Ultrastructure, molecular phylogenetics and chlorophyll *a* content of novel cyanobacterial symbionts in temperate sponges

Patrick M. Erwin[1*], Susanna López-Legentil[2], Xavier Turon[1]

[1]*Center for Advanced Studies of Blanes (CEAB-CSIC), Accés Cala S. Francesc 14, 17300 Blanes, Girona, Spain*

[2]*Department of Animal Biology, University of Barcelona, Diagonal Avenue 643, 08028 Barcelona, Spain*

*Corresponding author:

Phone: +34 972 336101

Fax: +34 972 337806

Email: erwin.patrickm@gmail.com

Abstract

Marine sponges often harbor photosynthetic symbionts that may enhance host metabolism and ecological success, yet little is known about the factors that structure the diversity, specificity and nature of these relationships. Here, we characterized the cyanobacterial symbionts in two congeneric and sympatric host sponges that exhibit distinct habitat preferences correlated with irradiance: *Ircinia fasciculata* (higher irradiance) and *I. variabilis* (lower irradiance). Symbiont composition was similar among hosts and dominated by the sponge-specific cyanobacterium *Synechococcus spongiarum*. Phylogenetic analyses of 16S-23S rRNA internal transcribed spacer (ITS) gene sequences revealed that Mediterranean *Ircinia* spp. host a specific, novel symbiont clade ("M") within the *S. spongiarum* species-complex. A second, rare cyanobacterium related to the ascidian symbiont *Synechocystis trididemni* was observed in low abundance in *I. fasciculata* and likewise corresponded to a new symbiont clade. Symbiont communities in *I. fasciculata* exhibited nearly twice the chlorophyll *a* concentrations of *I. variabilis*. Further, *S. spongiarum* clade M symbionts in *I. fasciculata* exhibited dense intracellular aggregations of glycogen granules, a storage product of photosynthetic carbon assimilation rarely observed in *I. variabilis* symbionts. In both host sponges, *S. spongiarum* cells were observed interacting with host archeocytes, although the lower photosynthetic activity of *Cyanobacteria* in *I. variabilis* suggests less symbiont-derived nutritional benefit. The observed differences in clade M symbionts among sponge hosts suggest that ambient irradiance conditions dictate symbiont photosynthetic activity and consequently may mediate the nature of host-symbiont relationships. In addition, the plasticity exhibited by clade M symbionts may be an adaptive attribute that allows for flexibility in host-symbiont interactions across the seasonal fluctuations in light and temperature characteristic of temperate environments.

Keywords: Sponge, Cyanobacterial Symbionts, Synechococcus spongiarum, Synechocystis, Electron Microscopy, 16S-23S rRNA ITS Phylogenetics

Introduction

Invertebrate-photosymbionts associations are common in shallow water marine environments, typically involving sponge or cnidarian hosts and cyanobacterial or algal symbionts [62, 69]. The photosynthetic capacity of these symbionts and translocation of fixed carbon to host organisms can boost invertebrate metabolism and increase overall holobiont fitness. Similar to photosymbionts in scleractinian corals [3] and ascidians [23], the relationship between host sponges and their associated *Cyanobacteria* are often mutually beneficial. Indeed, some host sponges acquire supplemental nutrition from the by-products of symbiont photosynthesis [16, 70]) while cyanobacterial symbionts receive a sheltered habitat within sponge tissue (e.g., reduced grazing pressure and UV exposure) and possibly benefit from the nitrogenous end products of host (animal) metabolism. In addition to nutrient translocation, symbiotic *Cyanobacteria* may also provide a source of defensive secondary metabolites [15, 63]. Accordingly, cyanobacterial symbionts appear to contribute to the competitive ability and ecological success of host sponges and represent a key functional component of the complex sponge microbiota.

Photosymbionts are prevalent in sponge communities of coastal ecosystems worldwide [64], accounting for one-third to three-fourths of coral reef sponges in the tropical regions [11, 54, 71] and over half of sponges from temperate ecosystems [27, 42]. In general, *Cyanobacteria* are the dominant photosynthetic symbiont group in sponge hosts [11, 64], although zooxanthellae and filamentous algae are also found in association with marine sponges [7, 22]. The genetic diversity of sponge-associated *Cyanobacteria* spans multiple phylogenetic lineages and form 10 monophyletic and

sponge-specific sequence clusters related to the genera *Synechococcus*, *Synechocystis*, *Oscillatoria*, *Lyngbya* and *Cyanobacterium* [53, 55, 60].

The most commonly reported and widespread cyanobacterial symbiont is "*Candidatus Synechococcus spongiarum*" [67], a single-celled cyanobacterium that occurs in peripheral (ectosomal) regions of the sponge body in diverse hosts from tropical and temperate marine environments across the globe [20, 53, 55, 60]. *S. spongiarum* symbionts account for up to 85% of sponge-photosymbiont associations in Caribbean reefs [11] and exhibit variable functional significance to host sponges [2, 12, 16, 31, 72]. Molecular evidence from 16S-23S ribosomal RNA (rRNA) internal transcribed spacer (ITS) sequences recently revealed cryptic diversity among populations of *S. spongiarum,* with 12 distinct symbiont clades structured by both geography and host phylogeny [13]. Additional studies targeting clade-level diversity in the *S. spongiarum* species-complex may shed new light on the variability of host-symbiont interactions described for this widespread cyanobacterium.

Cyanobacterial symbionts related to the genera *Synechocystis* and *Prochloron* have also been described from marine sponges, primarily based on microscopy observations and ultrastructural morphology [9, 45]. To date, molecular characterization of *Synechocystis* symbionts in marine sponges has been conducted for hosts in the genus *Lendenfeldia* from the Indo-Pacific [41] and Western Indian Ocean [55], *Spongia* sp. and *Mycale* sp. from Western Australian [27] and *Ectyoplasia ferox* from the Caribbean [51], while a single *Prochloron*-affiliated sequence has been reported in the Japanese sponge *Halichondria okadai* (GenBank acc. no. HM100971). In fact, the best studied *Synechocystis* and *Prochloron* symbionts, *S. trididemni* and *P. didemni*, are associated

with didemnid ascidian hosts [26, 28, 30, 33]. Among sponge hosts, the specificity and ecological importance of *Synechocystis* and *Prochloron* symbionts are currently unknown and further studies are needed to understand the biodiversity of these *Cyanobacteria* and their interactions with host sponges.

In this study, we examined the diversity and activity of cyanobacterial symbionts in Mediterranean *Ircinia* spp. using electron microscopy, molecular characterization and chlorophyll *a* quantification. The host sponges *I. fasciculata* and *I. variabilis* were chosen due to previous reports of cyanobacterial symbionts in these species [8, 47, 65, 66], their close phylogenetic relationship [14] and their distinct zonation patterns within the littoral benthos of the NW Mediterranean Sea [14]. Typical of a phototrophic sponge species, *I. fasciculata* occurs preferentially in exposed and high irradiance zones, while *I. variabilis* is more common in semi-sciophilous ('shade-loving') communities of vertical walls and shaded crevices. However, distribution patterns associated with light availability can also occur in non-phototrophic sponge species [4], necessitating detailed study of putative photosymbiont communities to confirm their presence and activity in host sponges. The objective of our study was to compare the genetic diversity, ultrastructural morphology and chlorophyll *a* content of cyanobacterial symbionts in two conspecific temperate sponges. By targeting both partial 16S rRNA and entire 16S-23S ITS gene sequences, our study allowed for both comparative phylogenetic analysis and fine-scale resolution of closely related cyanobacterial symbionts.

