Phylogenetic relationships among the filamentous cyanobacterial symbionts of Caribbean sponges and a comparison of photosynthetic production between sponges hosting filamentous and unicellular cyanobacteria

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Abstract: We investigated the filamentous cyanobacteria associated with two newly described species from the Caribbean coast of Panamá, *Haliclona walentinae* and *Xestospongia bocatorensis*. In addition to sequencing cyanobacterial 16S ribosomal RNA genes from *Hyrtios violaceus*, *H. walentinae* and *X. bocatorensis*, we measured the chlorophyll *a* content of *H. walentinae* and *X. bocatorensis* as an index of symbiont abundance. The photosynthetic and respiration rates of these two associations were compared to those of two sympatric sponges that host unicellular cyanobacteria, *Aplysina fulva* and *Neopetrosia subtriangularis*. A phylogeny of 16S ribosomal RNA genes reveals that the symbionts of *H. violaceus*, *H. walentinae* and *X. bocatorensis* are part of the *O. spongeliae* clade and that each sponge hosts a unique ribotype of this cyanobacterium. *H. walentinae* yielded the highest chlorophyll *a* concentrations, while *X. bocatorensis*, *A. fulva*, and *N. subtriangularis* were not significantly different. All sponges measured showed gross productivity to respiration (P:R) ratios greater than 1.5, indicating that cyanobacterial photosynthesis can compensate for host sponge respiration, and that all four species can be considered phototrophic. *X. bocatorensis* yielded the highest P:R ratios, while those of *H. walentinae*, *A. fulva,* and *N. subtriangularis* were not significantly different. Specialized associations with filamentous cyanobacteria may provide a valuable source of carbon to host sponges. These associations occur over a broader phylogenetic range of hosts than previously described, including representatives of the orders Dictyoceratida and Haplosclerida.

Keywords: cyanobacteria, molecular systematics, photosynthesis, phylogeny, symbioses

Introduction

Many marine sponges host diverse communities of extracellular symbiotic bacteria (Wilkinson *et al.* 1981, Hentschel *et al.* 2006). These symbionts may contribute to the host sponge's metabolism by providing nutrients such as fixed carbon or nitrogen (Wilkinson and Fay 1979, Rai 1990, Sarà *et al.* 1998). In particular, symbiotic cyanobacteria may provide their hosts with the products of photosynthesis. Cyanobacteria constitute nearly 50% of the biomass of some sponges, with up to 50% of the host's energy budget and 80% of the host's carbon budget derived from symbiont photosynthesis (Wilkinson 1983, Cheshire *et al.* 1997). Wilkinson (1987) suggested that these phototrophic symbioses were more prevalent in the Indo-Pacific region than in the Caribbean, concluding that Caribbean species were rarely phototrophic.

Two groups of cyanobacteria are most commonly reported as associates of marine sponges: unicellular cyanobacteria currently classified as *Candidatus Synechococcus spongiarum* and filamentous cyanobacteria classified as *Oscillatoria spongeliae* (Steindler *et al.* 2005, Diaz *et al.* 2007). Unicellular, *S. spongiarum*-like symbionts were previously described as *Aphanocapsa feldmani* by investigators using electron microscopy to identify sponge symbionts (Rützler 1990, Usher *et al.* 2006). Subsequent investigations based on molecular phylogenetic techniques have placed these symbionts into the genus *Synechococcus* (Usher *et al.* 2004, Thacker 2005). Although Usher *et al.* (2001) provided evidence for vertical transmission of these

symbionts, molecular phylogenetic analyses based on 16S ribosomal RNA (rRNA) gene sequences suggest there is no specialization of these symbionts for particular host species (Steindler *et al.* 2005, Thacker 2005). Common Caribbean hosts of *S. spongiarum* include *Aplysina fulva* (Pallas, 1766) and *Neopetrosia subtriangularis* (Duchassaing, 1850).

Filamentous *Oscillatoria spongeliae* have been previously reported from a variety of Indo-Pacific sponges based on both morphological (Rützler 1990, Diaz 1996) and molecular (Thacker and Starnes 2003, Thacker 2005, Ridley *et al.* 2005) evidence. These hosts include members of the dictyoceratid genera *Dysidea*, *Lamellodysidea*, *Lendenfeldia*, and *Phyllospongia*. *O. spongeliae* filaments are extracellular, approximately 10 μm wide, and range in length from 5 to 50 cells (Hinde *et al.* 1994). Previous research has documented the evolutionary specialization of these symbionts for particular host sponges. Molecular phylogenies of the Indo-Pacific hosts and their symbionts reveal that each sponge species hosts a unique clade of *O. spongeliae*, and suggest that cospeciation occurs between hosts and symbionts (Thacker and Starnes 2003, Ridley *et al.* 2005).

