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4 1 **Ultrastructure, molecular phylogenetics and**
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6 2 **chlorophyll a content of novel cyanobacterial**
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9 3 **symbionts in temperate sponges**
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17 Abstract

18 Marine sponges often harbor photosynthetic symbionts that may enhance host metabolism and ecological
19 success, yet little is known about the factors that structure the diversity, specificity and nature of these
20 relationships. Here, we characterized the cyanobacterial symbionts in two congeneric and sympatric host
21 sponges that exhibit distinct habitat preferences correlated with irradiance: *Ircinia fasciculata* (higher
22 irradiance) and *I. variabilis* (lower irradiance). Symbiont composition was similar among hosts and
23 dominated by the sponge-specific cyanobacterium *Synechococcus spongiarum*. Phylogenetic analyses of
24 16S-23S rRNA internal transcribed spacer (ITS) gene sequences revealed that Mediterranean *Ircinia* spp.
25 host a specific, novel symbiont clade (“M”) within the *S. spongiarum* species-complex. A second, rare
26 cyanobacterium related to the ascidian symbiont *Synechocystis trididemni* was observed in low abundance
27 in *I. fasciculata* and likewise corresponded to a new symbiont clade. Symbiont communities in *I.*
28 *fasciculata* exhibited nearly twice the chlorophyll *a* concentrations of *I. variabilis*. Further, *S. spongiarum*
29 clade M symbionts in *I. fasciculata* exhibited dense intracellular aggregations of glycogen granules, a
30 storage product of photosynthetic carbon assimilation rarely observed in *I. variabilis* symbionts. In both
31 host sponges, *S. spongiarum* cells were observed interacting with host archeocytes, although the lower
32 photosynthetic activity of *Cyanobacteria* in *I. variabilis* suggests less symbiont-derived nutritional benefit.
33 The observed differences in clade M symbionts among sponge hosts suggest that ambient irradiance
34 conditions dictate symbiont photosynthetic activity and consequently may mediate the nature of host-
35 symbiont relationships. In addition, the plasticity exhibited by clade M symbionts may be an adaptive
36 attribute that allows for flexibility in host-symbiont interactions across the seasonal fluctuations in light and
37 temperature characteristic of temperate environments.

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

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41 Introduction

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

56 Photosymbionts are prevalent in sponge communities of coastal ecosystems
57 worldwide [64], accounting for one-third to three-fourths of coral reef sponges in the
58 tropical regions [11, 54, 71] and over half of sponges from temperate ecosystems [27,
59 42]. In general, *Cyanobacteria* are the dominant photosynthetic symbiont group in
60 sponge hosts [11, 64], although zooxanthellae and filamentous algae are also found in
61 association with marine sponges [7, 22]. The genetic diversity of sponge-associated
62 *Cyanobacteria* spans multiple phylogenetic lineages and form 10 monophyletic and

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

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

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

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3 86 | with didemnid ascidian hosts [26, 28, 30, 33]. Among sponge hosts, the specificity and
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6 87 | ecological importance of *Synechocystis* and *Prochloron* symbionts are currently unknown
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8 88 | and further studies are needed to understand the biodiversity of these *Cyanobacteria* and
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10 89 | their interactions with host sponges.

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13 90 | In this study, we examined the diversity and activity of cyanobacterial symbionts
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15 91 | in Mediterranean *Ircinia* spp. using electron microscopy, molecular characterization and
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17 92 | chlorophyll *a* quantification. The host sponges *I. fasciculata* and *I. variabilis* were chosen
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19 93 | due to previous reports of cyanobacterial symbionts in these species [8, 47, 65, 66], their
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21 94 | close phylogenetic relationship [14] and their distinct zonation patterns within the littoral
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23 95 | benthos of the NW Mediterranean Sea [14]. Typical of a phototrophic sponge species, *I.*
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25 96 | *fasciculata* occurs preferentially in exposed and high irradiance zones, while *I. variabilis*
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27 97 | is more common in semi-sciophilous ('shade-loving') communities of vertical walls and
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29 98 | shaded crevices. However, distribution patterns associated with light availability can also
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31 99 | occur in non-phototrophic sponge species [4], necessitating detailed study of putative
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33 100 | photosymbiont communities to confirm their presence and activity in host sponges. The
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35 101 | objective of our study was to compare the genetic diversity, ultrastructural morphology
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37 102 | and chlorophyll *a* content of cyanobacterial symbionts in two conspecific temperate
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39 103 | sponges. By targeting both partial 16S rRNA and entire 16S-23S ITS gene sequences, our
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41 104 | study allowed for both comparative phylogenetic analysis and fine-scale resolution of
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43 105 | closely related cyanobacterial symbionts.
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106 **Methods**

107 **Sample Collection**

108 The marine sponges *Ircinia fasciculata* (PALLAS 1766) and *I. variabilis*
109 | (SCHMIDT, 1862) were collected from shallow (3 to 8 m and 8 to 12 m, respectively)
110 | littoral zones at 2 neighboring sites (< 12 km apart) along the Catalan Coast (Spain) in the
111 | northwestern Mediterranean Sea. *I. fasciculata* colonies (*n* = 6) were sampled at Punta de
112 | S'Agulla (Blanes; 41° 40' 54.87" N, 2° 49' 00.01" E) and *I. variabilis* (*n* = 6) at Mar
113 | Menuda (Tossa de Mar; 41° 43' 13.62" N, 2° 56' 26.90" E) by SCUBA in March 2010.
114 | Tissue samples were collected from sponges using a clean scalpel blade then preserved in
115 | 100% ethanol and stored at -20 °C for genetic analyses, or processed immediately for
116 | chlorophyll *a* analysis and electron microscopy (see below).