Methods

Sample Collection

The marine sponges *Ircinia fasciculata* (PALLAS 1766) and *I. variabilis* (SCHMIDT, 1862) were collected from shallow (3 to 8 m and 8 to 12 m, respectively) littoral zones at 2 neighboring sites (< 12 km apart) along the Catalan Coast (Spain) in the northwestern Mediterranean Sea. *I. fasciculata* colonies ($n = 6$) were sampled at Punta de S'Agulla (Blanes; 41º 40' 54.87" N, 2º 49' 00.01" E) and *I. variabilis* ($n = 6$) at Mar Menuda (Tossa de Mar; 41º 43' 13.62" N, 2º 56' 26.90" E) by SCUBA in March 2010. Tissue samples were collected from sponges using a clean scalpel blade then preserved in 100% ethanol and stored at -20 ºC for genetic analyses, or processed immediately for chlorophyll *a* analysis and electron microscopy (see below).

Chlorophyll *a* Quantification

Chlorophyll *a* (chl *a*) concentrations were determined for *Ircinia fasciculata* ($n = 3$) and *I. variabilis* ($n = 3$), following Erwin & Thacker [12]. Briefly, 0.25 g of freshly collected ectosomal tissue (blotted wet weight) from each individual was separately extracted in 5 ml of 90% acetone, held overnight at 4ºC. Absorbance values of supernatant aliquots were determined at 750, 664, 647 and 630 nm and chl *a* concentrations were calculated using the equations of Parsons et al. [36], standardized by sponge mass extracted. Chl *a* concentrations were compared between host sponge species with a Student's *t*-test using the software SigmaPlot (version 11).

Transmission Electron Microscopy

To visualize the diversity and ultrastructure of cyanobacterial symbionts in *I.*

fasciculata and *I. variabilis*, transmission electron microscopy (TEM) observations were conducted on small ectosome tissue pieces (ca. 4 mm^3), following the methods of Erwin et al. [14]. Briefly, tissue pieces were fixed and incubated (overnight at 4ºC) in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde (buffered with filtered seawater) then rinsed and stored in filtered seawater. Following dehydration in a graded ethanol series, samples were embedded in Spurr resin (room temperature), sliced into ultra-thin sections (ca. 60 nm) and contrasted with uranyl acetate and lead citrate for ultrastructural observation [40]. TEM observations were performed at the Microscopy Unit of the Scientific and Technical Services of the University of Barcelona on a JEOL JEM-1010 (Tokyo, Japan) coupled with a Bioscan 972 camera (Gatan, Germany).

Cell dimensions were measured by digital image analysis with ImageJ software (version 1.43) [37]. To avoid underestimating cell size, only cells that exhibited a clear cell center and peripheral thylakoids were measured. For *S. spongiarum* symbionts, a total of 65 and 44 cells were measured in *Ircinia fasciculata* and *I. variabilis*, respectively. For *Synechocystis* sp. symbionts, a total of 7 cells were recovered and measured in *I. fasciculata*. Two measurements were recorded for each cell: the maximum cell diameter (hereafter, 'length') and the cell diameter perpendicular to the maximum (hereafter, 'width'). Cell dimensions were compared between host sponge species with a Student's *t*-test using the software SigmaPlot (version 11).

DNA Extraction and PCR Amplification

Metagenomic DNA extracts were prepared from samples of sponge tissue (ectosome and choanosome) from *I. fasciculata* ($n = 3$) and *I. variabilis* ($n = 3$) using the DNeasy® Blood & Tissue Kit (Qiagen®), following the manufacturer's animal tissue

protocol. Diluted DNA extracts (1:10) were used as templates in PCR amplification with the universal cyanobacterial forward primer 359F [35] and reverse primer 23S1R [24] to amplify a cyanobacterial rRNA gene fragment corresponding to the 3' end of the 16S region (1140 to 1142 bp), the entire 16S-23S ITS region (258 to 443 bp) and the 5' end of the 23S region (25 bp). Total PCR reaction volume was 50 µl, including 10 pmol of each primer, 10 nmol of each dNTP, 1X Reaction Buffer (Ecogen) and 5 units of BIOTAQ™ polymerase (Ecogen). Thermocycler reaction conditions were an initial denaturing time of 2 min at 94ºC, followed by 30 cycles of 1 min at 94ºC, 0.5 min at 50ºC, and 1.5 min at 72ºC, and a final extension time of 2 min at 72ºC. To minimize PCR amplification biases, a low annealing temperature and low cycle number were used and 3 separate reactions were conducted for each sample. PCR amplification products were gel-purified and cleaned using the QIAquick Gel Extraction Kit (Qiagen®), then triplicate PCR products were combined and quantified using a Qubit™ fluorometer and Quant-iT™ dsDNA Assay Kit (Invitrogen™).

Clone Library Construction and Sequencing Analysis

Purified PCR products (ca. 75 ng) were ligated into plasmids using the pGEM®-T Vector System (Promega). Individual clones were PCR-screened using vector primers and clones with ca. 1,650 bp inserts were purified and sequenced at Macrogen, Inc. Bi-directional sequencing with vector primers provided two overlapping sequence reads per clone and allowed the retrieval of the entire cloned amplicons. Raw sequence data were processed in Geneious [10] by aligning high quality forward and reverse reads to yield a final consensus sequence for each clone. Quality-checked sequences are archived in GenBank under accession nos. JQ410235 to JQ410319. Consensus sequences were

subject to nucleotide-nucleotide BLAST searches [1] to recover closely related sequences in the GenBank database and pairwise genetic distance (uncorrected p-distance) among sequences and rarefaction analysis by individual sponge host were conducted using the software package mothur [50].

Phylogenetic Analyses

Phylogenetic reconstructions were based on different regions of the recovered rRNA gene fragments for *Synechococcus* and *Synechocystis*, due to the resolution required for each symbiont phylogeny and the availability of reference sequences in the GenBank database. For *S. spongiarum* clones, phylogenies were constructed using 16S-23S rRNA ITS sequences, since 16S rRNA genes sequences do not exhibit sufficient variability for clade-level resolution of *S. spongiarum* and ITS sequences from *S. spongiarum* are available for comparative analyses [12, 13]. For *Synechocystis* symbionts, phylogenies were constructed using 16S rRNA gene sequences, 16S rRNA gene sequences were sufficient to resolve a novel clade of *Synechocystis* symbionts in *Ircinia fasciculata* and few ITS sequences from *Synechocystis* spp. are available for comparative analyses.

Consensus 16S-23S rRNA ITS sequences from *Synechococcus spongiarum* clones recovered herein were compared with 12 previously described *S. spongiarum* clades (A to L) [13] to determine the sub-specific clade affiliations of *S. spongiarum* symbionts in *I. fasciculata* and *I. variabilis*. A single consensus sequence was used for identical clones (100% identity) from the same individual. Unique sequences from *I. fasciculata* ($n = 20$) and *I. variabilis* ($n = 26$), representative sequences from *S. spongiarum* clades A to L ($n = 39$) and congeneric outgroup sequences from cultures ($n =$

4) and environmental sources (n = 3) were aligned using MAFFT [25]. Maximum likelihood phylogenies were constructed in PHYML [19] with the Hasegawa-Kishino-Yano model of nucleotide substitution and a gamma distribution of variable substitution rates among sites (HKY+G), as suggested by FINDMODEL; data were resampled using 100 bootstrap replicates. Bayesian inference was used to calculate posterior probabilities of branch nodes in MrBayes [43] implemented with the HKY+G model. Markov Chain Monte Carlo Markov (MCMC) analysis was performed with 4 chains (temp = 0.2) and run for 2,000,000 generations, with a sampling frequency of 400 generations (burn-in value = 1,250). After 1,957,000 generations, the average standard deviation of split frequencies among chains reached less than 0.01.

