterranean (Gerovasileiou and Voultsiadou 2012; Harmelin 1997; Sket 1996; but see Ise et al. 2023; Schuster et al. 2021).

Sponges are among the most abundant and species-rich components of marine karst ecosystems, particularly in light-deprived, karst caves (Gerovasileiou et al. 2016; Pisera and Gerovasileiou 2021). Karst caves can also host taxa typically found in the tenebrous depths of the ocean, as exemplified by recent discoveries of carnivorous and glass sponges in Mediterranean caves (Bakran-Petricioli et al. 2007; Vacelet 1996; Vacelet et al. 1994). Another

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compelling aspect of marine karst ecosystems lies in their potential for unearthing rare and previously undocumented species, including a significant number of species unique to these environments (Gerovasileiou et al. 2015; Iliffe and Kornicker 2009; Manconi and Serusi 2008).

In August 2018, we encountered a highly interesting marine karst ecosystem while snorkeling in a large tidal pool at a small island called Orchid Island (also known as Lanyu). Orchid Island is located in the Pacific Ocean about 68 km off the East coast of Taiwan and about 140 km from the northern Philippines. The island harbours many endemic terrestrial species (Hsieh 2002). Furthermore, despite several research efforts focusing on the coral reefs of Orchid Island, the karst pool and cave systems have remained neglected and understudied (Chao 2002; Chao and Lee 2002; Chen et al. 2022a, b; Kao et al. 2007; Lee 1980; Lee and Lee 1981; Reigle 1963; Yu 1985).

The subtidal cave system consists of several, very shallow tunnels connected via intertidal pools. We only explored the tunnels close to the pools, and these were all very shallow. The tunnels and caves were decorated by an abundance of small calcareous sponges, in addition to lithistid sponges and sclerosponges with specimens of the sclerosponge *Acanthochaetetes wellsi* up to 20 cm in length. Several potentially new lithistid species were sampled, which belonged to relatively rare genera including *Manihinea* (family Theonellidae), *Neophrissospongia* (family Corallistidae), and *Scleritoderma* (family Scleritodermidae).

With respect to their associated prokaryotic communities, the serendipitous discovery of the sponges inhabiting the karst ecosystem of Orchid Island enabled us to compare several sponge species free from the confounding effects of large-scale spatial processes and differences in local environmental conditions. Despite initial findings, which suggested that sponge composition was temporally and spatially 'stable' (Cárdenas et al. 2018; Hentschel et al. 2002; Hill et al. 2006; Pita et al. 2013; Reveillaud et al. 2014; Taylor et al. 2005;), a number of studies have now shown that the prokaryotic compositions of several sponge species differ among localities and as a function of geographic distance. Cleary et al. (2022), for example, showed that 55% of the variation in prokaryotic composition of the sponge species *Hyrtios erectus* could be explained by geographic distance after controlling for variation due to environmental conditions.

The primary goal of the present study was to provide a preliminary description of the sponges of the karst ecosystem of Orchid Island and their associated prokaryotic communities. For the first time, the prokaryotic communities of two, sympatrically occurring sclerosponge species, *Acanthochaetetes wellsi* and *Astrosclera willeyana*, were compared. We also assessed prokaryotic communities of a range of lithistid sponges in order to explore the species-level variation in the microbiomes of this understudied group, in addition to comparing these species to the other sampled sponge taxa. Lithistid sponges have been generally neglected with the notable exception of the species *Theonella swinhoei,* which has been extensively studied and characterised as a high microbial abundance (HMA) species (Lurgi et al. 2019). Kuo et al. (2019) also studied the bacterial composition of *T. swinhoei* using pyrosequencing. We also examined two phototrophic sponge species and determined their dominant symbionts. For all studied species, we identified abundant OTUs and compared these with homologous sequences in the GenBank database.

Material and methods

Study area

Orchid Island, with an area of 48.4 km^2 and a coastline of 38.5 km, and the adjacent islet (Hsiao Lanyu) lie 70 km to the east of the southern tip of mainland Taiwan in the western Pacific Ocean (Fig. 1). Sponge samples were collected across a total of nine different sites. These two islands are the northward extension of the Luzon volcanic arch formed by volcanic eruptions between 3.5–1.4 and 0.04–0.02 Ma, respectively, which is driven by the convergence boundary between the Eurasian and the Philippine Sea plates (Chen et al. 1990 1992; Wang and Burnett 1990; Yang et al. 1996). Geologically, Orchid Island mainly consists of igneous andesite intermingled with basalt, foraminifera limestone, laterite platforms, and coral rocks uplifted at a rate of 3.2 mm annually during the Pleistocene and Holocene (Chen 1993; Richard et al. 1986; Wang and Burnett 1990). The outermost coral rock and fringing reefs, which have been exposed to intense wave erosion, have sculpted an intricate network of caves and tunnels interconnected to the open sea (Fig. 2a).

Part of the subtidal and wave-cut groove habitats, the karst ecosystem of Orchid Island is prominently featured along its coastline. Pools connected to the open sea via underwater tunnels and canyons experience regular tidal fluctuations (Fig. 2b). In addition to this, subsurface freshwater flows into the system from the island resulting in pronounced salinity fluctuations close to land. The surface areas of the pools range from several to hundreds of square meters and reach depths of 3 to 12 meters. Caves and tunnels, varying in diameter from less than a meter to a few meters, form complex mazes and encompass environments with limited to no light penetration.

Sampling

All sponge specimens were collected using snorkeling and SCUBA diving. All specimens were photographed in their natural environment, and a fragment was collected using a scalpel. Sponge specimens were initially identified as morphospecies

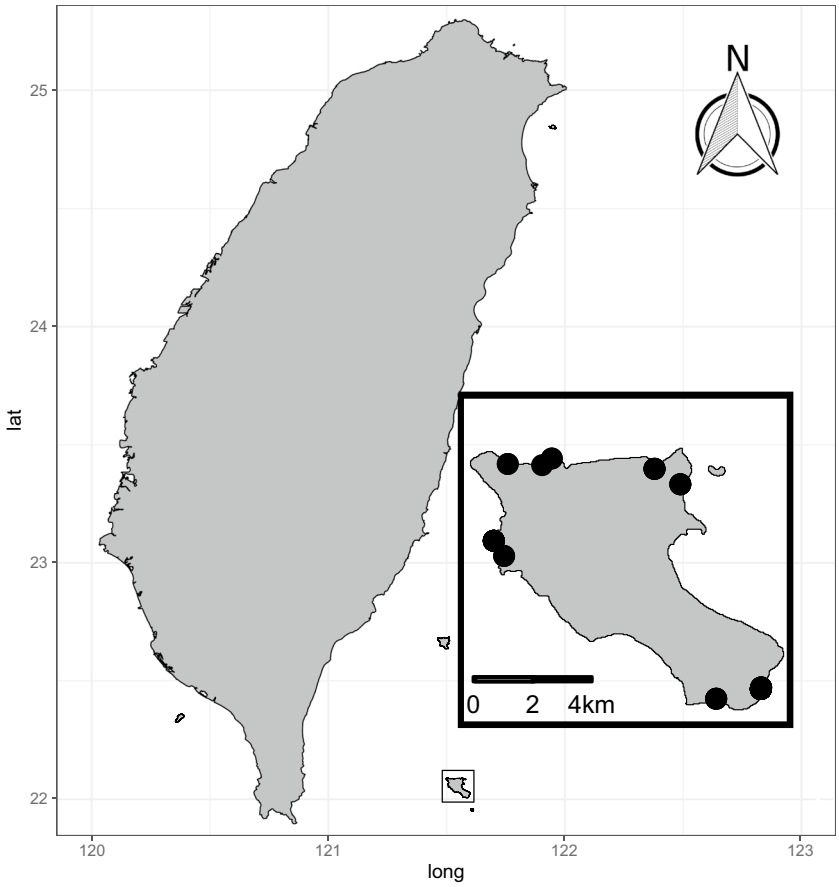

Fig. 1 Map of research area showing location of Orchid Island off the coast of Taiwan

in the field, and between 1 and 5 specimens were collected per morphospecies; in addition to sponge samples, sediment samples were collected from the top 5 cm surface layer using a sterile Falcon tube (Supplementary data 1). Upon removal from the water, a section from each sponge specimen was carefully excised and promptly placed in a vial containing 96% alcohol. Care was taken to include surface and interior tissue of each specimen. The vials were shipped to the Netherlands and stored in a − 20 °C freezer until subsequent DNA extraction. Sponge species identification was carried out using traditional taxonomic characters with a Leica microscope; samples from all species have been deposited at the Naturalis Biodiversity Center (as RMNH POR), Leiden, the Netherlands.