117 **Chlorophyll *a* Quantification**

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

126 **Transmission Electron Microscopy**

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

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3 128 *fasciculata* and *I. variabilis*, transmission electron microscopy (TEM) observations were
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5 129 conducted on small ectosome tissue pieces (ca. 4 mm³), following the methods of Erwin
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8 130 et al. [14]. Briefly, tissue pieces were fixed and incubated (overnight at 4°C) in a solution
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11 131 of 2.5% glutaraldehyde and 2% paraformaldehyde (buffered with filtered seawater) then
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13 132 rinsed and stored in filtered seawater. Following dehydration in a graded ethanol series,
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15 133 samples were embedded in Spurr resin (room temperature), sliced into ultra-thin sections
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17 134 (ca. 60 nm) and contrasted with uranyl acetate and lead citrate for ultrastructural
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20 135 observation [40]. TEM observations were performed at the Microscopy Unit of the
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22 136 Scientific and Technical Services of the University of Barcelona on a JEOL JEM-1010
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24 137 (Tokyo, Japan) coupled with a Bioscan 972 camera (Gatan, Germany).

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27 138 Cell dimensions were measured by digital image analysis with ImageJ software
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29 139 (version 1.43) [37]. To avoid underestimating cell size, only cells that exhibited a clear
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32 140 cell center and peripheral thylakoids were measured. For *S. spongiarum* symbionts, a
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34 141 total of 65 and 44 cells were measured in *Ircinia fasciculata* and *I. variabilis*,
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36 142 respectively. For *Synechocystis* sp. symbionts, a total of 7 cells were recovered and
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38 143 measured in *I. fasciculata*. Two measurements were recorded for each cell: the maximum
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40 144 cell diameter (hereafter, ‘length’) and the cell diameter perpendicular to the maximum
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42 145 (hereafter, ‘width’). Cell dimensions were compared between host sponge species with a
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44 146 Student’s *t*-test using the software SigmaPlot (version 11).

47 48 49 147 **DNA Extraction and PCR Amplification**

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52 148 Metagenomic DNA extracts were prepared from samples of sponge tissue
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54 149 (ectosome and choanosome) from *I. fasciculata* ($n = 3$) and *I. variabilis* ($n = 3$) using the
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56 150 DNeasy® Blood & Tissue Kit (Qiagen®), following the manufacturer’s animal tissue
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4 151 protocol. Diluted DNA extracts (1:10) were used as templates in PCR amplification with
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6 152 the universal cyanobacterial forward primer 359F [35] and reverse primer 23S1R [24] to
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8 153 amplify a cyanobacterial rRNA gene fragment corresponding to the 3' end of the 16S
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10 154 region (1140 to 1142 bp), the entire 16S-23S ITS region (258 to 443 bp) and the 5' end of
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12 155 the 23S region (25 bp). Total PCR reaction volume was 50 μ l, including 10 pmol of each
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14 156 primer, 10 nmol of each dNTP, 1X Reaction Buffer (Ecogen) and 5 units of BIOTAQ™
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16 157 polymerase (Ecogen). Thermocycler reaction conditions were an initial denaturing time
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18 158 of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 0.5 min at 50°C, and 1.5 min at
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20 159 72°C, and a final extension time of 2 min at 72°C. To minimize PCR amplification biases,
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22 160 a low annealing temperature and low cycle number were used and 3 separate reactions
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24 161 were conducted for each sample. PCR amplification products were gel-purified and
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26 162 cleaned using the QIAquick Gel Extraction Kit (Qiagen®), then triplicate PCR products
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28 163 were combined and quantified using a Qubit™ fluorometer and Quant-iT™ dsDNA
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30 164 Assay Kit (Invitrogen™).

37 165 **Clone Library Construction and Sequencing Analysis**

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40 166 Purified PCR products (ca. 75 ng) were ligated into plasmids using the pGEM®-T
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42 167 Vector System (Promega). Individual clones were PCR-screened using vector primers
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44 168 and clones with ca. 1,650 bp inserts were purified and sequenced at Macrogen, Inc. Bi-
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46 169 directional sequencing with vector primers provided two overlapping sequence reads per
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48 170 clone and allowed the retrieval of the entire cloned amplicons. Raw sequence data were
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50 171 processed in Geneious [10] by aligning high quality forward and reverse reads to yield a
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52 172 final consensus sequence for each clone. Quality-checked sequences are archived in
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54 173 GenBank under accession nos. JQ410235 to JQ410319. Consensus sequences were
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3 174 subject to nucleotide-nucleotide BLAST searches [1] to recover closely related sequences
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5 175 in the GenBank database and pairwise genetic distance (uncorrected p-distance) among
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8 176 sequences and rarefaction analysis by individual sponge host were conducted using the
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11 177 software package mothur [50].
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13 178 **Phylogenetic Analyses**