Consensus partial 16S rRNA gene sequences from *Ircinia*-derived clones related to the genera *Synechocystis* and *Prochloron* were compared with previously published sequences from top GenBank matches (n = 64) and outgroup sequences from related cyanobacterial genera (n = 17) to determine the phylogenetic affiliation of *Synechocystis*-like symbionts in *I. fasciculata* and *I. variabilis*. Sequence alignment and phylogenetic analyses were conducted as described above for *Synechococcus spongiarum* clones. During MCMC analysis, the average standard deviation of split frequencies among chains reach less than 0.01 after 1,278,000 generation cycles. Additional sponge-derived sequences related to *Synechocystis* have been reported from *Spongia* sp. (GenBank acc. nos. EU383035 and EU383036), *Mycale* sp. (EU383038) and *Ectyoplasia ferox* (EF159744); however, these partial sequences were excluded because their short length (<600 bp) precluded accurate phylogenetic placement, destabilized phylogenetic reconstructions and obscured relationships among the remaining sequences in the dataset.

Results

Chlorophyll *a* Concentrations

Chlorophyll *a* (chl *a*) concentrations in *I. fasciculata* ranged from 205.6 to 300.4 μg/g, averaging 248.1 ±27.8 μg/g (±SE). In *I. variabilis*, chl *a* concentrations ranged from 113.5 to 140.0 μg/g and averaged 131.0 ±15.1 μg/g. Differences in chl *a* concentrations between the two sponge species were significant (t = -4.022, df = 4, P < 0.05), with *I. fasciculata* averaging nearly twice the level of chl *a* of *I. variabilis* (89% increase).

Morphology, Abundance and Activity of Cyanobacterial Symbionts

Dense populations of cyanobacterial cells were observed in the ectosome of *Ircinia fasciculata* and *I. variabilis* (Figs. 1 and 2) and corresponded to two distinct symbiont cell morphologies. The dominant symbiont cells represent the sponge-specific symbiont, "*Candidatus* Synechococcus spongiarum" [67], diagnosed by the characteristic spiral thylakoids that occur in the cell perimeter surrounding a finely granulated cell center (Fig. 1). Few central cytoplasmic inclusions were identifiable in these cells. In both host sponges, *S. spongiarum* cells occurred in intercellular mesohyl areas and were actively reproducing via cell elongation, central constriction (yielding a figure 8 shape) and separation into two daughter cells. *S. spongiarum* cells also appeared to interact with host cells, often seen surrounding and interfacing with sponge archeocytes (Fig. 1). Symbiont cells were occasionally engulfed by host archeocytes, although no clear evidence of symbiont consumption (phagocytosis) was observed (Fig. S1).

Comparing *S. spongiarum* symbiont populations between the two host sponge species revealed two differentiating factors: cell size and glycogen abundance. Symbiont cells in *I. variabilis* were significantly larger than resident cells in *I. fasciculata* (Table1), in terms of both cell length ($t = 5.590$, df =107, $P < 0.001$) and cell width ($t = 9.467$, df = 107, $P < 0.001$). On average, *I. variabilis* symbionts were 18.8% larger than conspecific populations in *I. fasciculata*, although *S. spongiarum* cells exhibited overlapping values in cell length (1.35 to 2.78 μm in *I. fasciculata*, 1.72 to 3.03 μm in *I. variabilis*) and width (1.17 to 1.91 μm in *I. fasciculata*, 1.45 to 2.14 μm in *I. variabilis*) and were within previously reported cell size ranges from different host species (Table 1). A more consistent difference among symbiont populations occurred in the abundance of glycogen granules (fine black dots, 20 to 35 nm in diameter) between the lamellae of the thylakoids in *S. spongiarum* cells [46]. In *I. fasciculata*, symbionts exhibited a high abundance of glycogen granules and similar granules were observed in host cells interfacing with symbiont cells (Fig. 1f). In *I. variabilis*, glycogen granules were also present in *S. spongiarum* cells and neighboring sponge cells, though in much lower abundance (Fig. 1e).

A second morphotype of symbiotic *Cyanobacteria* was observed in *I. fasciculata* and occurred rarely ($n = 7$) within the host tissue (Fig. 2). Cell shape was spherical and cell size was over 3 times larger than *S. spongiarum* cells (Fig. 2a), averaging 7.11 ±1.36 μm in length and 7.11 ±1.43 μm in width. Parallel thylakoids occurred around the cell periphery and multiple cytoplasmic inclusions were observed in the cell center, including carboxysomes and polyphosphate bodies (Fig. 2b). These characteristics are diagnostic of *Cyanobacteria* in the genus *Synechocystis* and matched previous descriptions of sponge

symbionts [47, 66]. Unlike *S. spongiarum*, *Synechocystis* symbionts were not observed in reproductive processes and no close contact with sponge cells occurred, due to separation of symbionts from the sponge mesohyl by a lacunar space (0.5 to 2 μm) surrounding each *Synechocystis* cell (Fig. 2c).

Genetic Diversity of Cyanobacterial Symbionts

Consistent with electron microscopy observations, clone libraries revealed the presence of two distinct cyanobacterial symbionts in *I. fasciculata* and *I. variabilis* hosts. Rarefaction analysis revealed sufficient sampling to reach saturation in all host sponge individuals examined (Fig. S2). Analysis of the 16S rRNA gene regions (1140 to 1142 bp) revealed that the majority of clones from *I. fasciculata* (n =34, 85%) and all clones from *I. variabilis* (n = 45, 100%) corresponded to the sponge-specific cyanobacterium "*Candidatus* Synechococcus spongiarum" (99% sequence identity) [67]. The remaining clones (n = 6 from a single *I. fasciculata* individual) corresponded to the genus *Synechocystis*, matching most closely (> 97%) to uncultured *Synechocystis* symbionts from marine sponges and ascidians, including the cyanobacterium *S. trididemni*. In addition, *Synechocystis* clones matched nearly identically (> 99%) to symbiont clones derived from another dictyoceratid sponge, *Spongia* sp. (GenBank acc. nos. EU383035 and EU 383036); however, these partial 16S rRNA gene sequences were short (< 430 bp), precluding their inclusion in subsequent phylogenetic analyses.

Analysis of the 16S-23S rRNA ITS gene sequences confirmed the identification of *S. spongiarum*, matching closest (93.8%) to *S. spongiarum* (clade H) from the host sponge *Chondrilla nucula* (GenBank acc no. EU307451), and a *Synechocystsis*-like symbiont, matching closest (96.0%) to a *Synechocystis* symbiont in the ascidian host

Tridemnum solidum (GenBank acc. no. JF506243). ITS sequences from *S. spongiarum* symbionts (442 to 443 bp) contained 2 transfer RNA (tRNA) genes encoding tRNA-Ile (74 bp) and tRNA-Ala (73 bp). ITS sequences from *Synechocystis*-like symbionts were shorter (258 bp) and contained a single tRNA (tRNA-Ile), consistent with genome data from the congeneric, free-living cyanobacterium, *Synechocystis* sp. PCC6308 [57].

Phylogenetic analysis of 16S-23S rRNA ITS sequences recovered from *I. fasciculata* and *I. variabilis* revealed a novel clade of *Synechococcus spongiarum* distinct from all previously described symbiont clades (A to L; Fig. 3). This new symbiont clade, here labeled clade "M", exhibited reciprocal monophyly and greater than 3% sequence divergence (average = 9.6%, range = 6.2–21.5%) from sister clades, thus satisfying the precedent criteria for defining a new clade of *S. spongiarum* [13]. Within clade M, sequence divergence values were low (average = 0.41%, range = 0–1.3%) and no consistent genetic differentiation by host species was observed, as clones from *I. fasciculata* and *I. variabilis* formed a mixed cluster (Fig. 3).