DNA extraction

DNA was extracted using the Qiagen DNeasy Powersoil extraction kit (Qiagen, Venlo, the Netherlands). A maximum of 250 mg of sponge tissue was used per sample; tissue was taken from all sides of the specimen (outside to core, and if applicable top, middle, and bottom of the sample). Sediment DNA extraction utilised 250 mg of wet sediment. The manufacturer's protocol was followed with the exception of the initial vortexing step, which was carried out using the Qiagen TissueLyser II (Qiagen NV, Hilden, Germany). Sponge tissue was cut into small pieces using sterilised tweezers and scalpel blades and transferred to PowerBead Pro tubes containing ceramic and silica beads of different sizes. An extraction blank, in which no tissue was added to the PowerBead Pro tubes, was also included. Library preparation involved a two-step PCR protocol for all samples in addition to two negative controls (mQ water instead of template DNA) and the extraction blank. For the first PCR, the V3-V4 regions of the 16S rRNA gene were targeted and amplified using the primers 314F/ S-D-Bact-0785-a-A-21 (5′-CCTACGGGNGGCWGCAG-3′/5′-GACTACHVGGGTATCTAATCC-3′; Klindworth et al. 2013) with added 5′ Nextera transposase adaptors using the KAPA HiFi HotStart Ready Mix PCR Kit with a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The following PCR conditions were used: initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 98 °C for 20 s, annealing at 55 °C for 30 s, followed by extension at 72 °C for 30 s. The final extension was carried out at 72 °C for 1 min. PCR success was confirmed on an E-Gel™ (agarose gels at 2%), and the absence of amplification was validated for the negative controls and the extraction blank. PCR products were then cleaned with NucleoMag NGS-Beads (bead volume at

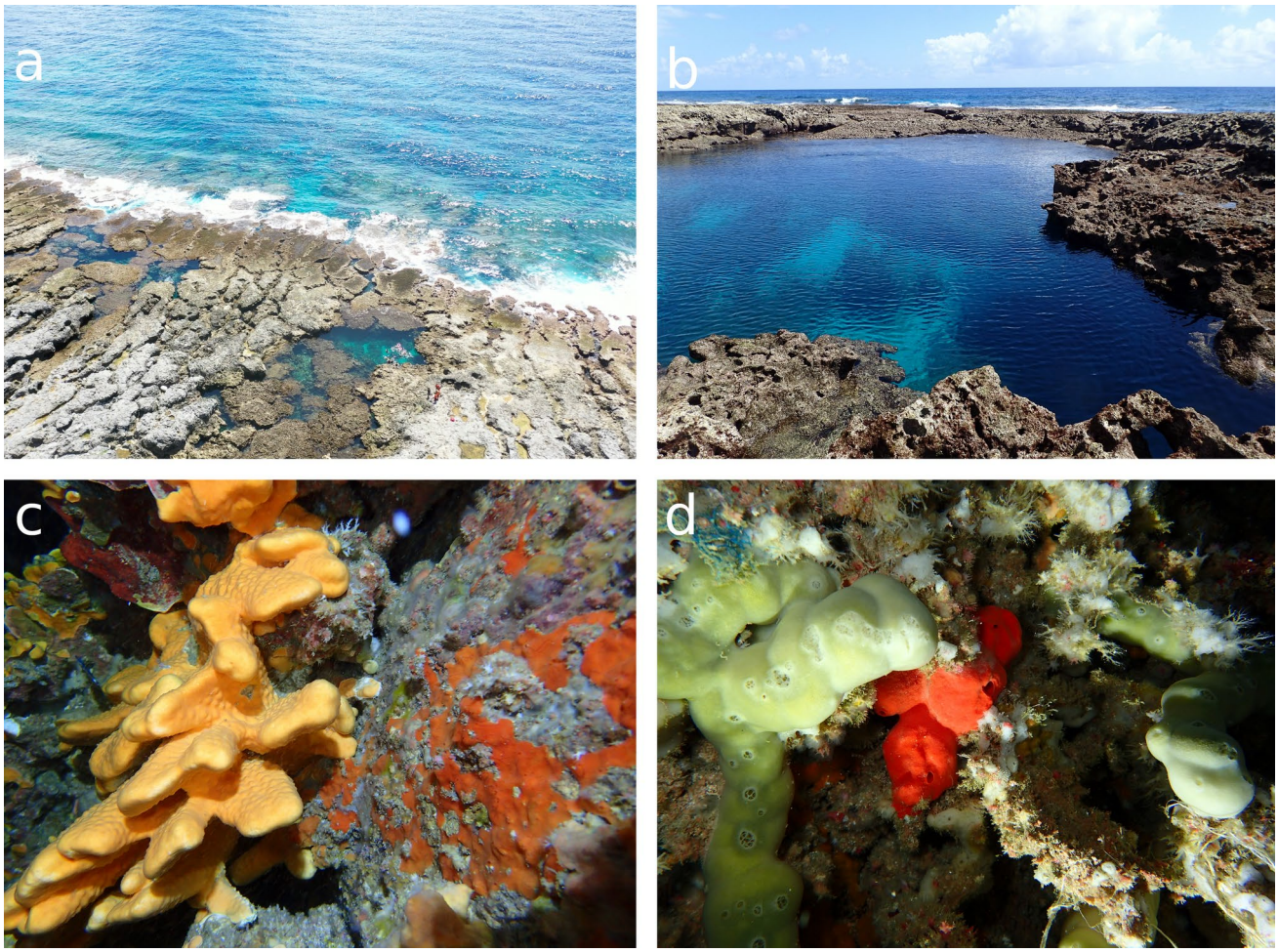

Fig. 2 **a** Drone image of submarine caves taken by JKZ Cleary; **b** photograph of pool habitat and entrance to submerged caves taken by DFR Cleary; **c** photograph of the sclerosponge *Acanthochaetetes wellsi* taken by DFR Cleary; **d** photograph of *Petrosia corticata* (the green-grey sponge) and *Manihinea* sp. (the red sponge) taken by DFR Cleary

0.9 times the total volume of the sample, Macherey Nagel, Düren, Germany) using the VP 407AM-N 96 Pin Magnetic Bead Extractor stamp (V&P Scientific, San Diego, CA, USA). For the second PCR, the cleaned PCR products (1 µL each) were amplified and labelled using the MiSeq Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) with the same thermal cycling scheme limited to 8 cycles. PCR products were then analysed with the Fragment Analyser Agilent 5300 using the DNF-910–33 dsDNA Reagent Kit (35–1500 bp) protocol (Agilent Technologies, Santa Clara, CA, USA) to confirm successful labelling of the DNA fragments. Negative controls and extraction blanks remained negative after this step. Pooling at equimolar concentration was performed with QIAgility 2 (Qiagen, Hilden, Germany). The final pool was then cleaned with NucleoMag NGSBeads, eluted in Milli-Q, and the DNA concentration was verified using Tapestation 4150 (Kit HSD 5000, Agilent Technologies, Santa Clara, CA, USA). Paired-end sequence reads were generated with an Illumina MiSeq v3 PE300 platform at BaseClear B.V. (Leiden, the Netherlands). FASTQ read sequence files were generated using bcl2fastq version 2.20 (Illumina). Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an in-house filtering protocol. In addition to this, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bps). The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.8.

Sequencing analysis

The 16S rRNA amplicon libraries were analysed using QIIME2 (version 2019.7; Bolyen et al. 2019). Raw data were imported yielding a demultiplexed 'qza' data file (artifact). The DADA2 plugin (Callahan et al. 2016) in QIIME 2 was

subsequently used to trim sequences (final length 400 nt). The DADA2 analysis yielded output archives containing an OTU (at a 100% similarity threshold, also known as amplicon sequence variant or 'ASV') table, denoising stats, and a fasta file of representative sequences. The feature-classifier plugin with the extract-reads method was then used with the i-sequences argument set to silva-138–99-seqs.qza. This was followed by the feature-classifier plugin with the fit-classifier-naive-bayes method, and the i-reference-taxonomy method set to silva-138–99-tax.qza. Both silva-138 files can be obtained from https://docs.qiime2.org/2020.8/data-resources/?highlight=silva. The feature-classifier plugin was then used with the classify-sklearn method, and the i-reads argument was set to the representative sequences file generated by the DADA2 analysis to produce a table with taxonomic classifications for all OTUs. Finally, mitochondria, chloroplasts, and Eukaryota were filtered out using the qiime taxa plugin with the filter-table method. All OTUs unclassified at domain and phylum level were also removed. The OTU and taxonomy tables were subsequently merged in R (R Core Team 2022). Accession numbers of closely related organisms to selected OTUs were obtained using NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). Sequences used in this study have been uploaded to the NCBI ShortRead Archive (BioProject nr: PRJNA865713 and BioSample nr: SUB11909922).

Statistical analyses

A table containing the OTU counts was imported into R. Supplementary data 2 contains all operational taxonomic unit (OTU) counts per sample and taxonomic classifications of all OTUs. The 50 most abundant OTUs are summarised in Supplementary data 3 including the results of the BLAST analyses. The raw OTU counts matrix was used to compare diversity and higher taxon abundance among groups. Diversity indices, namely, rarefied richness, evenness, Shannon's H′, and Fisher's alpha, were obtained using the *rarefy* and *diversity* functions from the *vegan* (Oksanen et al. 2020) package in R. Evenness (Pielou's J) was calculated by dividing Shannon's H′ by the number of OTUs in each sample. Variation in prokaryotic composition among groups (sponge species and sediment) was visualised with Principal Coordinates Analysis (PCO), and we tested for significant differences among groups with an *adonis* analysis from the *vegan* package. Subsequent statistical analyses were limited to groups with at least three samples. For the PCO, a Bray–Curtis distance matrix was first obtained using the *phyloseq* package (McMurdie and Holmes 2013) whereby the count data was rarefied using the *rarefy_even_depth* function with the sample.size argument set to the minimum sample size ($n=6978$) and subsequently $\log_{10}$ transformed.

Results

A preliminary description of several sponge species from Orchid Island's marine karst ecosystem

Prokaryotic communities were obtained from a total of 22 sponge species representing a total of nine orders and 17 families. All were members of the class Demospongiae. The majority of species were restricted to low-light, karst cave habitats. The following sponge species were sampled from submarine caves and canyons, *Acanthochaetetes wellsi* Hartman & Goreau, 1975, *Acanthostylotella cornuta* (Topsent, 1897), *Aciculites ciliata* Wilson, 1925, *Asteropus* cf. *simplex, Astrosclera willeyana* Lister, 1900, *Petrosia corticata* (Wilson, 1925), *Sollasipelta ornata* (Sollas, 1888), and *Vetulina incrustans* Schuster et al., 2018. Several species could not be conclusively identified to species level and are potentially new species. These are the following: *Theonella* aff. *timmi*, *Svenzea* aff. *devoogdae*, *Discodermia* sp., *Manihinea* sp., *Neophrissospongia* sp., *Penares* sp., *Scleritoderma* sp., *Xestospongia* sp., *Polymastia* sp., and *Theonella* sp.. Two species, *Lamellodysidea herbacea* (Keller, 1889) and *Lamellodysidea* sp., were sampled from pool habitat, while *Xestospongia testudinaria* Lamarck, 1815 and *Stylissa carteri* Dendy, 1889 were sampled from nearby coral reef habitat (Supplementary data 1). Formal descriptions of all potentially new species identified in this study are underway.