Filamentous cyanobacteria have previously been reported as symbionts from only two Caribbean sponges: *Hyrtios violaceus* (Duchassaing and Michelotti, 1864) (formerly *Oligoceras hemorrhages* de Laubenfels, 1936), which is common in the Bahamas and Belize (Wiedenmayer 1977, Rützler 1990), and an undescribed species of *Niphates*, also from the Bahamas and Belize (Diaz 1996). From the Bocas del Toro region of Panamá, Diaz *et al.* (2007) have described two additional Caribbean sponges that host filamentous cyanobacteria: *Haliclona walentinae* and *Xestospongia bocatorensis*. The objectives of our study were to PCR-amplify and sequence cyanobacterial 16S rRNA genes from three of these sponges: *H. violaceus*, *H. walentinae*, and *X. bocatorensis*. We compared these sequences to known sequences from *O. spongeliae* to examine their host specificity and phylogenetic relationships. For the two species from Panamá, we measured chlorophyll *a* concentrations to estimate the abundance of these cyanobacteria in their host sponges and measured photosynthetic and respiration rates to determine whether these sponges are phototrophic. We tested the hypotheses that sponges hosting filamentous cyanobacterial symbionts (1) contain higher concentrations of chlorophyll *a* and (2) have higher ratios of gross photosynthetic production to respiration than sympatric sponges hosting unicellular cyanobacterial symbionts.

Methods

Specimens of *Hyrtios violaceus* were collected from shallow reefs at Twin Cays, Belize, near the Smithsonian Institution's research station at Carrie Bow Cay, Belize. Specimens of *Haliclona walentinae*, *Xestospongia bocatorensis, Aplysina fulva* and *Neopetrosia subtriangularis* were collected from shallow reefs, between 2 and 5 m depth, near the Smithsonian Tropical Research Institute's Bocas Research Station, Bocas del Toro, Panamá. Sponges were preserved in 95% ethanol and RNAlater (Ambion, Inc.).

Genomic DNA extractions were prepared from preserved sponges (2 *H. walentinae* individuals, 4 *X. bocatorensis* individuals, and 3 *H. violaceus* individuals) using the Wizard Genomic DNA Purification Kit, following the manufacturer's protocol (Promega Corporation, Madison, WI). Nearly complete bacterial 16S rRNA genes were amplified from these extracts using universal bacterial primers (Martinez-Murcia *et al.* 1995). PCR products were inserted into plasmid vectors using the pGEM T-Easy Vector System (Promega). Representative clones from each individual sponge were screened for the presence of cyanobacterial 16S rRNA genes using cyanobacteria-specific PCR primers (Nübel *et al.* 1997). Three positive clones from each individual sponge were sequenced at the UAB CFAR DNA Sequencing Core Facility, using the plasmid's sequencing primers and internal sequencing primers (Nübel *et al.* 1997). Sequences were assembled and aligned using Sequencher 4.1 (GeneCodes, Ann Arbor, MI) and Se-Al (Rambaut, University of Oxford). For each individual sponge, a single consensus sequence was constructed from the three sequenced clones, since in all cases these clones showed less than 1% sequence divergence within an individual host.

Several reference sequences from GenBank were included in the 1,416 bp alignment, including *Oscillatoria spongeliae* from other marine sponges and two outgroup sequences, *O.* cf. *corallinae* and *Arthrospira* sp. PCC 7345 (Fig. 1). Phylogenetic analyses included a distance-based neighbor-joining approach conducted with MEGA version 3.1 (Kumar *et al.* 2004) using the Kimura 2-parameter model of nucleotide substitutions with 500 bootstrap replicates. We also performed a maximum likelihood (ML) phylogenetic analysis using GARLI version 0.951 (Zwickl 2006; download available at: http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html), including 100 bootstrap replicates. The hierarchical Akaike information criterion (AIC) implemented by Modeltest 3.7 (Posada and Crandall 1998) was used to select the best model of DNA substitution, the general time reversible model with proportion of invariable sites and a gamma distribution of variable substitution rates among variable sites (GTR+I+G). For Bayesian phylogenetic analyses, MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) was used to calculate the posterior probabilities of branch nodes, implementing the GTR+I+G likelihood model. The Monte Carlo Markov chain length was set at 500,000 generations with sampling every 100th generation and a burn-in value of 1250 cycles. After 250,000 generations, the average standard deviation of split frequencies reached less than 0.01.