Phylogenetic analysis of partial 16S rRNA gene sequences recovered from *I. fasciculata* revealed a novel symbiont clade within the *Synechocystis* evolutionary lineage (Fig. 4). Four robust and distinct *Synechocystis* symbiont clades were resolved: a sponge symbiont clade specific to the host species *I. fasciculata*, 2 closely related sponge symbionts clades specific to the host genus *Lendenfeldia*, and an ascidian symbiont clade corresponding to *Synechocystis tridemni* (Fig. 4). Additional sequences derived from sponges ($n = 2$) and ascidians ($n = 2$) were positioned within the *Synechocystis* lineage, although their relationships with other *Synechocystis* clades were unresolved. *Synechocystis* sequences clustered as a sister lineage to *Prochloron* sequences and

together formed a well-supported monophyletic clade comprised solely of symbiont-derived sequences (Fig. 4). *Prochloron* sequences were closely related to ascidian symbiont, *Prochloron didemni*, and recovered almost exclusively from ascidian hosts, with the exception of one sponge-derived (*Halichondria okadai*) and one coral-derived (*Muricea elongata*) sequence (Fig. 4).

Discussion

Symbiotic *Cyanobacteria* in the temperate sponge hosts, *Ircinia fasciculata* and *I. variabilis*, were shown to exhibit similar species composition yet different levels of photosynthetic pigments (chl *a*) and storage products (glycogen granules) that correlated with the irradiance conditions of preferred host habitats. In both hosts, symbiont communities were dominated by a novel clade ("M") of the unicellular cyanobacterium, *Synechococcus spongiarum*. A second single-celled cyanobacterium, *Synechocystis* sp., was also observed in *I. fasciculata*, though rarely (7 total cells) and sporadically (1 of the 3 host individuals). Symbiont communities associated with the photophilic host *I. fasciculata* exhibited nearly twice the chl *a* concentrations of *I. variabilis* and abundant accumulation of glycogen granules, a polysaccharide storage product of photosynthetic carbon assimilation. Notably, similar (putatively glycogen) granules were also observed in host cells interfacing with active symbionts in *I. fasciculata*, indicating the potential transfer of surplus carbon stores to the host sponge. These results suggest that ambient irradiance conditions play a role in dictating the photosynthetic activity of sponge-associated *Cyanobacteria*, and possibly mediate the nature of host-symbiont interactions among different host sponge species.

The phylogenetic signature of cyanobacterial symbionts in temperate *Ircinia* spp. was quite different from congeneric species in the Caribbean, which lack *Synechocystis* symbionts and host distinct *S. spongiarum* clades [13]. The fine-scale phylogenetic resolution afforded by 16S-23S rRNA ITS sequence data has important implications in host-specificity, as the interpretation of symbiont specificity varies with molecular marker resolution. For example, based on 16S rRNA gene sequences, clade M symbionts recovered from Mediterranean *Ircinia* spp. herein matched nearly identically (99.1–99.5% sequence identity) to 16S rRNA gene sequences from clade J symbionts described in Caribbean *Ircinia* hosts. In contrast, 16S-23S rRNA ITS gene sequences showed that clade M symbionts were clearly differentiated from clade J symbionts based on sequence similarity (90.2–91.1% identity) and phylogenetic analysis (distinct monophyletic clades), revealing cryptic biogeographic trends in symbiont structure among *Ircinia* hosts.

The biogeographic distribution of *S. spongiarum* suggests that unique clades inhabit hosts from different regions. In addition to the Mediterranean clade M symbionts described herein, distinct *S. spongiarum* clades have also been reported in the Indo-Pacific (Palau, clade F) and eastern Atlantic (Canary Islands, clade E) [13]. In fact, the majority of clades described to date are specific to a single geographic region (Fig. 5). However, the clade diversity within a region is strongly correlated with the number of host species surveyed (Fig. 5), indicating that additional sampling is required to fully elucidate the diversity and distribution of *S. spongiarum* clades. Indeed, even in the well-studied coral-zooxanthellae symbioses, the sampling of new hosts and environments continues to reveal novel subclades and expand the distribution of known clades of *Symbiodinium* symbionts [6].

Clade-level differentiation of *S. spongiarum* symbionts is a recently described phenomenon [13] and whether this cryptic genetic diversity relates to differences in symbiont functioning and host benefit is currently unknown. The presence of a single, shared *S. spongiarum* clade in *Ircinia* spp. provides new insight into clade-level symbiont physiology through the comparative analyses of cellular ultrastructure and photosynthetic pigment concentrations in clade M symbionts from hosts in high (*I. fasciculata*) and low (*I. variabilis*) irradiance habitats. Clade M symbionts were smaller in *I. fasciculata* compared to *I. variabilis*, suggesting morphological plasticity in cell size in response to the ambient irradiance levels. *I. fasciculata* exhibited greater chl *a* concentrations compared to *I. variabilis* and dense aggregations of glycogen granules in symbiont cells, indicators of higher photosynthetic activity. Glycogen accumulation is consistent with high photosynthetic output, representing a key storage polysaccharide for fixed carbon in *Cyanobacteria* [34, 58]. Together, these data suggest flexibility among populations of clade M symbionts and acclimation to environmental irradiance gradients, rather than expulsion and compositional shifts as reported for coral-zooxanthellae symbioses [44].

Previous investigations of sponges hosting *S. spongiarum* have reported high variability in the functional role of symbiotic *Cyanobacteria* and dependence of host sponges on photosymbiont communities. Among some host sponges, symbiont loss has little effect on host growth rates [12, 61, 72], secondary metabolite production [18], stress response [31] and host mortality [32]. In contrast, other host species exhibit decreased growth rates [12], metabolic collapse [2] and mass mortality of local host populations [17] in response to the reduction or loss of *S. spongiarum* symbionts. Similar data and experiments are unavailable for *Ircinia* hosts; however, symbiont loss due to temperature

extremes has been suggested to contribute to mass mortality events of *Ircinia fasciculata* in the Mediterranean [8]. Further, the sponge *Petrosia ficiformis* hosts a related, facultative cyanobacterium, *Synechococcus feldmanni* [67], that occurs in hosts from light-exposed habitats yet is absent in conspecific sponges from dark caves [48]. The plasticity of this unique sponge-cyanobacteria symbiosis allows for comparative analyses of symbiotic and aposymbiotic *P. ficiformis* individuals and has yielded insight into the genetic regulation and metabolic implications of host-symbiont interactions [48, 56].

Notably, our results also indicate high photosynthetic capacity in some temperate sponge-cyanobacteria symbioses, as chl *a* levels in *I. fasciculata* were consistent with values reported for tropical *Ircinia* spp. [11] and similar glycogen accumulation has been observed in symbionts of the tropical sponge *Chondrilla nucula* [46]. In contrast, low symbiont activity in *I. variabilis* hosts may indicate less dependence on symbiont photosynthetic output. Alternatively, symbiont populations may be actively regulated by *I. variabilis* to avoid specialist predators attracted to cyanobacteria-rich sponges [5] and reduce the oxidative stress of reactive oxygen species produced by symbiont photosynthesis [38, 39]. Additional studies are required to resolve specific host-symbiont interactions, with emphasis on fine-scale symbiont characterization, as well as, more refined metrics of host-symbiont metabolic interactions [16, 59].

Synechocystis symbionts formed a monophyletic clade specific to the Mediterranean host *I. fasciculata* and distinct from related sponge and ascidian-associated symbionts. The morphology of these symbionts matched previous descriptions of symbionts characterized as *Aphanocapsa raspaigellae* in *I. variabilis* [47, 66]. Reclassification of this cyanobacterium to the genus *Synechocystis* was suggested by

previous authors [26, 64] and is supported by the phylogenetic analyses herein. *Synechocystis* symbionts formed a monophyletic cyanobacterial lineage and exhibited a close phylogenetic relationship with *Prochloron* symbionts, consistent with previous molecular phylogenies [52] and the ultrastructural similarity of these symbiont genera [9, 21].