Only three specimens of the sclerosponge *Astrosclera willeyana* were observed from three different sites, and they all had the usual pyriform, half-spherical growth form. *Acanthochaetetes wellsi* was found in a wide range of shapes, and formed large, stalactite-like formations and was highly abundant and dominant in certain caves (Fig. 2c).

The larger sclerosponges coexisted within their cave and canyon habitats with a large number of smaller species, including a diverse array of calcareous and lithistid sponge species. Lithistid demosponges emerged as dominant and species-rich components of the cave fauna. The specimens sampled encompassed species belonging to the orders Tetractinellida and Sphaercladina representing five different families. Aside from the tetractinellid, lithistid sponges, we also sampled a single non-tetractinellid, lithistid sponge species, *Vetulina incrustans.*

To date, we have successfully identified nine tetractinellid species of which five are potentially novel. Of the order Tetractinellida, the most species-rich group belonged to the family Theonellidae with four potentially new species in the genera *Theonella*, *Maninihea*, and *Discodermia*. Within the family Scleritodermidae, two species were observed, the large flabelliform *Aciculites ciliata* and a potentially new species of *Scleritoderma* with an unusual

tube-forming morphology. A small globular specimen of *Sollasipelta ornata* (Neopeltidae) and a potentially new species of *Neophrissospongia* (Corallistidae) were sampled. The latter species was abundant at many different sites and formed cylindrical, fused masses and had a brownish colour. Apart from the lithistid tetractinellids, two non-lithistid sponge species belonging to the order Tetractinellida were sampled, namely, *Asteropus* cf. *simplex* and a potentially novel species of *Penares*.

In addition to the sclerosponges and tetractinellids, an array of other sponge taxa across a range of orders were identified including potentially novel species. Three species belonging to the order Haplosclerida (Petrosiidae) were observed. The giant barrel sponge, *Xestospongia testudinaria*, was found at water depths exceeding 20 meters in coral reefs nearby the karst ecosystem. The green/grey *Petrosia* (*Strongylophora*) *corticata* (Wilson, 1925) was relatively abundant in overhangs and caves (Fig. 2d), and formed large cylindrical branches and unlike other Petrosiidae was parchment-like and soft. An unusual, potentially new Petrosiidae species was observed from crevices and caves; it had a pink colouration and turned dark brown/black upon preservation and has been tentatively identified as *Xestospongia* sp.. Two species belonging to the Scopalinidae family were observed, the very common, orange reef species *Stylissa carteri* from nearby coral reefs and a potentially new species of *Svenzea*. One species belonging to the order Polymastiida was observed, namely, *Polymastia* sp.. The cyanobacterial sponge *Lamellodysidea herbacea* (Dictyoceratida: Dysideidae) was found in shallow-water pools together with another, as-yet-unidentified, species of *Lamellodysidea*.

Sponge-associated prokaryotic communities

Prokaryotic communities were retrieved from 68 samples belonging to 23 groups, namely 22 sponge species and sediment (Supplementary data 1). After quality control, the dataset consisted of 727,262 sequences and 6636 OTUs. In terms of sequences the most abundant phyla were Chloroflexi (198,432 sequences, 1209 OTUs accounting for 18.2% of all OTUs), Proteobacteria (172,986 sequences, 1878 OTUs accounting for 28.3% of all OTUs), Actinobacteriota (81,928 sequences, 484 OTUs accounting for 7.3% of all OTUs), and Cyanobacteria (63,630 sequences, 166 OTUs accounting for 2.5% of all OTUs). The 50 most abundant OTUs are presented in Supplementary data 3. Biodiversity indices exhibited significant variations among groups (Fig. 3 and Supplementary data 4). Evenness was highest in sediment followed by species of tetractinellid sponges, all of which had high evenness. *Stylissa carteri*, *A. willeyana* and the phototrophic sponges *L. herbacea* and *Lamellodysidea* sp. had significantly lower evenness than all other sponge species (Supplementary data 4). As with evenness, richness was highest in the tetractinellid sponge species. Richness was significantly higher in the tetractinellid *Aciculites ciliata* than in all non-tetractinellid species (Supplementary data 4).

Proteobacterial abundance was significantly higher in *S. carteri* and *A. wellsi* than in all other sponge species except for the highly variable *L. herbacea* (Fig. 4). *Aciculites ciliata* was the only tetractinellid cave species in which cyanobacterial sequences were recorded. Cyanobacterial abundance was, furthermore, significantly higher in *L. herbacea and Lamellodysidea sp.* than in all other sponge species. The relative abundances of Chloroflexi and Actinobacteriota were significantly lower in *L. herbacea, Lamellodysidea* sp., and *S. carteri* than all other sponge species (Supplementary data 5). No Acidobacteriota members were recorded in *S. carteri.* In addition to this, the relative abundance of this phylum was significantly lower in *Lamellodysidea herbacea, Lamellodysidea* sp., *Acanthochaetetes wellsi*, and *Vetulina incrustans* than in the remaining sponge species. No Gemmatimonadota members were recorded in *S. carteri or Lamellodysidea sp*. The relative abundance of Gemmatimonadota was also significantly lower in *A. wellsi*, *V. incrustans*, and *Xestospongia* sp. than all other sponge species.

The most abundant class, the Dehalococcoidia (Chloroflexota), was highly variable among sponge species ranging from no recorded sequences in *L. herbacea* and *Lamellodysidea* sp. to its greatest abundance in the sponge *A. wellsi*, which was significantly greater than in all other sponge species where the taxon was recorded (Fig. 5 and Supplementary data 6). The class Alphaproteobacteria was most abundant in the sponges *A. wellsi* and *L. herbacea.* For *A. wellsi*, the difference was significant for all sponge species except *L. herbacea*. The class Gammaproteobacteria was significantly more abundant in *S. carteri* than in all remaining sponge species. Results for the Acidimicrobiia and Cyanobacteria were basically the same as those at phylum level considering their overwhelming dominance. The Anaerolineae differed markedly from the Dehalococcoidia with Anaerolineae members reaching greatest abundance in *X. testudinaria*, significantly greater than in all other sponge species where the taxon was recorded. No members of the Anaerolineae were recorded in the species *A. wellsi*, *L. herbacea*, *Lamellodysidea* sp., or *S. carteri*.

PCO ordinations of the first and second axes including all samples are shown in Fig. 6. The first axis separated tetractinellid species belonging to the Ancorinidae, Corallistidae, Geodiidae, and Theonellidae families from all other sponge species, while the second axis separated tetractinellid species belonging to the Sclerotidermidae family from all other species. There was a highly significant difference in