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16 179 Phylogenetic reconstructions were based on different regions of the recovered
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18 180 rRNA gene fragments for *Synechococcus* and *Synechocystis*, due to the resolution
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20 181 required for each symbiont phylogeny and the availability of reference sequences in the
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22 182 GenBank database. For *S. spongiarum* clones, phylogenies were constructed using 16S-
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24 183 23S rRNA ITS sequences, since 16S rRNA genes sequences do not exhibit sufficient
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26 184 variability for clade-level resolution of *S. spongiarum* and ITS sequences from *S.*
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28 185 *spongiarum* are available for comparative analyses [12, 13]. For *Synechocystis*
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30 186 symbionts, phylogenies were constructed using 16S rRNA gene sequences, 16S rRNA
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32 187 gene sequences were sufficient to resolve a novel clade of *Synechocystis* symbionts in
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34 188 *Ircinia fasciculata* and few ITS sequences from *Synechocystis* spp. are available for
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36 189 comparative analyses.
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42 190 Consensus 16S-23S rRNA ITS sequences from *Synechococcus spongiarum*
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44 191 clones recovered herein were compared with 12 previously described *S. spongiarum*
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46 192 clades (A to L) [13] to determine the sub-specific clade affiliations of *S. spongiarum*
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48 193 symbionts in *I. fasciculata* and *I. variabilis*. A single consensus sequence was used for
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50 194 identical clones (100% identity) from the same individual. Unique sequences from *I.*
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52 195 *fasciculata* ($n = 20$) and *I. variabilis* ($n = 26$), representative sequences from *S.*
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54 196 *spongiarum* clades A to L ($n = 39$) and congeneric outgroup sequences from cultures ($n =$
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3 197 | 4) and environmental sources ($n = 3$) were aligned using MAFFT [25]. Maximum
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5 198 | likelihood phylogenies were constructed in PHYML [19] with the Hasegawa-Kishino-
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8 199 | Yano model of nucleotide substitution and a gamma distribution of variable substitution
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10 200 | rates among sites (HKY+G), as suggested by FINDMODEL; data were resampled using
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12 201 | 100 bootstrap replicates. Bayesian inference was used to calculate posterior probabilities
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14 202 | of branch nodes in MrBayes [43] implemented with the HKY+G model. Markov Chain
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16 203 | Monte Carlo Markov (MCMC) analysis was performed with 4 chains (temp = 0.2) and
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18 204 | run for 2,000,000 generations, with a sampling frequency of 400 generations (burn-in
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20 205 | value = 1,250). After 1,957,000 generations, the average standard deviation of split
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27 207 | Consensus partial 16S rRNA gene sequences from *Ircinia*-derived clones related
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29 208 | to the genera *Synechocystis* and *Prochloron* were compared with previously published
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31 209 | sequences from top GenBank matches ($n = 64$) and outgroup sequences from related
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33 210 | cyanobacterial genera ($n = 17$) to determine the phylogenetic affiliation of *Synechocystis*-
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35 211 | like symbionts in *I. fasciculata* and *I. variabilis*. Sequence alignment and phylogenetic
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37 212 | analyses were conducted as described above for *Synechococcus spongiarum* clones.
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39 213 | During MCMC analysis, the average standard deviation of split frequencies among
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41 214 | chains reach less than 0.01 after 1,278,000 generation cycles. Additional sponge-derived
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43 215 | sequences related to *Synechocystis* have been reported from *Spongia* sp. (GenBank acc.
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45 216 | nos. EU383035 and EU383036), *Mycale* sp. (EU383038) and *Ectyoplasia ferox*
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47 217 | (EF159744); however, these partial sequences were excluded because their short length
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49 218 | (<600 bp) precluded accurate phylogenetic placement, destabilized phylogenetic
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51 219 | reconstructions and obscured relationships among the remaining sequences in the dataset.
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220 Results

221 Chlorophyll *a* Concentrations

222 Chlorophyll *a* (chl *a*) concentrations in *I. fasciculata* ranged from 205.6 to 300.4
223 $\mu\text{g/g}$, averaging $248.1 \pm 27.8 \mu\text{g/g}$ ($\pm\text{SE}$). In *I. variabilis*, chl *a* concentrations ranged
224 from 113.5 to 140.0 $\mu\text{g/g}$ and averaged $131.0 \pm 15.1 \mu\text{g/g}$. Differences in chl *a*
225 concentrations between the two sponge species were significant ($t = -4.022$, $df = 4$, $P <$
226 0.05), with *I. fasciculata* averaging nearly twice the level of chl *a* of *I. variabilis* (89%
227 increase).

228 Morphology, Abundance and Activity of Cyanobacterial Symbionts

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

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4 241 Comparing *S. spongiarum* symbiont populations between the two host sponge
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6 242 species revealed two differentiating factors: cell size and glycogen abundance. Symbiont
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8 243 cells in *I. variabilis* were significantly larger than resident cells in *I. fasciculata* (Table1),
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10 244 in terms of both cell length ($t = 5.590$, $df = 107$, $P < 0.001$) and cell width ($t = 9.467$, $df =$
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12 245 107 , $P < 0.001$). On average, *I. variabilis* symbionts were 18.8% larger than conspecific
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14 246 populations in *I. fasciculata*, although *S. spongiarum* cells exhibited overlapping values
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16 247 in cell length (1.35 to 2.78 μm in *I. fasciculata*, 1.72 to 3.03 μm in *I. variabilis*) and
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18 248 width (1.17 to 1.91 μm in *I. fasciculata*, 1.45 to 2.14 μm in *I. variabilis*) and were within
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20 249 previously reported cell size ranges from different host species (Table 1). A more
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22 250 consistent difference among symbiont populations occurred in the abundance of glycogen
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24 251 granules (fine black dots, 20 to 35 nm in diameter) between the lamellae of the thylakoids
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26 252 in *S. spongiarum* cells [46]. In *I. fasciculata*, symbionts exhibited a high abundance of
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28 253 glycogen granules and similar granules were observed in host cells interfacing with
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30 254 symbiont cells (Fig. 1f). In *I. variabilis*, glycogen granules were also present in *S.*
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32 255 *spongiarum* cells and neighboring sponge cells, though in much lower abundance (Fig.
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41 257 A second morphotype of symbiotic *Cyanobacteria* was observed in *I. fasciculata*
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43 258 and occurred rarely ($n = 7$) within the host tissue (Fig. 2). Cell shape was spherical and
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45 259 cell size was over 3 times larger than *S. spongiarum* cells (Fig. 2a), averaging 7.11 ± 1.36
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47 260 μm in length and $7.11 \pm 1.43 \mu\text{m}$ in width. Parallel thylakoids occurred around the cell
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49 261 periphery and multiple cytoplasmic inclusions were observed in the cell center, including
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51 262 carboxysomes and polyphosphate bodies (Fig. 2b). These characteristics are diagnostic of
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53 263 *Cyanobacteria* in the genus *Synechocystis* and matched previous descriptions of sponge
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3 264 | symbionts [47, 66]. Unlike *S. spongiarum*, *Synechocystis* symbionts were not observed in
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6 265 | reproductive processes and no close contact with sponge cells occurred, due to separation
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8 266 | of symbionts from the sponge mesohyl by a lacunar space (0.5 to 2 μm) surrounding each
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10 267 | *Synechocystis* cell (Fig. 2c).