The ecological significance of *Synechocystis* symbionts in marine sponges remains unclear, as these photosymbionts exhibit variable incidence and abundance among different populations of *Ircinia* host sponges. In the current study, *Synechocystis* symbionts in *I. fasciculata* occurred rarely and sporadically among the sponge individuals studied and were observed to have limited interactions with host cells. Previous investigations of *Ircinia* spp. from different regions of the Mediterranean have reported more abundant *Synechocystis* populations in host sponges from Bari, Italy [47] and Marseille, France [66], suggested biogeographic variability in these photosymbiont communities. Consistent among these previous reports and the present study is the physical separation of *Synechocystis* from host sponge tissue by a lacunar space surrounding each symbiont cell. The lack of direct contact with sponge tissue may limit host-symbiont interactions, although cellular secretion from intact *Synechocystis* symbionts and leakage from disintegrating cells has been observed [47]. Further, the occurrence of *Synechocystis* symbionts as secondary to dominant cyanobacterial populations in *Ircinia* spp. [47, 66, this study] and *Lendenfeldia* spp. hosts [41] suggests that these symbionts may be opportunistic and exploiting host habitats receptive to photosymbionts (e.g., distributed in high irradiance zones, tolerant of oxidative stress) while providing minimal ecological benefit. Additional study is clearly required to test

such hypotheses as well as assess potential contributions to host ecology beyond symbiont-derived photosynthates (e.g., secondary metabolite production).

Environmental irradiance gradients represent an important factor in structuring invertebrate-photosymbiont associations and may dictate the nature of host-symbiont interactions. In the symbiosis between cnidarian hosts and zooxanthellae, irradiance exposure and intensity have been shown to influence the density, physiology and composition of photosymbiont communities [3, 49]. Similarly, the cyanobacterial symbionts studied herein exhibited physiological and morphological differences in related host sponges from different light environments, including photosynthetic pigment content (chl *a* concentrations), fixed carbon accumulation (glycogen granules), and symbiont cell size. In contrast to cnidarian symbionts, whose cladal composition can shift across small-scale irradiance gradients [44], sponge photosymbiont communities were dominated by clade M symbionts in both host sponges regardless of irradiance conditions, suggesting that less dynamic and more versatile host-symbiont interactions occur in *Ircinia-Synechococcus* associations. Thus, the temperate clade M of *S. spongiarum* described herein appears to represent a flexible symbiont able to survive in sponge hosts under different environmental conditions, a potential hallmark of symbiotic *Cyanobacteria* occurring in temperate ecosystems that must tolerate large seasonal fluctuations in light and temperature. Future research on temperate sponge-cyanobacteria interactions targeting the fine-scale characterization of symbiont communities and temporal monitoring of host-symbiont interactions are required to test such hypotheses and determine the contributions of these symbiotic systems to host ecology and microbial biodiversity.

Acknowledgements

We thank Dr. M. Uriz (CEAB) for help with sponge identification and F. Crespo (CEAB) for field assistance. This research was supported by the Spanish Government projects CTM2010-17755 and CTM2010-22218, the Catalan Government grant 2009SGR-484 for Consolidated Research Groups, and by the US National Science Foundation under grant 0853089.

References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403-410.
2. Arillo A, Bavestrello G, Burlando B, Sarà M (1993) Metabolic integration between symbiotic cyanobacteria and sponges: a possible mechanism. Mar Biol 117: 159-162.
3. Baker AC (2003) Flexibility and specificity in coral-algal symbioses: diversity, ecology and biogeography of *Symbiodinium*. Ann Rev Ecol Evol Syst 34: 661-689.
4. Bannister RJ, Hoogenboom MO, Anthony KRN, Battershill CN, Whalan S, et al. (2011) Incongruence between the distribution of a common coral reef sponge and photosynthesis. Mar Ecol Prog Ser 423: 95-100.
5. Becerro MA, Turon X, Uriz MJ, Templado J (2003) Can a sponge feeder be a herbivore? *Tylodina perversa* (Gastropoda) feeding on *Aplysina aerophoba* (Demospongiae) Biol J Linn Soc 78: 429-438.
6. Bongaerts P, Sampayo EM, Bridge TCL, Ridgway T, Vermeulen F, et al. (2011) *Symbiodinium* diversity in mesophotic coral communities on the Great Barrier Reef: a first assessment. Mar Ecol Prog Ser 439: 117-126.
7. Carballo JL, Ávila E (2004) Population dynamics of a mutualistic interaction between the sponge *Haliclona caerulea* and the red alga *Jania adherens*. Mar Ecol Prog Ser 279: 93-104.
8. Cebrian E, Uriz MJ, Garrabou J, Ballesteros E (2011) Sponge mas mortalities in a warming Mediterranean Sea: are cyanobacteria-harboring species worse off? *PLoS ONE* 6: e20211.
9. Cox GC, Hiller RG, Larkum AWD (1985) An unusual cyanophyte, containing phycourobilin and symbiotic with ascidians and sponges. Mar Biol 89: 149-163.
10. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, et al. (2010) Geneious v5.3. Available from http://www.geneious.com.controlled public data. Nucleic Acids Res. 35: D169-D172.
11. Erwin PM, Thacker RW (2007) Incidence and identity of photosynthetic symbionts in Caribbean coral reef sponge assemblages. J Mar Biol Ass UK 87: 1683-1692.
12. Erwin PM, Thacker RW (2008a) Phototrophic nutrition and symbiont diversity of two Caribbean sponge-cyanobacteria symbioses. Mar Ecol Prog Ser 362: 139-147.
13. Erwin PM, Thacker RW (2008b) Cryptic diversity of the symbiotic cyanobacterium *Synechococcus spongiarum* among sponge hosts. Mol Ecol 17: 2937-2947.
14. Erwin PM, López-Legentil S, Gonzalez-Pech R, Turon X (In press) A specific mix of generalists: Bacterial symbionts in Mediterranean *Ircinia* spp. FEMS Microbiology Ecology DOI 10.1111/j.1574-6941.2011.01243.x
15. Flatt PM, Gautschi JT, Thacker RW, Musafija-Girt M, Crews P, Gerwick WH (2005) Identification of the cellular site of polychlorinated peptide biosynthesis in the marine sponge *Dysidea* (*Lamellodysidea*) *herbacea* and symbiotic cyanobacterium *Oscillatoria spongeliae* by CARD-FISH analysis. Mar Biol 147: 761-774.