For *H. walentinae* and *X. bocatorensis*, chlorophyll *a* concentrations and photosynthetic production were compared to two sympatric sponges, *A. fulva* and *N. subtriangularis*. Microscopic examinations of all four sponges did not reveal the presence of photosynthetic eukaryotes; thus, chlorophyll *a* concentrations are directly correlated with the abundance of cyanobacteria within a sponge, as reported by other investigators (Wilkinson 1983, Rai 1990). For each specimen, approximately 0.25 g (wet mass) of finely chopped sponge ectosome was placed in 10 ml of a 90% acetone:water mixture and held overnight at 4°C. Each sample was briefly spun in a centrifuge to remove suspended solids, after which the supernatant was transferred to a cuvette and absorbance measured at 750, 664, 647, and 630 nm in a spectrophotometer. Chlorophyll *a* concentrations were calculated based on

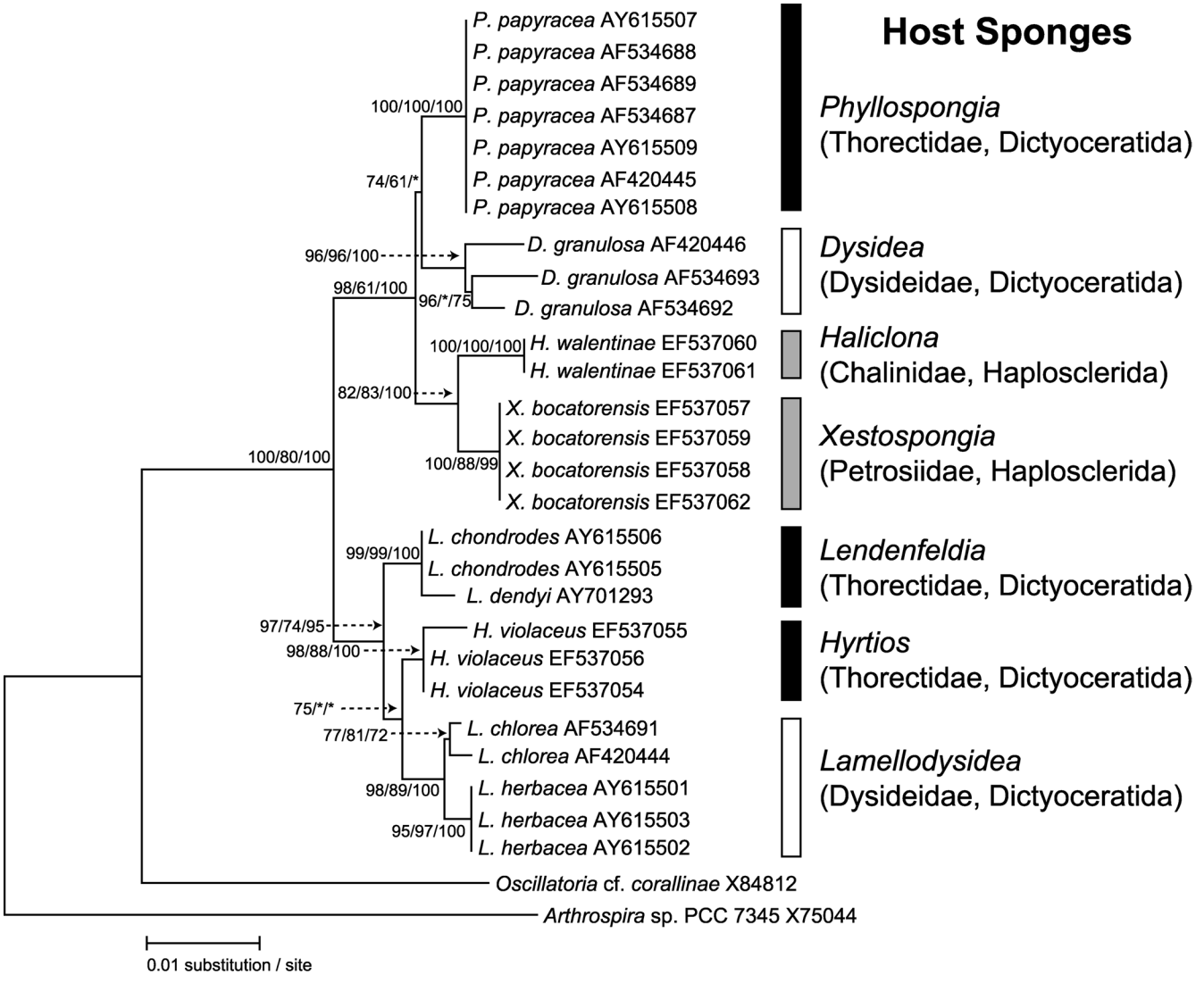

Fig. 1: Neighbor-joining phylogeny of 16S ribosomal RNA gene sequences amplified from *Oscillatoria spongeliae* symbionts of marine sponges. Numbers at each node represent percentage bootstrap support from 500 replicates of a neighbor-joining analysis, percentage bootstrap support from 100 replicates of a maximum-likelihood analysis, and percentage Bayesian posterior probabilities, respectively. Asterisks indicate less than 50% support. Branch tips are labeled with the identity of the host sponge and GenBank accession number, while shaded bars indicate the genus, family, and order of the host sponges. Scale bar indicates number of substitutions per site based on the Kimura two-parameter model of nucleotide substitutions.

equations provided by Parsons *et al.* (1984) and standardized to sponge mass. Differences in chlorophyll *a* concentrations among sponge species and symbiont type (unicellular vs. filamentous) were compared using a nested analysis of variance (ANOVA), with species nested within symbiont type. *Post hoc* comparisons among species were conducted using Fisher's least significant difference test.

Photosynthetic and respiration rates were measured for fragments of sponges that were incubated in 0.5 l bottles filled with filtered seawater. Each fragment was incubated sequentially in a light bottle, which allowed approximately 75% of ambient light (an average of 1000 µmol quanta / m^2 / s during this experiment) to reach the sponge, and a dark bottle, which allowed no light to reach the sponge. Immediately prior to each incubation, the initial oxygen concentration in each bottle was measured using a YSI Model 85 oxygen meter. After 1 hour of incubation in a water bath, which maintained a temperature of 30°C (reflecting the ambient water temperature), final oxygen concentrations