14 268 | **Genetic Diversity of Cyanobacterial Symbionts**

16 269 | Consistent with electron microscopy observations, clone libraries revealed the
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18 270 | presence of two distinct cyanobacterial symbionts in *I. fasciculata* and *I. variabilis* hosts.
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21 271 | Rarefaction analysis revealed sufficient sampling to reach saturation in all host sponge
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23 272 | individuals examined (Fig. S2). Analysis of the 16S rRNA gene regions (1140 to 1142
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25 273 | bp) revealed that the majority of clones from *I. fasciculata* ($n = 34$, 85%) and all clones
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28 274 | from *I. variabilis* ($n = 45$, 100%) corresponded to the sponge-specific cyanobacterium
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30 275 | “*Candidatus Synechococcus spongiarum*” (99% sequence identity) [67]. The remaining
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32 276 | clones ($n = 6$ from a single *I. fasciculata* individual) corresponded to the genus
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35 277 | *Synechocystis*, matching most closely (> 97%) to uncultured *Synechocystis* symbionts
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37 278 | from marine sponges and ascidians, including the cyanobacterium *S. trididemni*. In
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39 279 | addition, *Synechocystis* clones matched nearly identically (> 99%) to symbiont clones
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41 280 | derived from another dictyoceratid sponge, *Spongia* sp. (GenBank acc. nos. EU383035
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43 281 | and EU 383036); however, these partial 16S rRNA gene sequences were short (< 430
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45 282 | bp), precluding their inclusion in subsequent phylogenetic analyses.

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49 283 | Analysis of the 16S-23S rRNA ITS gene sequences confirmed the identification
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51 284 | of *S. spongiarum*, matching closest (93.8%) to *S. spongiarum* (clade H) from the host
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53 285 | sponge *Chondrilla nucula* (GenBank acc no. EU307451), and a *Synechocystis*-like
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55 286 | symbiont, matching closest (96.0%) to a *Synechocystis* symbiont in the ascidian host
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3 287 *Trididemnum solidum* (GenBank acc. no. JF506243). ITS sequences from *S. spongiarum*
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5 288 symbionts (442 to 443 bp) contained 2 transfer RNA (tRNA) genes encoding tRNA-Ile
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8 289 (74 bp) and tRNA-Ala (73 bp). ITS sequences from *Synechocystis*-like symbionts were
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10 290 shorter (258 bp) and contained a single tRNA (tRNA-Ile), consistent with genome data
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12 291 | from the congeneric, free-living cyanobacterium, *Synechocystis* sp. PCC6308 [57].
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15 292 Phylogenetic analysis of 16S-23S rRNA ITS sequences recovered from *I.*
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17 293 *fasciculata* and *I. variabilis* revealed a novel clade of *Synechococcus spongiarum* distinct
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19 294 from all previously described symbiont clades (A to L; Fig. 3). This new symbiont clade,
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21 295 here labeled clade “M”, exhibited reciprocal monophyly and greater than 3% sequence
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23 296 divergence (average = 9.6%, range = 6.2–21.5%) from sister clades, thus satisfying the
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25 297 | precedent criteria for defining a new clade of *S. spongiarum* [13]. Within clade M,
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27 298 sequence divergence values were low (average = 0.41%, range = 0–1.3%) and no
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29 299 consistent genetic differentiation by host species was observed, as clones from *I.*
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31 300 *fasciculata* and *I. variabilis* formed a mixed cluster (Fig. 3).
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34 301 Phylogenetic analysis of partial 16S rRNA gene sequences recovered from *I.*
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36 302 *fasciculata* revealed a novel symbiont clade within the *Synechocystis* evolutionary
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38 303 lineage (Fig. 4). Four robust and distinct *Synechocystis* symbiont clades were resolved: a
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40 304 sponge symbiont clade specific to the host species *I. fasciculata*, 2 closely related sponge
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42 305 symbionts clades specific to the host genus *Lendenfeldia*, and an ascidian symbiont clade
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44 306 corresponding to *Synechocystis trididemni* (Fig. 4). Additional sequences derived from
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46 307 sponges ($n = 2$) and ascidians ($n = 2$) were positioned within the *Synechocystis* lineage,
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48 308 although their relationships with other *Synechocystis* clades were unresolved.
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50 309 *Synechocystis* sequences clustered as a sister lineage to *Prochloron* sequences and
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3 310 together formed a well-supported monophyletic clade comprised solely of symbiont-
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5 311 derived sequences (Fig. 4). *Prochloron* sequences were closely related to ascidian
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8 312 symbiont, *Prochloron didemni*, and recovered almost exclusively from ascidian hosts,
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10 313 with the exception of one sponge-derived (*Halichondria okadai*) and one coral-derived
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12 314 (*Muricea elongata*) sequence (Fig. 4).