16. Freeman CJ, Thacker RW (2011) Complex interactions between marine sponges and their symbiotic microbial communities. Limnol Oceanogr 56: 1577-1586.
17. Fromont J, Garson M (1999) Sponge bleaching on the West and East coasts of Australia. Coral Reefs 18: 340.
18. Gómez R, Erpenbeck D, van Dijk T, Richelle-Maurer E, Devijver C, Braekman JC, Woldringh C, van Soest RWM (2002) Identity of cyanobacterial symbiont of *Xestospongia muta*. Boll Mus Ist Biol Univ Genova 66-67: 82-83.
19. Guidon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696-704.
20. Hentschel U, Usher KM, Taylor MW (2006) Marine sponges as microbial fermenters. FEMS Microbiol Ecol 55: 167-177.
21. Hernández-Mariné M, Turon X, Catalan J (1990) A marine *Synechocystis* (Chroococcales, Cyanophyta) epizoic on didemnid ascidians from the Medterranean Sea. Phycologia 29: 275-284.
22. Hill M, Allenby A, Ramsby B, Schönberg C, Hill A (2011) *Symbiodinium* diversity among host clionaid sponges from Caribbean and Pacific reefs: evidence of heteroplasmy and putative host-specific symbiont lineages. Mol Phylogenet Evol 59: 81-88.
23. Hirose E, Maruyama T (2004) What are the benefits in the ascidian-*Prochloron* symbiosis? Endocytobiosis Cell Res 15: 51-62.
24. Iteman I, Rippka R, Tandeau de Marsac N, Herdman M (2000) Comparison of conserved structural and regulatory domains within divergent 16S rRNA–23S rRNA spacer sequences of cyanobacteria. Microbiology 146: 1275-1286.
25. Katoh K, Misawa K, Kum K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30: 3059-3066.
26. Lafargue F, Duclaux G (1979) Premier exemple, en Atlantique tropical, d'une association symbiotique entre une ascidie Didemnidae et une Cyanophycée Chroococcale: *Tridemnum cyanophorum* nov. sp. et *Synechocystis tridemni* nov. sp. Annls. Inst. Océanogr. Paris 55: 163-184.
27. Lemloh ML, Fromont J, Brümmer F, Usher KM (2009) Diversity and abundance of photosynthetic sponges in temperate Western Australia. BMC Ecology 9: 4.
28. Lewin RA (1975) A marine *Synechocystis* (Cyanophyta, Chroococcales) epizoic on ascidians. Phycologia 14: 153-160.
29. López-Legentil S, Erwin PM, Pawlik JR, Song B (2010) Effects of sponge bleaching on ammonia oxidizing Archaea: Distribution and relative expression of ammonia monooxygenase genes associated with the barrel sponge *Xestospongia muta*. Microb Ecol 60(3): 561-571.
30. López-Legentil S, Song B, Bosch M, Pawlik JR, Turon X (2011) Cyanobacterial diversity and a new *Acaryochloris*-like symbiont from Bahamian sea-squirts. PLoS ONE 6: e23938.
31. López-Legentil S, Song B, McMurray SE, Pawlik JR (2008) Bleaching and stress in coral reef ecosystems: *hsp*70 expression by the giant barrel sponge *Xestospongia muta*. Mol Ecol 17: 1840-1849.

32. McMurray SE, Blum JE, Leichter JJ, Pawlik JR (2011) Bleaching of the giant barrel sponge *Xestospongia muta* in the Florida Keys. Limnol Oceanogr 56: 2243-2250.
33. Münchhoff J, Hirose E, Maruyama T, Sunairi M, Burns BP, et al. (2007) Host specificity and phylogeography of the prochlorophyte *Prochloron* sp., an obligate symbiont in didemnid ascidians. Environ Microbiol 9: 890-899.
34. Nakamura Y, Takahashi J, Sakurai A, Inaba Y, Suzuki E, Nihei S, Fujiwara S, Tsuzuki M, Miyashita H, Ikemoto H, Kawachi M, Sekiguchi H, Kurano H (2005) Some cyanobacteria synthesize semi-amylopectin type α-polyglucans instead of glycogen. Plant Cell Physiol 46: 539-545.
35. Nübel U, Garcia-Pichel F, Muyzer G (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl Environ Microbiol 63: 3327-3332.
36. Parsons TR, Maita Y, Lalli CM (1984) A manual of chemical and biological methods for seawater analysis. Pergamon Press, New York.
37. Rasband WS (2011) ImageJ. US National Institute of Health, Bethesda, Maryland, USA. http://imagej.nih.gov/ij/. Accessed 27 Apr 2010.
38. Regoli F, Cerrano C, Chierici E, Bompadre S, Bavestrello G (2000) Susceptibility to oxidative stress of the Mediterranean demosponge *Petrosia ficiformis*: role of endosymbionts and solar irradiance. Mar Biol 137: 453-461.
39. Regoli F, Cerrano C, Chierici E, Chiantore MC, Bavestrello G (2004) Seasonal variability of prooxidant pressure and antioxidant adaptation to symbiosis in the Mediterranean demosponge *Petrosia ficiformis*. Mar Ecol Prog Ser 275: 129-137.
40. Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208-212.
41. Ridley CP, Faulkner DJ, Haygood MG (2005) Investigation of *Oscillatoria spongeliae*-dominated bacterial communities in four dictyoceratid sponges. Appl Environ Microbiol 71: 7366-7375.
42. Roberts DE, Cummins SP, Davis AR, Pangway C (1999) Evidence for symbiotic algae in sponges from temperate coastal reefs in New South Wales Australia. Mem Queensl Mus 44: 493-497.
43. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.
44. Rowan R, Knowlton N, Baker A, Jara J (1997) Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. Nature 388: 265-269.
45. Rützler K (1981) An unusual bluegreen alga symbiotic with two new species of *Ulosa* (*Porifera: Hymeniacidonidae*) from Carrie Bow Cay, Belize. Mar Ecol 2: 35-50.
46. Rützler K (1990) Associations between Caribbean sponges and photosynthetic organisms. In *New perspectives in sponge biology* (ed. K. Rützler), pp. 455-466. Washington DC: Smithsonian Institute Press.
47. Sarà M (1971) Ultrastructural aspects of the symbiosis between two species of the genus *Aphanocapsa* (Cyanophyceae) and *Ircinia variabilis* (Demospongiae). Mar Biol 11: 214-221.

48. Sarà M, Bavestrello G, Cattaneo-Vietti R, Cerrano C (1998) Endosymbiosis in sponges: Relevance for epigenesis and evolution. Symbiosis 25: 57-70.
49. Saunders BK, Muller-Parker G (1997) The effects of temperature and light on two algal populations in the temperate sea anemone *Anthopleura elegantissim* (Brandt, 1835). J Exp Mar Biol Ecol 211: 213-224.
50. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537-7541.
51. Schmitt S, Angermeier H, Schiller R, Lindqust N, Hentschel U (2008) Molecular microbial diversity of sponge reproductive stages and mechanistic insight into vertical transmission of microbial symbionts. Appl Environ Microbiol 74: 7694-7708.
52. Shimada A, Yano N, Kanai S, Lewin RA, Maruyama T (2003) Molecular phylogenetic relationship between two symbiotic photo-oxygenic prokaryotes, *Prochloron* sp. and *Synechocystis trididemni*. Phycologia 42: 193-197.
53. Simister RL, Deines P, Botté ES, Webster NS, Taylor MW (2012) Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. Environ Microbiol 14: 517-524.
54. Steindler L, Beer S, Ilan M (2002) Photosymbiosis in intertidal and subtidal tropical sponges. Symbiosis 33: 263-273.
55. Steindler L, Huchon D, Avni A, Ilan M (2005) 16S rRNA phylogeny of sponge-associated cyanobacteria. Appl Environ Microbiol 71: 4127-4131.
56. Steindler L, Schuster S, Ilan M, Avni A, Cerrano C, Beer S (2007) Differential gene expression in a marine sponge in relation to its symbiotic state. Mar Biotech 9: 543-549.
57. Stewart FJ, Cavanaugh CM (2007) Intragenomic variation and evolution of the internal transcribed spacer of the rRNA operon in bacteria. J Mol Evol 65: 44-67.
58. Suzuki E, Ohkawa H, Moriya K, Matsubara T, Nagaike Y, Iwasaki I, Fujiwara S, Tsuzuki M, Nakamura Y (2010) Carbohydrate metabolism in mutants of the cyanobacterium *Synechococcus elongatus* PCC 7942 defective in glycogen synthesis. Appl Environ Microbiol 76: 3153-3159.
59. Tanaka Y, Miyajima T, Koike I, Hayashibara T, Ogawa H (2006) Translocation and conservation of organic nitrogen within the coral-zooxanthella symbiotic system of *Acropora pulchra*, as demonstrated by dual isotope-labeling techniques. J Exp Mar Biol Ecol 336: 110-119.
60. Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev 71: 295-347.
61. Thacker RW (2005) Impacts of shading on sponge-cyanobacteria symbioses: a comparison between host-specific and generalist associations. Integr Comp Biol 45: 369-376.
62. Thacker RW, Freeman CR (*in press*) Sponge-microbe symbioses: recent advances and new directions. Adv Mar Biol