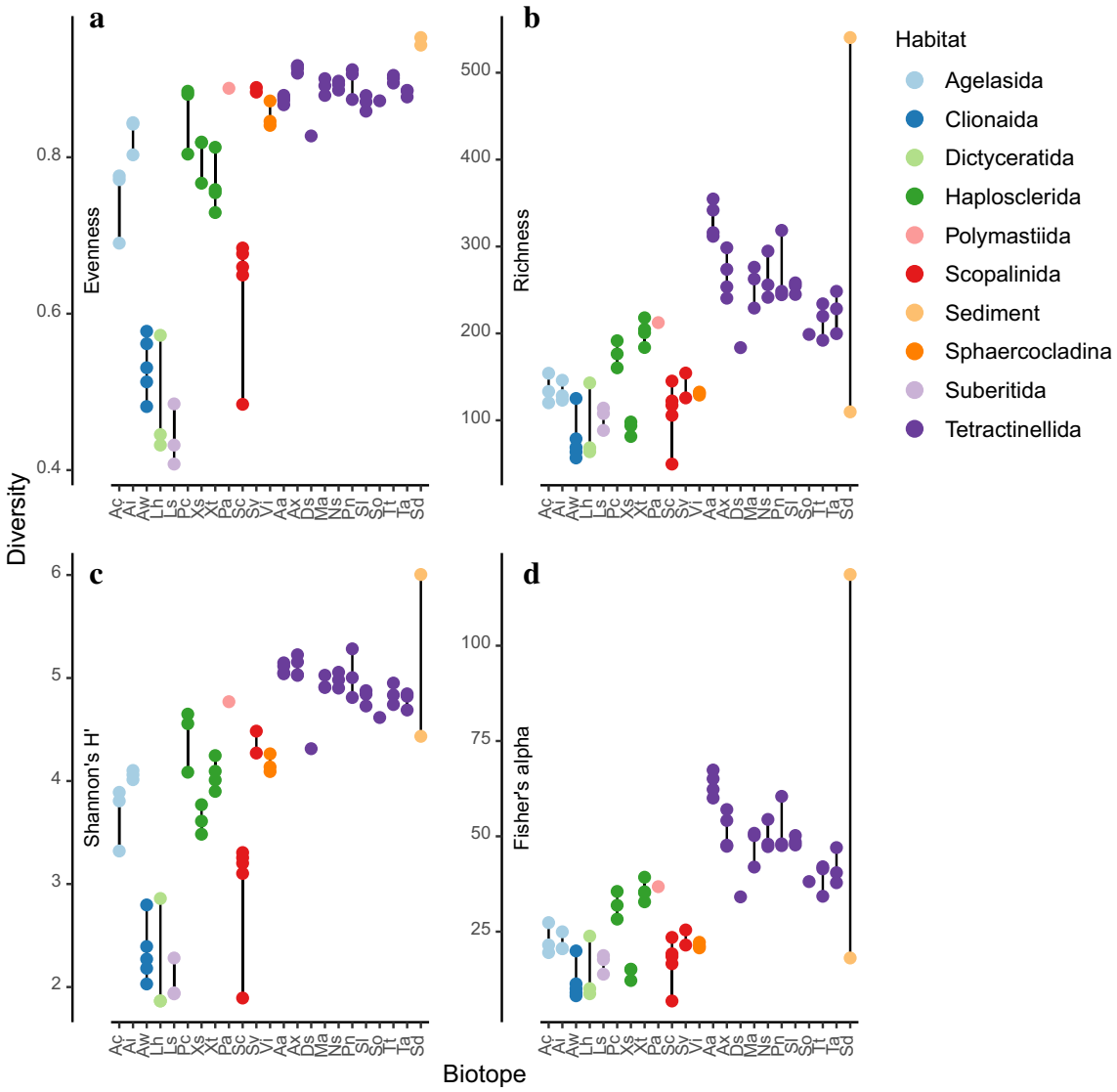

Fig. 3 Diversity values and results of glm analyses for selected indices. **a** Evenness: $F_{17,43}=35.9$, $P<0.001$; **b** Richness: $F_{17,43}=10.49$, $P<0.001$; **c** Shannon's H': $F_{17,43}=25.54$, $P<0.001$; **d** Fisher's alpha: $F_{17,43}=11.55$, $P<0.001$. The groups sampled were as follows: Ac, *Acanthostylotella cornuta*; Ai, *Astrosclera willeyana*; Aw, *Acanthochaetetes wellsi*; Lh, *Lamellodysidea herbacea*; Ls, *Lamellodysidea* sp.; Pc, *Petrosia corticata*; Xs, *Xestospongia* sp.; Xt, *Xestospongia testudinaria*; Pa, *Polymastia* sp.; Sc, *Stylissa carteri*; Sv, *Svenzea* aff. *devoogdae*; Vi, *Vetulina incrustans*; Aa, *Aciculites ciliata*; Ax, *Asteropus* cf. *simplex*; Ds, *Discodermia* sp.; Ma, *Manihinea* sp.; Ns, *Neophrissospongia* sp.; Pn, *Penares* sp.; Sl, *Scleritoderma* sp.; So, *Sollasipelta ornuta*; Tt, *Theonella* aff. *timmi*; Ta, *Theonella* sp.; and Sd, sediment. Bonferroni corrected P values <0.0125 indicate significant variation among groups

composition among groups (sponge species and sediment) (adonis, $F_{17,43}=7.87$, $P<0.001$, $R^2=0.757$). Species identity/group, thus explained more than 75% of the variation in composition. The eigenvalues for the first and second axes of the full dataset were 4.48 and 2.36, respectively, and accounted for 15.03% and 7.90% of the variation in the data set or 22.93% of the total variation.

Excluding the tetractinellid species, the major axis of variation in the PCO analysis separated the known low microbial abundance (LMA) sponge species *S. carteri* from the known HMA sponge species *X. testudinaria* with the other sponge species intermediate (Fig. 7). There, however, appeared to be two clusters of samples of these intermediate species with one, closer to *X. testudinaria*, consisting of the species *P. corticata*, *Polymastia* sp., *A. willeyana*, *Xestospongia* sp., and *Svenzea* aff. *devoogdae* and the other, closer to *S. carteri*, consisting of the species *A. cornuta*, *V. incrustans*, *L. herbacea*, and *Lamellodysidea* sp. in addition to sediment. The second axis of variation separated the sclerosponge *A. wellsi* from all other sponge species.

Tetractinellid species shared a similar subset of abundant OTUs compared to the other host sponge species

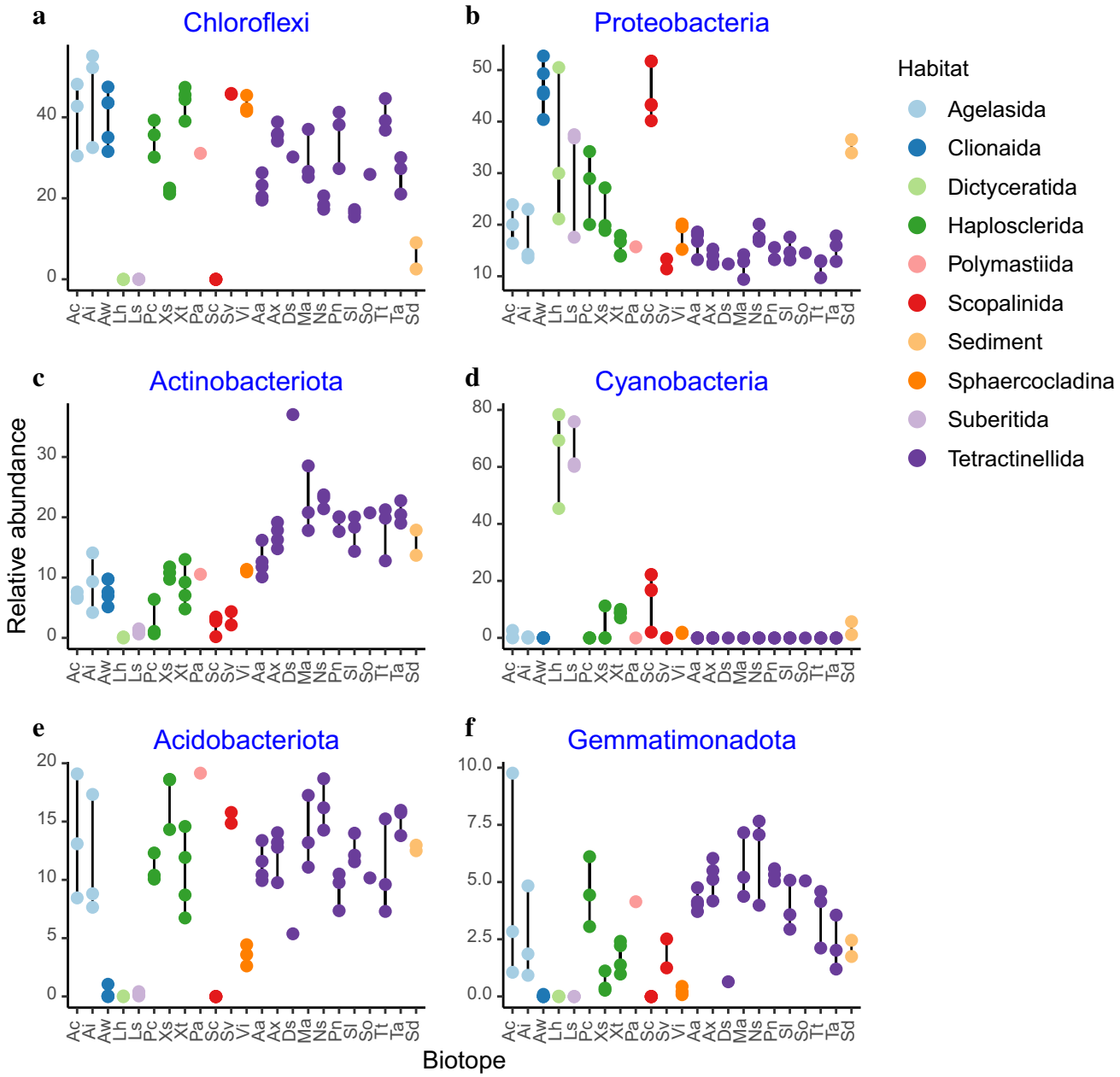

Fig. 4 Relative abundances and results of glm analyses for the most abundant phyla: **a** Chloroflexi: $F_{17,43}=34.71$, $P<0.001$; **b** Proteobacteria: $F_{17,43}=22.18$, $P<0.001$; **c** Actinobacteriota: $F_{17,43}=20.47$, $P<0.001$; **d** Cyanobacteria: $F_{17,43}=65.55$, $P<0.001$; **e** Acidobacteriota: $F_{17,43}=31.92$, $P<0.001$, and **f** Gemmatimonadota: $F_{17,43}=19.77$, $P<0.001$. The groups sampled were as follows: Ac, *Acanthostylotella cornuta*; Ai, *Astrosclera willeyana*; Aw, *Acanthochaetetes wellsi*; Lh, *Lamellodysidea herbacea*; Ls, *Lamellodysidea* sp.; Pc, *Petrosia corticata*; Xs, *Xestospongia* sp.; Xt, *Xestospongia testudinaria*; Pa, *Polymastia* sp.; Sc, *Stylissa carteri*; Sv, *Svenzea* aff. *devoogdae*; Vi, *Vetulina incrustans*; Aa, *Aciculites ciliata*; Ax, *Asteropus* cf. *simplex*; Ds, *Discodermia* sp.; Ma, *Manihinea* sp.; Ns, *Neophrissospongia* sp.; Pn, *Penares* sp.; Sl, *Scleritoderma* sp.; So, *Sollasipelta ornuta*; Tt, *Theonella* aff. *timmi*; Ta, *Theonella* sp.; and Sd: sediment

(Fig. 8). In addition to this, OTUs 41, 42, and 50 were abundant or restricted to the tetractinellid species *Aciculites ciliata* and *Scleritoderma* sp., which clustered together in the PCO analysis. OTU-84, assigned to the Caldilineales, in turn, was recorded in all tetractinellid species with the exception of the aforementioned species.