16 315 Discussion

19 316 Symbiotic *Cyanobacteria* in the temperate sponge hosts, *Ircinia fasciculata* and *I.*
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21 317 *variabilis*, were shown to exhibit similar species composition yet different levels of
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23 318 photosynthetic pigments (chl *a*) and storage products (glycogen granules) that correlated
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26 319 with the irradiance conditions of preferred host habitats. In both hosts, symbiont
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28 320 communities were dominated by a novel clade (“M”) of the unicellular cyanobacterium,
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30 321 *Synechococcus spongiarum*. A second single-celled cyanobacterium, *Synechocystis* sp.,
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32 322 was also observed in *I. fasciculata*, though rarely (7 total cells) and sporadically (1 of the
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35 323 3 host individuals). Symbiont communities associated with the photophilic host *I.*
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37 324 *fasciculata* exhibited ed nearly twice the chl *a* concentrations of *I. variabilis* and abundant
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39 325 accumulation of glycogen granules, a polysaccharide storage product of photosynthetic
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41 326 carbon assimilation. Notably, similar (putatively glycogen) granules were also observed
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44 327 in host cells interfacing with active symbionts in *I. fasciculata*, indicating the potential
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46 328 transfer of surplus carbon stores to the host sponge. These results suggest that ambient
47
48 329 irradiance conditions play a role in dictating the photosynthetic activity of sponge-
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50 330 associated *Cyanobacteria*, and possibly mediate the nature of host-symbiont interactions
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53 331 among different host sponge species.
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3 332 The phylogenetic signature of cyanobacterial symbionts in temperate *Ircinia* spp.
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5 333 was quite different from congeneric species in the Caribbean, which lack *Synechocystis*
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8 334 | symbionts and host distinct *S. spongiarum* clades [13]. The fine-scale phylogenetic
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10 335 | resolution afforded by 16S-23S rRNA ITS sequence data has important implications in
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12 336 | host-specificity, as the interpretation of symbiont specificity varies with molecular
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15 337 | marker resolution. For example, based on 16S rRNA gene sequences, clade M symbionts
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17 338 | recovered from Mediterranean *Ircinia* spp. herein matched nearly identically (99.1–
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19 339 | 99.5% sequence identity) to 16S rRNA gene sequences from clade J symbionts described
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21 340 | in Caribbean *Ircinia* hosts. In contrast, 16S-23S rRNA ITS gene sequences showed that
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23 341 | clade M symbionts were clearly differentiated from clade J symbionts based on sequence
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25 342 | similarity (90.2–91.1% identity) and phylogenetic analysis (distinct monophyletic
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27 343 | clades), revealing cryptic biogeographic trends in symbiont structure among *Ircinia* hosts.
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32 344 The biogeographic distribution of *S. spongiarum* suggests that unique clades
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34 345 inhabit hosts from different regions. In addition to the Mediterranean clade M symbionts
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36 346 described herein, distinct *S. spongiarum* clades have also been reported in the Indo-
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38 347 | Pacific (Palau, clade F) and eastern Atlantic (Canary Islands, clade E) [13]. In fact, the
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40 348 | majority of clades described to date are specific to a single geographic region (Fig. 5).
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43 349 | However, the clade diversity within a region is strongly correlated with the number of
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45 350 | host species surveyed (Fig. 5), indicating that additional sampling is required to fully
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47 351 | elucidate the diversity and distribution of *S. spongiarum* clades. Indeed, even in the well-
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49 352 | studied coral-zooxanthellae symbioses, the sampling of new hosts and environments
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51 353 | continues to reveal novel subclades and expand the distribution of known clades of
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55 354 | *Symbiodinium* symbionts [6].
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3 355 Clade-level differentiation of *S. spongiarum* symbionts is a recently described
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6 356 phenomenon [13] and whether this cryptic genetic diversity relates to differences in
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8 357 symbiont functioning and host benefit is currently unknown. The presence of a single,
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10 358 shared *S. spongiarum* clade in *Ircinia* spp. provides new insight into clade-level symbiont
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12 359 physiology through the comparative analyses of cellular ultrastructure and photosynthetic
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14 360 pigment concentrations in clade M symbionts from hosts in high (*I. fasciculata*) and low
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16 361 (*I. variabilis*) irradiance habitats. Clade M symbionts were smaller in *I. fasciculata*
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18 362 compared to *I. variabilis*, suggesting morphological plasticity in cell size in response to
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20 363 the ambient irradiance levels. *I. fasciculata* exhibited greater chl *a* concentrations
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22 364 compared to *I. variabilis* and dense aggregations of glycogen granules in symbiont cells,
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24 365 indicators of higher photosynthetic activity. Glycogen accumulation is consistent with
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26 366 high photosynthetic output, representing a key storage polysaccharide for fixed carbon in
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28 367 *Cyanobacteria* [34, 58]. Together, these data suggest flexibility among populations of
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30 368 clade M symbionts and acclimation to environmental irradiance gradients, rather than
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32 369 expulsion and compositional shifts as reported for coral-zooxanthellae symbioses [44].