63. Unson MD, Holland ND, Faulkner DJ (1994) A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. Mar Biol 119: 1-11.
64. Usher KM (2008) The ecology and phylogeny of cyanobacterial symbionts in sponges. Mar Ecol 29: 178-192.
65. Usher KM, Fromont J, Sutton DC, Toze S (2004a) The biogeography and phylogeny of unicellular cyanobacterial symbionts in sponges from Australia and the Mediterranean. Microb Ecol 48: 167-177.
66. Usher KM, Kuo J, Fromont J, Toze S, Sutton DC (2006) Comparative morphology of five species of symbiotic and non-symbiotic coccoid cyanobacteria. Eur J Phycol 41: 179-188.
67. Usher KM, Toze S, Fromont J, Kuo J, Sutton DC (2004b) A new species of cyanobacterial symbiont from the marine sponge *Chondrilla nucula*. Symbiosis 36: 183-192.
68. Vacelet J, Donadey C (1977) Electron microscope study of the association between some sponges and bacteria. J Exp Mar Biol Ecol 30: 301-314.
69. Venn AA, Loram JE, Douglas AE (2008) Photosynthetic symbionts in animals. J Exp Bot 59: 1069-1080.
70. Wilkinson CR (1979) Nutrient translocation from symbiotic cyanobacteria to coral reef sponges. In: Léci C, Boury-Esnault N (eds), *Biologie de Spongiarires*, Vol. 291. Colloques Internationaux du Centre National Recherche Scientifique, Paris, pp. 373-380.
71. Wilkinson CR (1987) Interocean differences in size and nutrition of coral reef sponges populations. Science 236: 1654-1657.
72. Wilkinson CR, Vacelet J (1979) Transplantation of marine sponges to different conditions of light and current. J Exp Mar Biol Ecol 37: 91-104.

FIGURE LEGENDS

Figure 1. Electron micrographs of *Synechococcus spongiarum* symbionts in the host sponges *Ircinia fasciculata* (*left panel*) and *I. variabilis* (*right panel*). **a, b** Dense aggregations of intercellular *S. spongiarum* cells in host sponge tissue, often undergoing cell division (*white arrowheads*) and occurring among sponge fibers (*f*). **c, d** Sponge amoebocytes (*am*) interacting with *S. spongiarum* cells (*s*). **e, f** Individual *S. spongiarum* cells exhibiting the characteristic spiral thylakoid membrane (*t*) with glycogen granules (*g*) very abundant in *S. spongiarum* cells from *I. fasciculata* and nearby host cells. **g, h** Reproducing *S. spongiarum* cells in host sponge mesohyl. Scale bars equal 5 μm (**a-d**) and 1 μm (**e-h**).

Figure 2. Electron micrographs of *Synechocystis* sp. symbionts in the host sponge *Ircinia fasciculata*. **a** *Synechocystis* sp. cell (*black arrowheads*) size compared to the co-occurring cyanobacterium *Synechococcus spongiarum* cells (*white arrowheads*) **b** *Synechocystis* sp. cell with thylakoid membranes (*t*) occurring on the periphery of the cell and cytoplasmic inclusions resembling carboxysomes (*c*) and polyphosphate bodies (*pb*), surrounded by a lacunar space (*la*) **c** Close-up view of the peripheral thylakoid membranes (*t*) and lacunar space between the cyanobacterial cells and sponge mesohyl cells. Scale bars denote 5 μm (**a**), 2 μm (**b**) and 1 μm (**c**).

Figure 3. Phylogeny of cyanobacterial 16S-23S rRNA ITS gene sequences from the sponge-specific symbiont *Synechococcus spongiarum* highlighting a new sub-specific clade ("M") from *Ircinia fasciculata* and *I. variabilis*. Terminal node labels denote the host species of each sequence, followed by the number of clones (in parenthesis) and the sponge individual for sequences from this study (bold) or the GenBank accession nos. for representative clones from the 12 previously described clades. Tree topology was constructed using maximum likelihood (ML) inference. Bootstrap support values for ML analyses (upper) and posterior probabilities (PP) for Bayesian inference (lower) are shown on internal nodes, with double asterisks (**) indicating bootstrap values < 50% or PP < 0.50. Bold values indicate support for distinct symbiont clades (gray boxes), with clade labels shown on the right (dark bars). Outgroup sequences include three environmental and four cultured *Synechococcus* sequences. Scale bar represents 0.04 substitutions per site.

Figure 4. Phylogeny of cyanobacterial 16S rRNA gene sequences from sponge and ascidian-associated symbionts in the genera *Synechocystis* and *Prochloron*. Terminal nodes denote the host species of each sequence and the sponge individual for sequences from this study (bold) or the GenBank accession nos. for related sequences. Black diamonds highlight sponge-derived sequences; the star highlights a coral-derived sequence. Tree topology was constructed using maximum likelihood (ML) inference. Bootstrap support values for ML analysis (upper) and posterior probabilities (PP) for Bayesian inference (lower) are shown on internal nodes, with double asterisks (**) indicating bootstrap values < 50% or PP < 0.50. Bold values indicate support for distinct symbiont clades (gray boxes), with clade labels shown on the right (dark bars);

values less than 70% for both ML and PP analyses are not shown. Scale bar represents 0.01 substitutions per site.

Figure 5. Linear regression of the clade-level diversity in the sponge-associated cyanobacterium, *S. spongiarum*, and the number of host species studied for 5 geographic regions.

Table 1. Cellular dimensions of the sponge-associated cyanobacterium *Synechococcus spongiarum* from multiple sponge hosts (n.a. = sample sizes not available).

Region	Host Sponges	Number of Cells Measured	Length in µm (range or average ±SD)	Width in µm (range or average ±SD)	Citation
Mediterranean	*Petrosia ficiformis*	n.a.	4 – 5	1.5 – 2	[68]
Mediterranean	*Ircinia variabilis*	n.a.	2 – 3	n.a.	[47]
	Multiple species[1]	75	1.52 ±0.17	1.24 ±0.13	[66]
	Ircinia variabilis	44	2.18 ±0.31	1.81 ±0.16	This study
	Ircinia fasciculata	65	1.85 ±0.29	1.51 ±0.16	This study
Caribbean	Multiple species[2]	n.a.	1.1 – 2.0	0.6 – 1.0	[46]
Australia	*Chondrilla nucula*	13	0.95 ±0.2	0.68 ±0.15	[67]
Med & Australia	Multiple species[3]	207	0.96 ±0.2	0.70 ±0.15	[66]

[1] *I. variabilis* and *P. ficiformis*; cyanobacterium described by authors as *Aphanocapsa feldmannii*

[2] *Geodia* (2 spp.), *Spheciospongia* (1), *Chondrilla* (1), *Ircinia* (2), *Aplysina* (4), *Verongula* (3), *Cribochalina* (2), *Xestospongia* (3) and *Neofibularia* (1)