Several abundant OTUs were only recorded from single sponge species. OTU-91 was restricted to the sponge *V. incrustans*, OTU-97 to the sponge *P. corticata*, OTU-21 to *A. cornuta*, and OTU-104 to *A. willeyana*. Apart from these sponges, some sponge species harboured multiple OTUs that were unique to those species, ranging from two

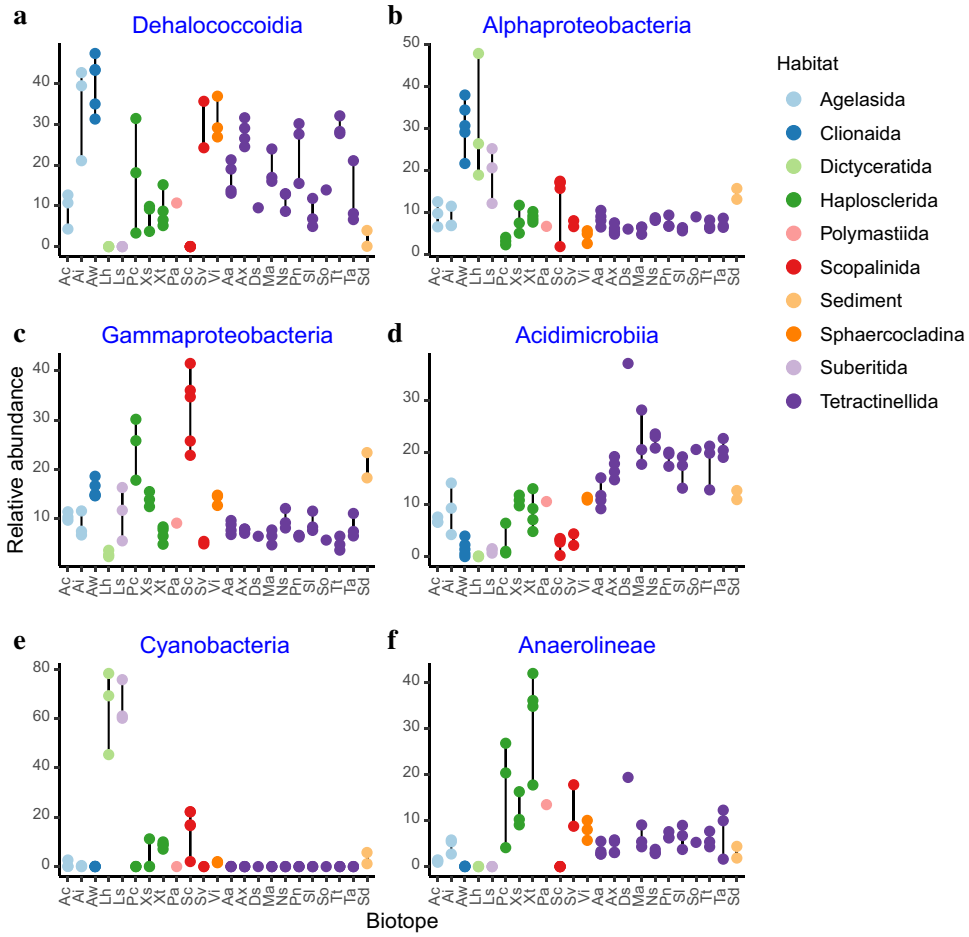

Fig. 5 Relative abundances and results of glm analyses for the most abundant classes: **a** Dehalococcoidia: $F_{17,43}=23.39$, $P<0.001$; **b** Alphaproteobacteria: $F_{17,43}=24.11$, $P<0.001$; **c** Gammaproteobacteria: $F_{17,43}=16.82$, $P<0.001$; **d** Acidimicrobiia: $F_{17,43}=14.91$, $P<0.001$; **e** Cyanobacteria: $F_{17,43}=65.55$, $P<0.001$; and **f** Anaerolineae: $F_{17,43}=32.79$, $P<0.001$. The groups sampled were as follows: Ac, *Acanthostylotella cornuta*; Ai, *Astrosclera willeyana*; Aw, *Acanthochaetetes wellsi*; Lh, *Lamellodysidea herbacea*; Ls, *Lamellodysidea* sp.; Pc, *Petrosia corticata*; Xs, *Xestospongia* sp.; Xt, *Xestospongia testudinaria*; Pa, *Polymastia* sp.; Sc, *Stylissa carteri*; Sv, *Svenzea* aff. *devoogdae*; Vi, *Vetulina incrustans*; Aa, *Aciculites ciliata*; Ax, *Asteropus* cf. *simplex*; Ds, *Discodermia* sp.; Ma, *Manihinea* sp.; Ns, *Neophrissospongia* sp.; Pn, *Penares* sp.; Sl, *Scleritoderma* sp.; So, *Sollasipelta ornuta*; Tt, *Theonella* aff. *timmi*; Ta, *Theonella* sp.; and Sd, sediment

OTUs (5 and 88) for *Lamellodysidea* sp., to three OTUs for *S. carteri* (10, 15, and 24), four OTUs for *L. herbacea* (4, 12, 62, and 68), and five OTUs (2, 8, 37, 38, and 64) for *A. wellsi*. OTUs 4, 5, and 12, all classified as the cyanobacterial species *Hormoscilla spongeliae*, all exhibited close relationships to organisms obtained from sponges identified as Dysideidae sp. from Guam. OTU-88 only had 91% sequence similarity to an organism detected in a sponge identified as *Lamellodysidea* sp. sampled from China. OTU-62 had > 99% sequence similarity to an organism detected in a sponge identified as *Phyllospongia papyracea*. OTU-68 had 96% sequence similarity to an organism detected in a sponge identified as *Lendenfeldia chondrodes* (Supplementary data 3).

The five OTUs (2, 8, 37, 38, and 64) restricted to the sponge *A. wellsi* were classified as belonging to the SAR202 clade (Chloroflexi), Defluviicoccales (Alphaproteobacteria), HOC36 (Gammaproteobacteria), Actinobacteriota, and Gammaproteobacteria, respectively. OTU-2 had 95% sequence similarity to an organism detected in a sponge identified as *Coelocarteria singaporensis* sampled from the Great Barrier Reef. OTU-8 had 96% sequence similarity to an organism detected in a coral identified as *Pocillopora damicornis*. OTU-37 had 94% sequence similarity to an organism detected in a coral identified as *Siderastrea stellata* sampled from Brazil. OTU-38 had 94% sequence similarity to an organism detected in a sponge identified as *Poecillastra compressa*. OTU-64 had 96% sequence similarity to an organism detected in biofilm of an artificial substrate from the Great Barrier Reef (Supplementary data 3).

Discussion

Here, we assessed the prokaryotic communities of several sponge species sampled from waters surrounding Orchid Island, Taiwan. In addition to sampling sponges from a karst pool and cave ecosystem, two species of sponges (*X. testudinaria* and *S. carteri*) were also sampled from nearby coral reefs. Both *X. testudinaria* and *S. carteri* have been extensively studied in previous publications as pertains to their microbial symbionts (Cleary et al. 2015, 2018, 2020, 2021, 2022; de Voogd et al. 2015, 2019; Polónia et al. 2015, 2016, 2017, 2018, 2021). Below, we go into more detail with respect to specific groups.

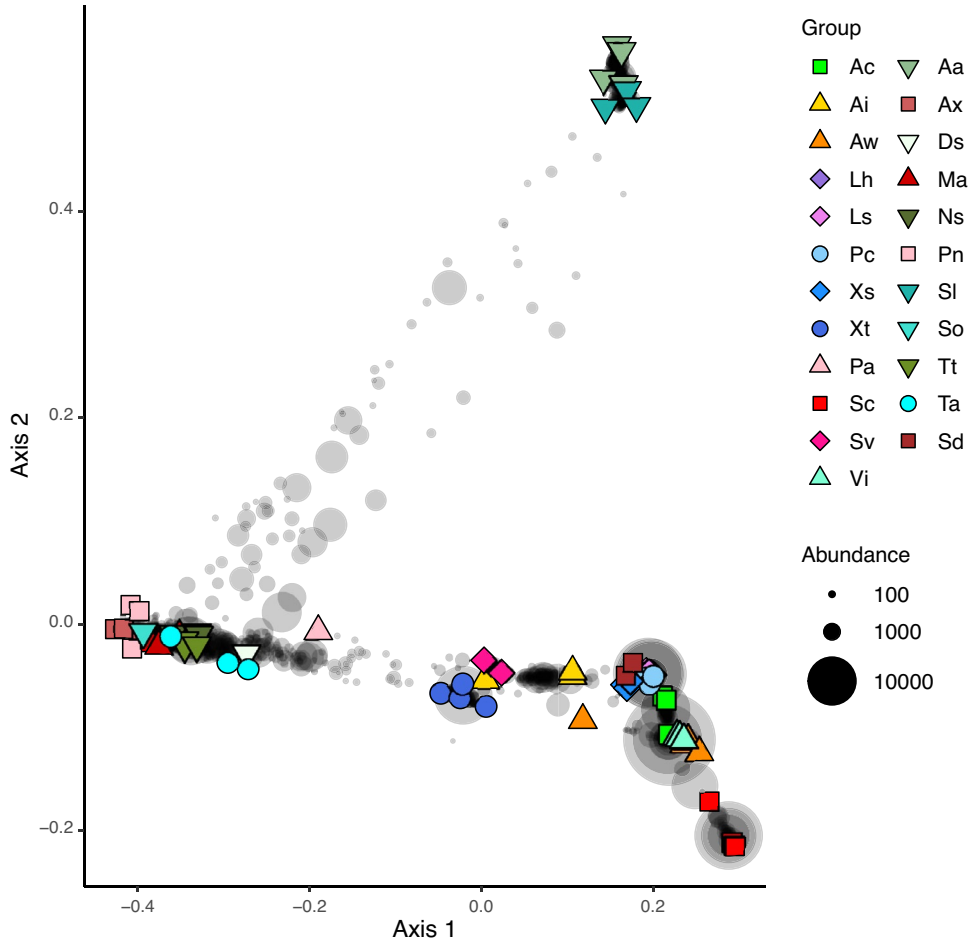

Fig. 6 Ordination showing the first two axes of the principal coordinates analysis (PCO) of OTU composition. Symbols are colour coded and represent samples belonging to different groups as shown in the legend on the right side of the figure. Grey symbols denote weighted averages scores for OTUs. The symbol size corresponds to group abundance (number of sequence reads). The legend symbols represent the following groups: Ac, *Acanthostylotella cornuta*; Ai, *Astrosclera willeyana*; Aw, *Acanthochaetetes wellsi*; Lh, *Lamellodysidea herbacea*; Ls, *Lamellodysidea* sp.; Pc, *Petrosia corticata*; Xs, *Xestospongia* sp.; Xt, *Xestospongia testudinaria*; Pa, *Polymastia* sp.; Sc, *Stylissa carteri*; Sv, *Svenzea* aff. *devoogdae*; Vi, *Vetulina incrustans*; Aa, *Aciculites ciliata*; Ax, *Asteropus* cf. *simplex*; Ds, *Discodermia* sp.; Ma, *Manihinea* sp.; Ns, *Neophrissospongia* sp.; Pn, *Penares* sp.; Sl, *Scleritoderma* sp.; So, *Sollasipelta ornuta*; Tt, *Theonella* aff. *timmi*; Ta, *Theonella* sp.; and Sd, sediment