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39 370 Previous investigations of sponges hosting *S. spongiarum* have reported high
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41 371 variability in the functional role of symbiotic *Cyanobacteria* and dependence of host
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43 372 sponges on photosymbiont communities. Among some host sponges, symbiont loss has
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45 373 little effect on host growth rates [12, 61, 72], secondary metabolite production [18], stress
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47 374 response [31] and host mortality [32]. In contrast, other host species exhibit decreased
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49 375 growth rates [12], metabolic collapse [2] and mass mortality of local host populations
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51 376 [17] in response to the reduction or loss of *S. spongiarum* symbionts. Similar data and
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53 377 experiments are unavailable for *Ircinia* hosts; however, symbiont loss due to temperature
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3 378 extremes has been suggested to contribute to mass mortality events of *Ircinia fasciculata*
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6 379 in the Mediterranean [8]. Further, the sponge *Petrosia ficiformis* hosts a related,
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8 380 facultative cyanobacterium, *Synechococcus feldmanni* [67], that occurs in hosts from
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10 381 light-exposed habitats yet is absent in conspecific sponges from dark caves [48]. The
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12 382 plasticity of this unique sponge-cyanobacteria symbiosis allows for comparative analyses
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14 383 of symbiotic and aposymbiotic *P. ficiformis* individuals and has yielded insight into the
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16 384 genetic regulation and metabolic implications of host-symbiont interactions [48, 56].
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20 385 Notably, our results also indicate high photosynthetic capacity in some temperate
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22 386 sponge-cyanobacteria symbioses, as chl *a* levels in *I. fasciculata* were consistent with
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24 387 values reported for tropical *Ircinia* spp. [11] and similar glycogen accumulation has been
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26 388 observed in symbionts of the tropical sponge *Chondrilla nucula* [46]. In contrast, low
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28 389 symbiont activity in *I. variabilis* hosts may indicate less dependence on symbiont
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30 390 photosynthetic output. Alternatively, symbiont populations may be actively regulated by
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32 391 *I. variabilis* to avoid specialist predators attracted to cyanobacteria-rich sponges [5] and
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34 392 reduce the oxidative stress of reactive oxygen species produced by symbiont
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36 393 photosynthesis [38, 39]. Additional studies are required to resolve specific host-symbiont
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38 394 interactions, with emphasis on fine-scale symbiont characterization, as well as, more
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40 395 refined metrics of host-symbiont metabolic interactions [16, 59].
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46 396 *Synechocystis* symbionts formed a monophyletic clade specific to the
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48 397 Mediterranean host *I. fasciculata* and distinct from related sponge and ascidian-
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50 398 associated symbionts. The morphology of these symbionts matched previous descriptions
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52 399 of symbionts characterized as *Aphanocapsa raspaigellae* in *I. variabilis* [47, 66].
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55 400 Reclassification of this cyanobacterium to the genus *Synechocystis* was suggested by
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3 401 | previous authors [26, 64] and is supported by the phylogenetic analyses herein.
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5 402 | *Synechocystis* symbionts formed a monophyletic cyanobacterial lineage and exhibited a
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7 403 | close phylogenetic relationship with *Prochloron* symbionts, consistent with previous
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9 404 | molecular phylogenies [52] and the ultrastructural similarity of these symbiont genera [9,
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15 406 | The ecological significance of *Synechocystis* symbionts in marine sponges
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17 407 | remains unclear, as these photosymbionts exhibit variable incidence and abundance
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19 408 | among different populations of *Ircinia* host sponges. In the current study, *Synechocystis*
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21 409 | symbionts in *I. fasciculata* occurred rarely and sporadically among the sponge individuals
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23 410 | studied and were observed to have limited interactions with host cells. Previous
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25 411 | investigations of *Ircinia* spp. from different regions of the Mediterranean have reported
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27 412 | more abundant *Synechocystis* populations in host sponges from Bari, Italy [47] and
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29 413 | Marseille, France [66], suggested biogeographic variability in these photosymbiont
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31 414 | communities. Consistent among these previous reports and the present study is the
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33 415 | physical separation of *Synechocystis* from host sponge tissue by a lacunar space
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35 416 | surrounding each symbiont cell. The lack of direct contact with sponge tissue may limit
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37 417 | host-symbiont interactions, although cellular secretion from intact *Synechocystis*
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39 418 | symbionts and leakage from disintegrating cells has been observed [47]. Further, the
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41 419 | occurrence of *Synechocystis* symbionts as secondary to dominant cyanobacterial
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43 420 | populations in *Ircinia* spp. [47, 66, this study] and *Lendenfeldia* spp. hosts [41] suggests
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45 421 | that these symbionts may be opportunistic and exploiting host habitats receptive to
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47 422 | photosymbionts (e.g., distributed in high irradiance zones, tolerant of oxidative stress)
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49 423 | while providing minimal ecological benefit. Additional study is clearly required to test
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3 424 | such hypotheses as well as assess potential contributions to host ecology beyond
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5 425 | symbiont-derived photosynthates (e.g., secondary metabolite production).
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8 426 | Environmental irradiance gradients represent an important factor in structuring
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10 427 | invertebrate-photosymbiont associations and may dictate the nature of host-symbiont
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12 428 | interactions. In the symbiosis between cnidarian hosts and zooxanthellae, irradiance
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14 429 | exposure and intensity have been shown to influence the density, physiology and
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16 430 | composition of photosymbiont communities [3, 49]. Similarly, the cyanobacterial
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18 431 | symbionts studied herein exhibited physiological and morphological differences in
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20 432 | related host sponges from different light environments, including photosynthetic pigment
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22 433 | content (chl *a* concentrations), fixed carbon accumulation (glycogen granules), and
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24 434 | symbiont cell size. In contrast to cnidarian symbionts, whose cladal composition can shift
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26 435 | across small-scale irradiance gradients [44], sponge photosymbiont communities were
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28 436 | dominated by clade M symbionts in both host sponges regardless of irradiance
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30 437 | conditions, suggesting that less dynamic and more versatile host-symbiont interactions
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32 438 | occur in *Ircinia-Synechococcus* associations. Thus, the temperate clade M of *S.*
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34 439 | *spongiarum* described herein appears to represent a flexible symbiont able to survive in
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36 440 | sponge hosts under different environmental conditions, a potential hallmark of symbiotic
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38 441 | Cyanobacteria occurring in temperate ecosystems that must tolerate large seasonal
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40 442 | fluctuations in light and temperature. Future research on temperate sponge-cyanobacteria
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42 443 | interactions targeting the fine-scale characterization of symbiont communities and
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44 444 | temporal monitoring of host-symbiont interactions are required to test such hypotheses
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46 445 | and determine the contributions of these symbiotic systems to host ecology and microbial
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48 446 | biodiversity.
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For Peer Review

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623 **FIGURE LEGENDS**

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

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

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

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

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3 657 values less than 70% for both ML and PP analyses are not shown. Scale bar represents 0.01 substitutions
4 658 per site.

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7 659 **Figure 5.** Linear regression of the clade-level diversity in the sponge-associated cyanobacterium, *S.*
8 660 *spongiarum*, and the number of host species studied for 5 geographic regions.