[3] *Chondrilla nucula*, *C. australiensis* and *I. variabilis*

FIGURES

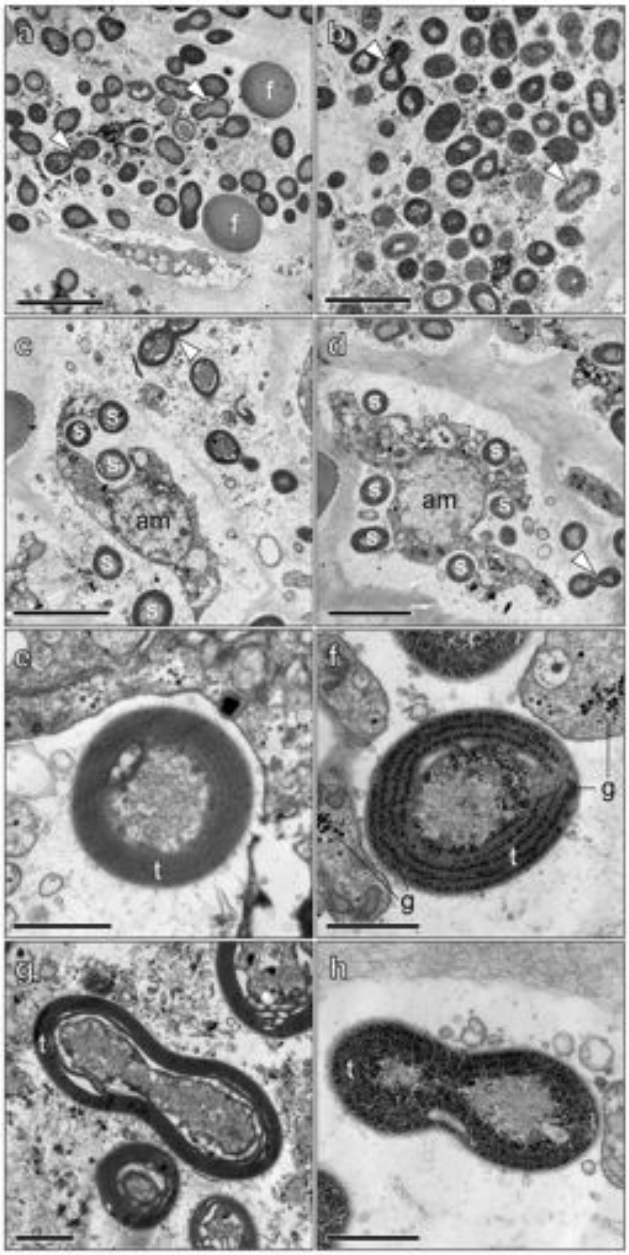

FIG. 1

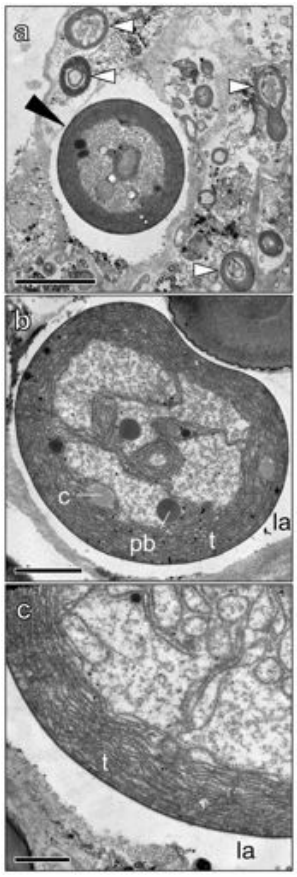

FIG. 2


Clade M

Ircinia fasciculata
Ircinia variabilis

Mediterranea Sea
Synechococcus spongiarum
Clade A
Clade D
Clade E
Clade F
Clade B
Clade J
Clade G
Clade C
Clade K
Clade H
Clade I
Clade L
0.04

FIG. 3

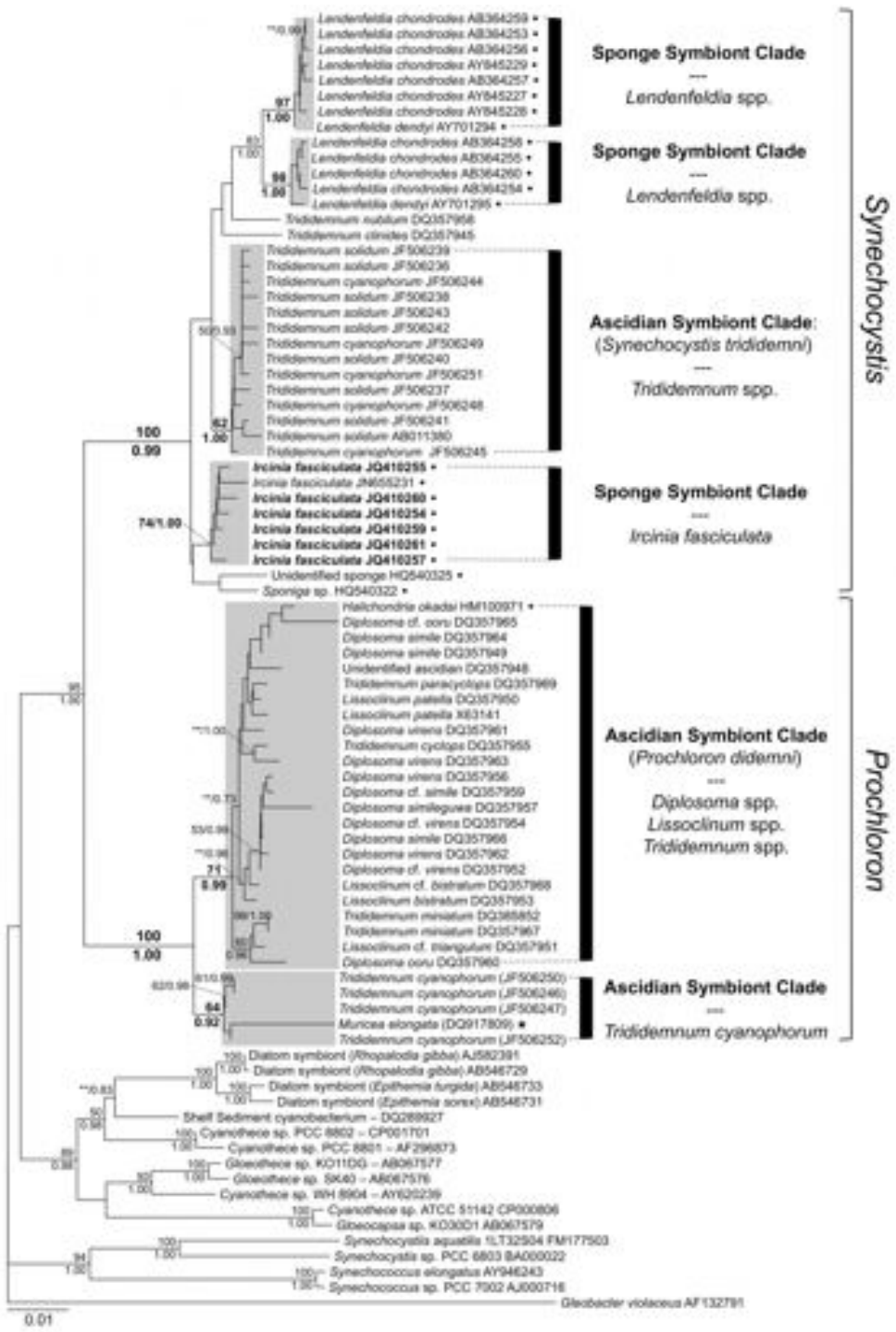

FIG. 4

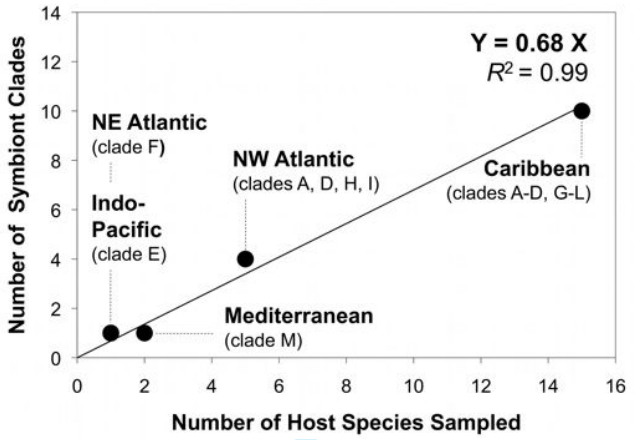

FIG. 5