Sclerosponges

Two sclerosponge species, namely, *A. willeyana* (order Agelasida) and *A. wellsi* (order Clionaida) were sampled from cave and canyon habitats. Sclerosponges form a hypercalcified, coral-like skeleton surrounded by a very thin layer of live tissue embedded with siliceous spicules and exhibit superficial similarities, despite belonging to distantly related orders. Extant sclerosponges, relicts of a bygone age, bear morphological resemblance to now extinct stromatoporids that once formed extensive reefs before and alongside the emergence of coral reefs (Basile et al. 1984). They prevailed as the main reef-building marine organisms throughout the Phanerozoic, but can still be found in modern marine environments (Vacelet 1985; Asami et al. 2021 and references therein). They are long-lived, slow growing, often mushroom-shaped, and primarily inhabit caves and deeper waters; in some locations, they contribute to reef formation (Asami et al. 2020; Lang et al. 1975; Macartney et al. 2020). These characteristics, together with the fact that their exoskeleton is formed by the slow deposition of calcium carbonate in sequential layers through time, render them valuable as paleo-proxy recorders of environmental conditions at depths and environments where photosynthetic scleractinian corals are not present (Asami et al. 2020; Grottoli et al. 2020).

Acanthochaetetes wellsi is a member of the Acanthochaetetidae family, an ancient family with a predominantly extinct lineage (Rützler and Vacelet 2002). Only a handful of species persist within two extant genera, including, *A. wellsi* and *Willardia caicosensis*. The genus *Acanthochaetetes* was established based on the extinct fossil species *A. seunesi* Fischer, 1970; *A. wellsi* is considered a living fossil. It was originally discovered in shallow-water caves of Guam but has since been recorded in other Indo-Pacific locations in similar, cave habitats. In addition to *A. wellsi*, another agelasid, the non-sclerosponge *Acanthostylotella cornuta* (Topsent, 1897), was also observed. This mono-specific, poorly-known species (de Voogd et al. 2010) was originally described from Indonesia and was recently placed in the Agelasida (Morrow and Cárdenas 2015).

Our results demonstrated that, despite their hypercalcified skeletal similarity, the sclerosponge species harboured distinct prokaryotic communities. Species richness and evenness were higher in *A. willeyana* than in *A. wellsi*. Chloroflexi were, however, relatively abundant in both host species. Acidobacteriota, in turn, were more abundant in

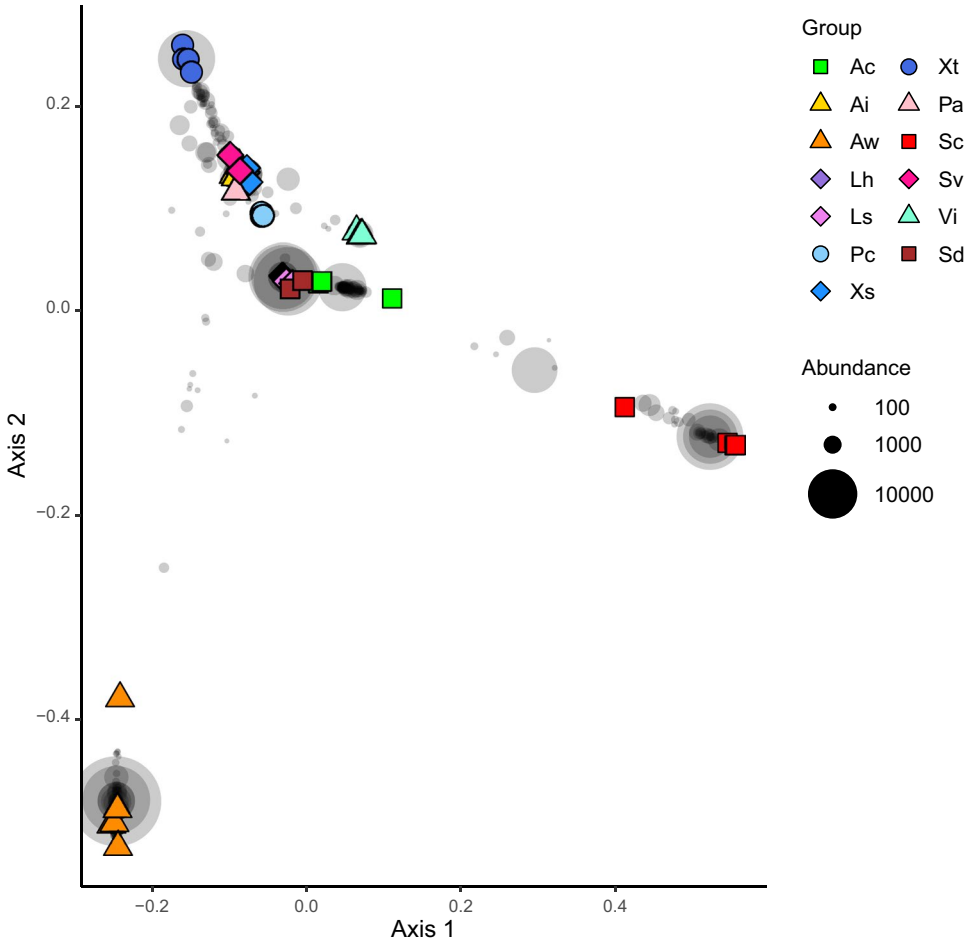

Fig. 7 Ordination showing the first and second axes of the principal coordinates analysis (PCO) of OTU composition excluding tetractinellid host species. Symbols are colour coded and represent samples belonging to different groups as shown in the legend on the right side of the figure. Grey symbols denote weighted averages scores for OTUs. The symbol size corresponds to group abundance (number of sequence reads). The legend symbols represent the following groups: Ac, *Acanthostylotella cornuta*; Ai, *Astrosclera willeyana*; Aw, *Acanthochaetetes wellsi*; Lh, *Lamellodysidea herbacea*; Ls, *Lamellodysidea* sp.; Pc, *Petrosia corticata*; Xs, *Xestospongia* sp.; Xt, *Xestospongia testudinaria*; Pa, *Polymastia* sp.; Sc, *Stylissa carteri*; Sv, *Svenzea* aff. *devoogdae*; Vi, *Vetulina incrustans*; and Sd, sediment

A. willeyana than in *A. wellsi*. In terms of composition, *A. willeyana* clustered closer to the known HMA sponge species *X. testudinaria*. Our results for *A. willeyana* align with those of Karlińska-Batres and Wörheide (2013a, 2015) for *A. willeyana* specimens sampled from the Great Barrier Reef and from sites across the Indo-Pacific region, respectively, namely, prokaryotic communities dominated by Chloroflexi with major Actinobacteriota, Acidobacteriota, and Proteobacteria components and minor Gemmatimonadota and Poribacteria components. In a previous study, the coralline *Ceratoporella nicholsoni* (order Agelasida) maintained a stable microbiome through a depth gradient (37 to 97 m) consisting mainly of Chloroflexi, Thaumarcheaota, Proteobacteria, Acidobacteria, and Actinobacteria (Macartney et al. 2020). Similar results were obtained for the coralline sponge *Vaceletia crypta* (order Dictyoceratida) with Chloroflexi the most abundant phylum (35%) (Karlińska-Batres and Wörheide 2013b). Some studies have suggested that the symbiotic microbial communities of coralline members may be involved in calcification (Jackson et al. 2011; Jackson and Wörheide 2014). Karlińska-Batres and Wörheide (2013a) also noted that their samples separated according to sampling location suggesting an important role of geographic distance in structuring prokaryotic community composition in line with our study for two HMA sponge species, *X. testudinaria* and *Hyrtios erectus* (Cleary et al. 2022).