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For Peer Review

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

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

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

666 ²*Geodia* (2 spp.), *Sphaciospongia* (1), *Chondrilla* (1), *Ircinia* (2), *Aplysina* (4), *Verongula* (3), *Cribochalina*
 667 (2), *Xestospongia* (3) and *Neofibularia* (1)

668 ³*Chondrilla nucula*, *C. australiensis* and *I. variabilis*

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FIGURES

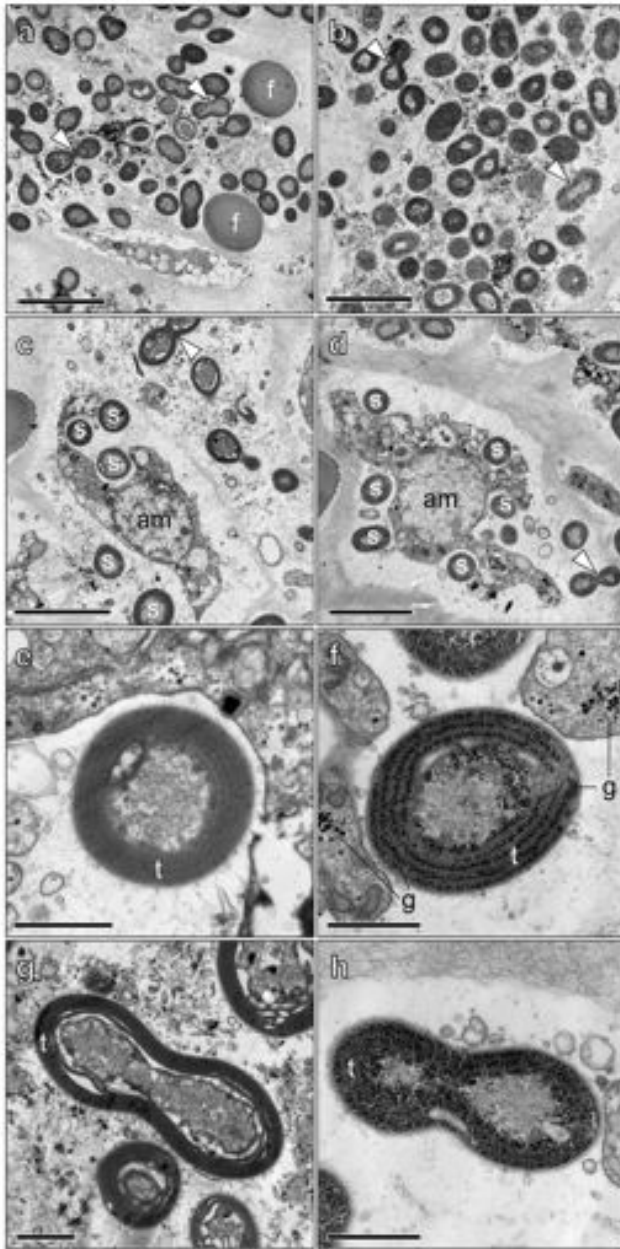


FIG. 1

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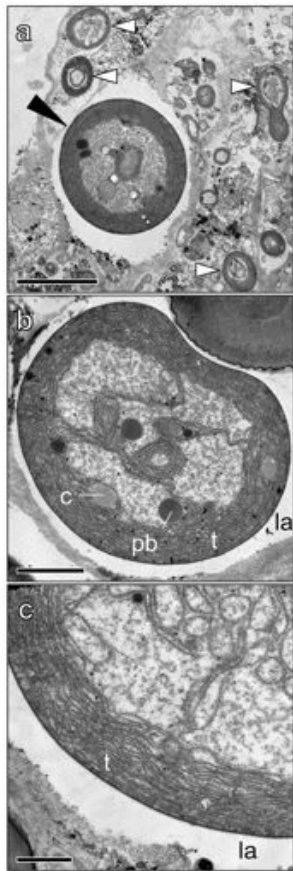


FIG. 2

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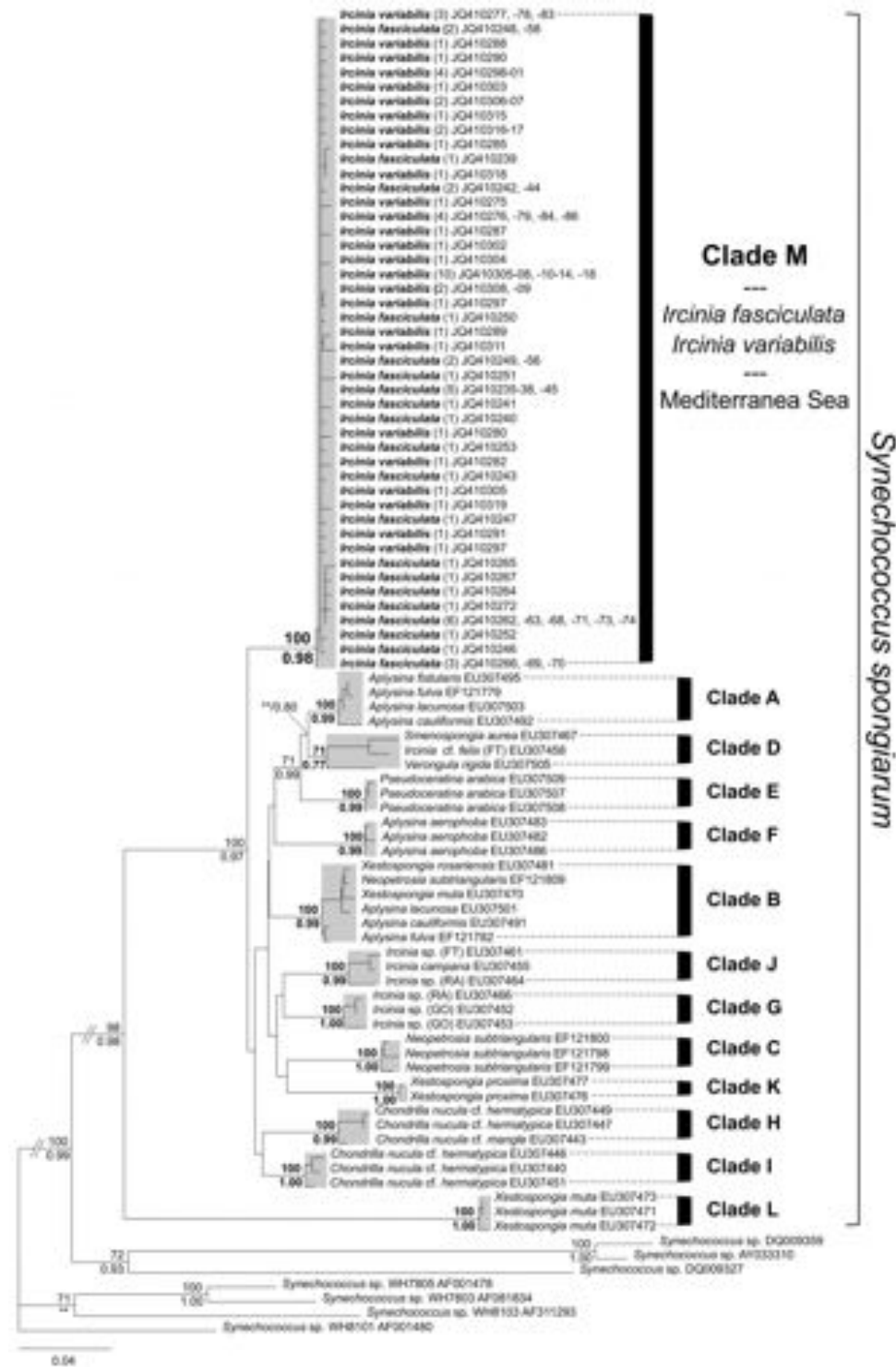