Acanthochaetetes wellsi harboured the most compositionally distinct prokaryotic community of the two coralline sponges sampled, had the lowest richness of all sponge species, and harboured the most taxon-restricted abundant OTUs. These OTUs, furthermore, had relatively low sequence similarities to organisms in GenBank. Moreover, the Chloroflexi in *A. wellsi* were exclusively classified as Dehalococcoidia with no OTUs classified as Anaerolineae. To the best of our knowledge, this is the first next-generation assessment of the prokaryotic community of the sclerosponge *Acanthochaetetes wellsi.*

Lithistid sponges

In addition to sclerosponges, we encountered a diverse array of other sponge species, among which lithistids were particularly notable. Lithistids, also known as desma-bearing or rock sponges, are characterised by their exceptionally dense (hypersilicified) skeletal structure and articulated skeletons composed of desma megascleres (Pisera and Gerovasileiou 2021). Lithistids, an ancient lineage of sponges, were

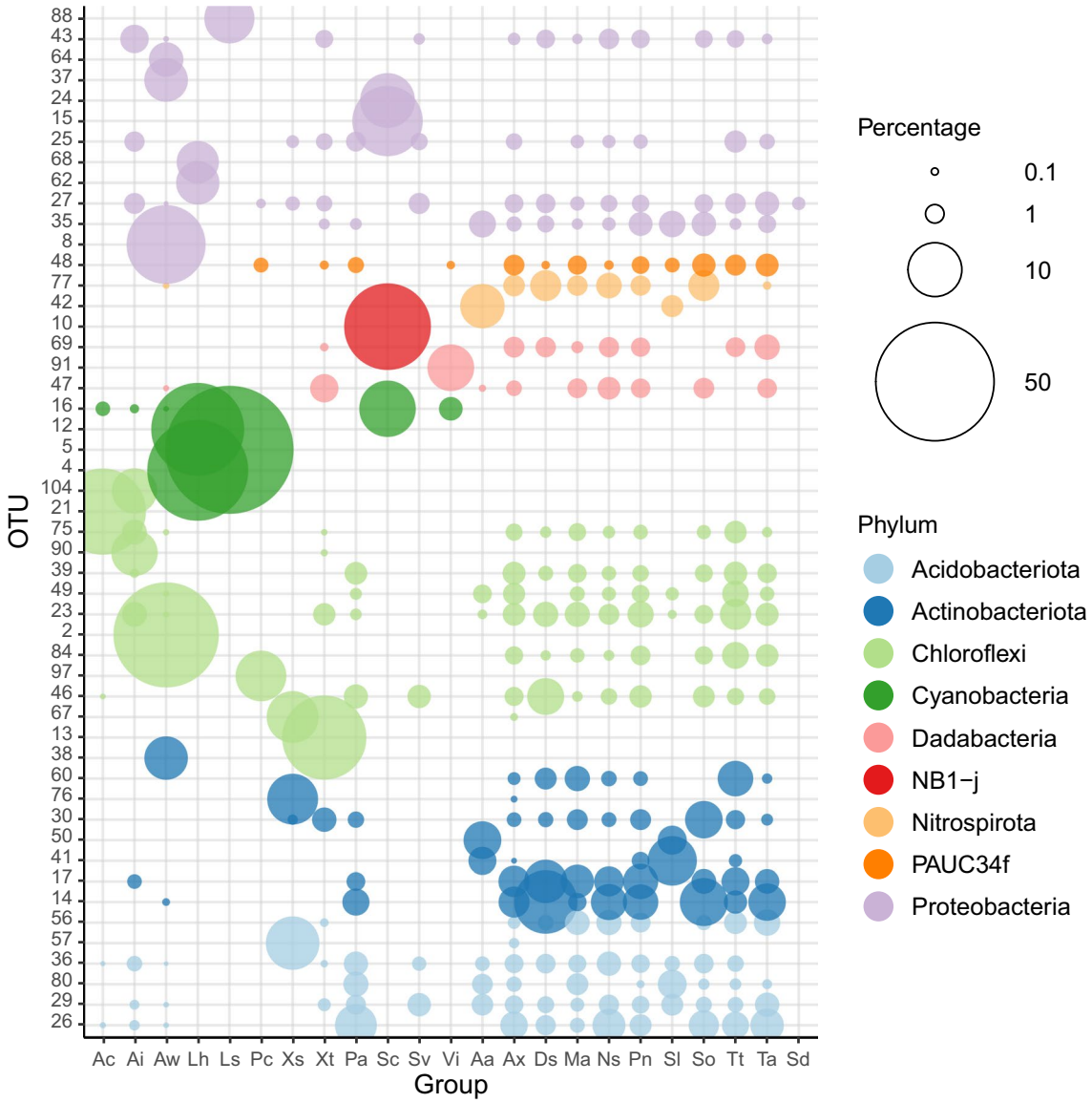

Fig. 8 Percentage abundance of the 50 most abundant OTUs represented by different colours based on prokaryotic phylum for the following groups: Ac, *Acanthostylotella cornuta*; Ai, *Astrosclera willeyana*; Aw, *Acanthochaetetes wellsi*; Lh, *Lamellodysidea herbacea*; Ls, *Lamellodysidea* sp.; Pc, *Petrosia corticata*; Xs, *Xestospongia* sp.; Xt, *Xestospongia testudinaria*; Pa, *Polymastia* sp.; Sc, *Stylissa carteri*; Sv, *Svenzea* aff. *devoogdae*; Vi, *Vetulina incrustans*; Aa, *Acicu-lites ciliata*; Ax, *Asteropus* cf. *simplex*; Ds, *Discodermia* sp.; Ma, *Manihinea* sp.; Ns, *Neophrissospongia* sp.; Pn, *Penares* sp.; Sl, *Scleritoderma* sp.; So, *Sollasipelta ornuta*; Tt, *Theonella* aff. *timmi*; Ta, *Theonella* sp.; and Sd, sediment. The size of each OTU circle is proportional to the mean percentage of sequences per group as shown by the symbol legend in the upper right corner of the figure

integral components of Upper Cambrian reefs, and alongside microbial biofilms, they contributed to the formation of columnar structures known as stromatolites (Coulson and Brand 2016). Valeria D'Auria et al. (2002) identified them as a 'spectacular source of new metabolites'. A diverse array of compounds, including alkaloids, pigments, novel sterols, cyclic and linear peptides, polyketides, and macrolides, have been isolated from these sponges. Drawing parallels between the structures of many of these compounds and those produced by microorganisms, Valeria D'Auria et al. (2002) suggested that some of these compounds may be synthesised by microbial symbionts residing within the sponges.

Lithistids can form dense aggregations in shallow-water caves where freshwater influx occurs (Pisera and Gerovasileiou 2021) and at greater depths on sponge mounds where they provide habitat to a diverse array of vertebrate and invertebrate species and contribute to nutrient cycling and bentho-pelagic exchange (Cathalot et al. 2015; Hawkes

et al. 2019; Hourigan et al. 2017; Maldonado et al. 2015, 2020; Manconi et al. 2006; Rooks et al. 2020; Xavier et al. 2021). They were previously classified in their own order, the Lithistida. The reclassification of lithistids into different orders was driven by molecular and morphological data that suggested polyphyletic origins (Morrow and Cárdenas 2015). The tetracladinid and dicranocladinid lithistids, however, remained monophyletic and branched with the choristid demosponges. Based on molecular systematics, most lithistid sponges have been placed in the Tetractinellida order, which presently includes three suborders, the Astrophorina, Spirophorina, and Thoosina (Schuster et al. 2021). Tetractinellid sponges have a subradial or radial skeletal configuration, both monactine and triaene megascleres, aster, sigma, microxea, raphide and microrhabd microscleres, and desmas are sometimes present (Morrow and Cárdenas 2015). In contrast to the tetracladinid lithistids, rhizomorinid families were identified as polyphyletic (Kelly-Borges and Pomponi 1994). The only non-tetracladinid lithistid sampled in the present study was the bright yellow *Vetulina incrustans* (Vetulinidae: Sphaerocladina), recently described from deep crevices of caves in the Philippines (Schuster et al. 2018). A single specimen of *S. ornata* was also sampled, the only representative of the monogeneric dicranocladinid family Neopeltidae. Originally found at a depth of 236 m in the Banda Sea, Indonesia (Sollas 1888), it was the only *Sollasipelta* species inhabiting both deep-sea and shallow cave/dark habitats. Recently, a new species of *Sollasipelta* was described from a submarine cave in the Ryukyu Islands in Japan (Ise et al. 2023). The authors compared their newly observed species with three other extant species, and although the location of the new species *S. subterranea* is very close to Orchid Island, our species aligns with the description of *S. ornata* and not *S. subterranea.*

Lithistid species belonging to the Tetractinellida order formed two distinct clusters in the PCO analysis with one cluster encompassing both species from the Scleritodermidae family (*Aciculites ciliata* and *Scleritoderma* sp.) while the other cluster encompassed all remaining species from the other families. The Scleritodermidae is the only tetractinellid family recorded in the present study belonging to the suborder Spirophorina. All the other tetractinellid families (Ancorinidae, Corallistidae, Geodiidae, Neopeltidae, and Theonellidae) belonged to the suborder Astrophorina. This suggests a phylogenetic signal on sponge-associated prokaryotic composition. Lithistid species belonging to the Tetractinellida order generally exhibited high evenness and richness and relatively high abundances of HMA-indicator taxa, such as Chloroflexi, Actinobacteriota, and Acidobacteriota (Moitinho-Silva et al. 2017). The microbial community of the tetractinellid cold water *Geodia barretti* (Tetractinellida, Demospongiae) was shown to be dominated by Chloroflexi (SAR202), Poribacteria, and Acidobacteria (Radax et al. 2012). Proteobacteria, particularly Gammaproteobacteria, constituted the most abundant taxon, followed by Chloroflexi, Firmicutes, and Alphaproteobacteria in the lithistid sponge *Discodermia* spp. sampled across a depth range of 24 to 161 meters in the Bahamas Archipelago (Brück et al. 2012).

The tetractinellid species shared a large number of dominant OTUs, many of which were not recorded in other sponge taxa in the present study with the exception of *Polymastia* sp. (Order: Polymastiida), which clustered intermediate to the tetractinellid species and the other sponge species. Unfortunately, our sampling for this species was limited to a single specimen. Despite their markedly distinct composition, the dominant OTUs recorded in the tetractinellids had relatively high sequence similarities to organisms in GenBank, in contrast to the sclerosponge species *A. wellsi*. Samples of the non-tetractinellid, lithistid *V. incrustans* clustered distinct from the tetractinellid, lithistids and only shared a single abundant OTU with them.

Petrosiidae

Petrosiid sponges exhibit a wide geographical distribution, thrive in a range of depths, and tolerate a wide range of water temperatures, from cold to warm (Desqueyroux-Faúndez and Valentine 2002). They typically form massive, bulbous structures, but can also exhibit branching or encrusting growth forms, possess a stony, brittle texture and a smooth surface, and may assume a vase-like shape reminiscent of a volcano crater. The family encompasses four genera and two subgenera. The *Petrosia* genus is characterized by a network of free spicules composed of oxea, exhibiting three distinct size classes within the subgenus *Petrosia* and strongyle megascleres within the subgenus *Strongylophora*. Their distribution spans the North, Central, and South Pacific, Central Atlantic, and Indian Oceans (Desqueyroux-Faúndez and Valentine 2002).

Three sampled host sponge species belonged to the family Petrosiidae including the known HMA species *X. testudinaria* as well as *Xestospongia* sp. and *P. corticata*. Previous studies have identified several species within the Petrosiidae family as HMA species (Gloeckner et al. 2014; Moitinho-Silva et al. 2017). For example, *X. testudinaria* has consistently been shown to harbour a HMA-type prokaryotic community characterised by a predominance of HMA-indicator taxa (Cleary et al. 2018, 2019a, b, 2020). Likewise, in a compositional analysis, the sponge species *Petrosia elephantotus* collected from the Red Sea clustered together with other known HMA species such as *X. testudinaria*, and had a relatively high abundance of Poribacteria compared to non-HMA species sampled from the same area (Cleary et al. 2020). In Mayotte, a species tentatively identified as *Petrosia* aff. *spheroida* clustered together with the known

HMA species *H. erectus* and *X. testudinaria* (de Voogd et al. 2019). The Mediterranean *Petrosia ficiformis* was also recognised as a HMA species (Ribes et al. 2015). Interestingly, *P. ficiformis* occurs in two distinct colour morphs. The white/pink morph was found in dark/shady environments, including cave interiors and entrances, and lacked phototrophic symbionts. A violet morph, in contrast, inhabiting illuminated habitats such as rocky cliffs, harboured a diverse assemblage of intracellular cyanobacteria. The presence of the cyanobacterium *Synechococcus feldmannii* has, furthermore, been confirmed (with the aid of TEM observation and Chlorophyll a measurements) in the pink and violet morphs, but not in the white morph (Burgsdorf et al. 2014). A 16S rRNA pyrosequencing analysis of these two different colour morphs also revealed a stable bacterial community dominated by Chloroflexi, Gammaproteobacteria, and Acidobacteria in all colour morphs. Geographical variation was also shown to be a more important structural component of variation in microbial diversity than host-genetic variability (Burgsdorf et al. 2014).

Phototrophic sponges

Phototrophic sponges were another notable component of the sponge fauna in the present study. As the name implies, they depend on photosynthetic symbionts for a substantial portion of their energy needs. Previous research has documented varying distributions of photo- and heterotrophic sponges with phototrophic sponges more abundant in the Indo-Pacific region (e.g., the Great Barrier Reef) and primarily inhabiting outer reefs, located further from the shoreline, while heterotrophic sponges are relatively more prevalent in the Caribbean (Bell et al. 2018, 2020; Erwin and Thacker 2007). Phototrophic sponges also predominantly occur in clearer, oligotrophic environments.

Cyanobacteria are among the most abundant photosynthetic organisms present in symbiotic associations with sponges in general and particularly in phototrophic sponges (Konstantinou et al. 2018). *Lamellodysidea* species, for example, have been consistently shown to host the cyanobacterial symbiont, *Hormoscilla spongeliae* (previously known as *Oscillatoria spongeliae*), and appear unable to survive without the species. This obligate relationship has also made it hitherto impossible to culture *H. spongeliae* owing to the symbiont's apparent inability to survive independently (Schorn et al. 2019; Usher 2008). *Hormoscilla spongeliae* is known to produce high amounts of several halogenated compounds and polybrominated diphenyl ethers (PBDEs), which are chemically identical to anthropogenic pollutants, but with potential antimicrobial and antipredator properties (Agarwal et al. 2017; Flatt et al. 2005). Alongside Cyanobacteria, the microbial community of *Lamellodysidea* members and more specifically *L. herbacea* also consisted of Bacteroidetes, Alphaproteobacteria, Gammaproteobacteria, and Oligoflexia members (Podell et al. 2020). Both phototrophic sponges in this study exhibited low evenness and richness. Unlike many of the other sponge species, both phototrophic sponge species were only found in the high-light intensity pool environments growing over rock surfaces. In addition to *L. herbacea*, *H. spongeliae*, recorded as *Oscillatoria spongeliae*, has also been found to be a prominent member of the microbiomes of the phototrophic sponges *Phyllospongia papyracea* and *Lendenfeldia chondrodes* (Ridley et al. 2005).

SAR202 were enriched in cave sponges

The bacterial phylum Chloroflexi exhibited the highest overall abundance and Chloroflexi members were only rare in species from the high-light intensity pools or adjacent coral reefs, namely, the phototrophic species or *S. carteri*, a known LMA sponge species. Several studies have documented a high abundance of Chloroflexi members in HMA species (Cleary et al. 2020, 2021; Cleary et al2019a; Moitinho-Silva et al. 2017; Schmitt et al. 2011; Swierts et al. 2018). Despite the scarcity or absence of Chloroflexi members in numerous LMA species, they can flourish in others. For example, we previously observed Chloroflexi members in the sponge species *Paratetilla bacca* from Mayotte, *Acanthella cavernosa* from Taiwan, and *Ectyoplasia coccinea*, *Cinachyrella* sp., and *Topsentia aqabaensis* from the Red Sea, all of which were otherwise compositionally similar to samples from known LMA species from the same locations (Cleary et al. 2019a; Cleary et al. 2020; de Voogd et al. 2019). Chloroflexi abundance was high in both the agelasid *A. cornuta* and the sclerosponge *A. wellsi*. In *A. wellsi*, this was mainly due to high levels of Dehalococcoidia and in *A. cornuta* high levels of Dehalococcoidia (mainly SAR202) and TK17. In the Silva 138 database, the SAR202 clade is an order of the Dehalococcoidia. In both sponge species, levels of Anaerolineae were very low in contrast to the known HMA species *X. testudinaria* sampled from nearby coral reefs, which had the highest Anaerolineae levels. SAR202 members have been shown to be abundant in bathypelagic waters and Anaerolinae members in a wide range of habitats varying from arctic permafrost to the mammalian gastrointestinal tract (Campbell et al. 2014; Hug et al. 2013; Varela et al. 2008). In humans, Anaerolinae members scavenge material from human tissue and lysed bacterial cells and may perform a similar function in sponges given their rapid cell turnover.

Prior research indicates that HMA sponges are specialised for the uptake of dissolved organic matter (DOM), whereas LMA sponge species are specialised for the uptake of particulate organic matter (Bayer et al. 2018; McMurray et al. 2018). Chloroflexi, in particular, are postulated to play a key role in DOM dynamics. SAR202, for

example, have been implicated in the degradation of recalcitrant or refractory DOM (Colatriano et al. 2018; Landry et al. 2017). Landry et al. (2017) identified enzymes involved in recalcitrant compound oxidation within the genomes of deep-sea Chloroflexi members, some of which were also detected in SAR202 genomes from sponge symbionts (Bayer et al. 2018). The aforementioned evidence suggests a potential role for SAR202 members in DOM degradation within sponges, particularly in environments with elevated DOM levels, such as areas close to rivers or submarine groundwater discharge (Kim and Kim 2017). The overwhelming abundance of Chloroflexi in cave-dwelling sponges including tetractinellids, other putative HMA species, and species such as *A. cornuta* and *A. wellsi* hints that DOM-rich groundwater discharge from Orchid Island may constitute the primary environmental factor shaping the cave sponge communities. While further research is needed to substantiate this hypothesis, the implications are intriguing and suggest that the sponge-filled cave systems surrounding Orchid Island and other similar areas could potentially function as DOM filters with important repercussions for nutrient dynamics across the land-sea interface.

In summary, this study delves into a poorly studied ecosystem with a number of conspicuous sponge species potentially new to science. In addition to the species in the present study, a rich calcareous sponge fauna was also encountered. While these specimens have been collected, their species-level identification remains pending. Formal descriptions of the potentially new species are forthcoming. In addition to sponges, other groups of organisms including corals and algae were observed. Surveys should also be undertaken to discover potentially similar systems in adjacent insular areas with rocky coasts. Intriguing and otherwise rare species have already been identified in cave systems in Japan and the Philippines (Schuster et al. 2018; Asami et al. 2020, 2021). Aside from the present karst cave system, these other cave systems potentially harbour a wealth of new and rare species. For instance, we collected three different specimens of the bright, yellow coloured, encrusting *V. incrustans*. This species was recently described based on a single specimen from a crevice in the Philippines (Schuster et al. 2018). Species belonging to this genus are considered relict species, representing the sole surviving members of a once diverse Mesozoic group. Previously, they were only recorded from the Caribbean region (Pisera et al., 2017). Ise et al. (2023) recently described a new lithistid species, *Sollasipelta subterranea* from an anchialine cave in the Ruykyus Islands, and an earlier report showed that many more species await description including new species from the particular cave in Japan (Ise 2019). In the Mediterranean, caves support a large part of total poriferan diversity in addition to the large number of deep-sea, relict, and living fossils that are recorded in these caves (Gerovasileiou and Voultsiadou 2012); marine cave systems, however, remain understudied in other regions of the world. With regard to the prokaryotic communities, we we observed a marked enrichment of Dehalococcoidia members (primarily SAR202) in all cave sponge species.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval No animal testing was performed during this study.

Sampling and field studies All necessary permits for sampling and observational field studies have been obtained by the authors from the competent authorities.

Data availability Sequences used in this study have been uploaded to the NCBI ShortRead Archive (BioProject nr: PRJNA865713 and BioSample nr: SUB11909922).

Author contribution DFRC collected specimens in the field, analysed the data, and wrote the manuscript. NJdeV collected specimens in the field, identified the sponge specimens, and helped to write the manuscript. MvdP performed the laboratory analyses. ARMP and NCMG helped to write the manuscript.

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