FIG. 3

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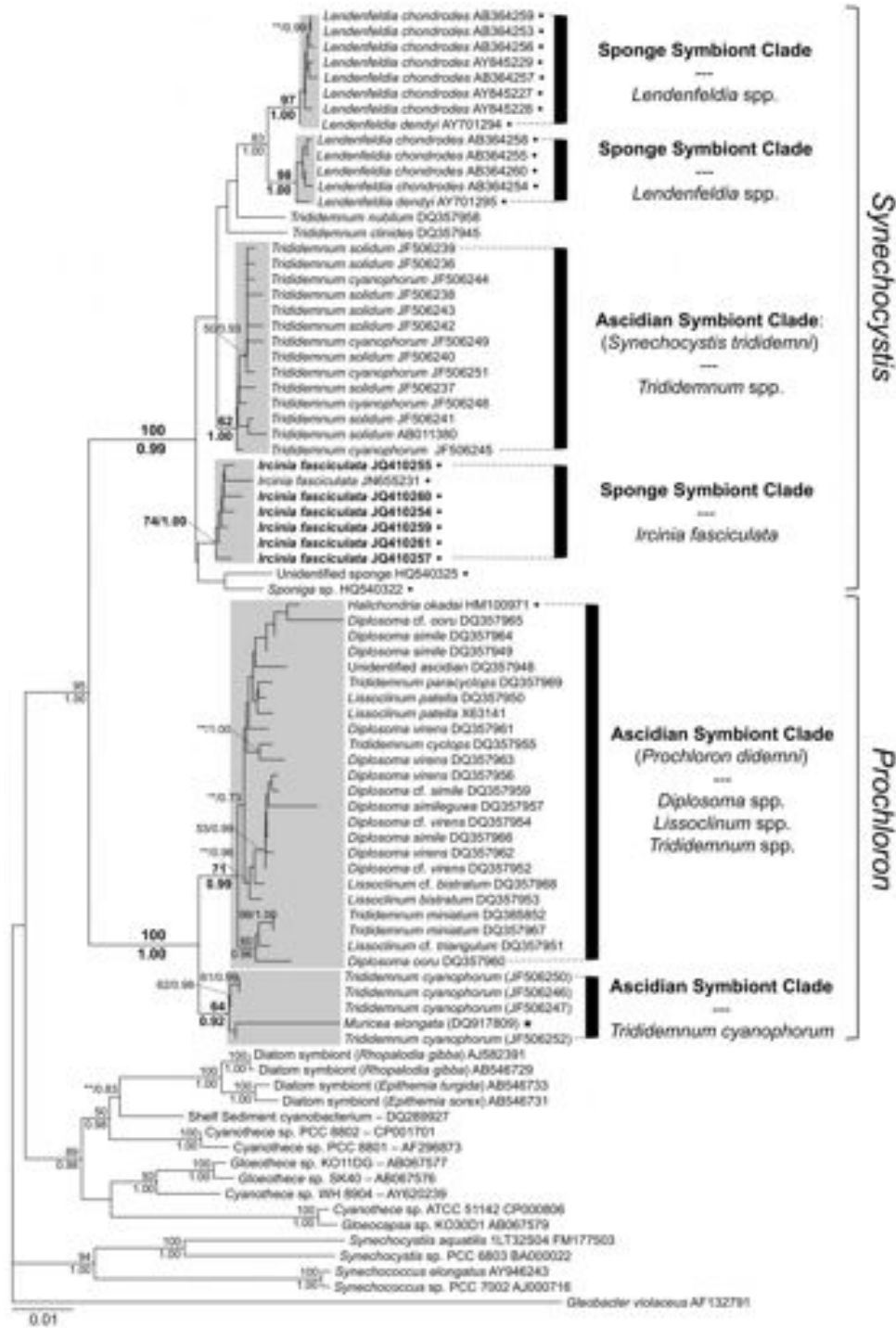


FIG. 4

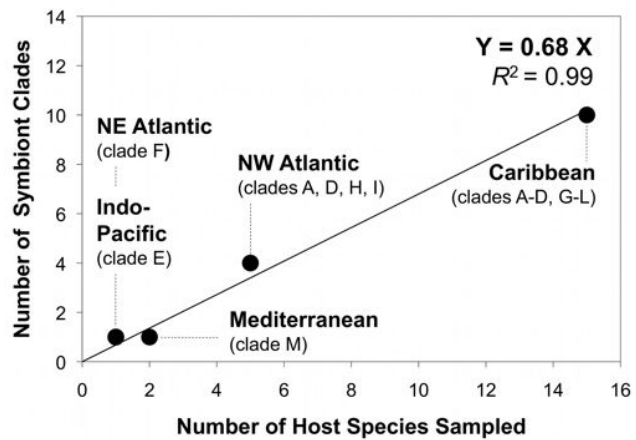


FIG. 5