were measured. Respiration was calculated as the change in oxygen concentrations in the dark bottles, standardized by sponge wet mass, while net photosynthesis was calculated from the change in oxygen concentrations in the light bottles, standardized by sponge wet mass. Gross photosynthesis was calculated as the difference between respiration and net photosynthesis. The gross production to respiration ratio (P:R; Wilkinson 1983) was calculated as gross photosynthesis divided by respiration. Differences in P:R ratios among sponge

species and symbiont type (unicellular vs. filamentous) were compared using a nested analysis of variance (ANOVA), with species nested within symbiont type. Post hoc comparisons among species were conducted using Fisher's least significant difference test.

Results

The cyanobacterial symbionts of *Hyrtios violaceus*, *Haliclona walentinae*, and *Xestospongia bocatorensis* yielded 16S rRNA gene sequences that shared 96.7% to 99.4% identity with other sequences obtained from *Oscillatoria spongeliae*. Sequences have been deposited in GenBank under accession numbers EF537054 to EF537062. Each sponge species hosts a unique and well-supported monophyletic clade of *O. spongeliae* (Fig. 1), clearly illustrating the high degree of host-specificity observed for this symbiont. The *H. violaceus* symbiont is most similar to that of *Lendenfeldia chondrodes*, and is part of a monophyletic clade that includes the symbionts of *Lamellodysidea chlorea* and *Lamellodysidea herbacea*. The symbionts hosted by *H. walentinae* and *X. bocatorensis* were very similar, showing only 0.78% sequence divergence. These symbionts formed a monophyletic clade with those of *Dysidea granulosa* and *Phyllospongia papyracea*.

Chlorophyll *a* concentrations did not vary significantly among sponge species nested within symbiont types ($F = 3.405$, $df = 2$, $P = 0.059$), but did vary significantly between symbiont types ($F = 5.548$, $df = 1$, $P = 0.032$). However, post hoc tests revealed that this pattern was driven by a single species. *H. walentinae* yielded significantly higher chlorophyll *a* concentrations than the other three sponges, creating the significant difference between symbiont types (Fig. 2).

Gross photosynthetic production to respiration (P:R; Fig. 3) ratios varied significantly among sponge species nested within symbiont types ($F = 7.314$, $df = 2$, $P = 0.007$) and between sponges hosting filamentous and unicellular cyanobacteria ($F = 18.508$, $df = 1$, $P = 0.001$). Post hoc tests revealed that this pattern was also driven by a single species, since *X. bocatorensis* yielded the highest P:R ratio, while those of the other three sponges were not significantly different. All of these sponges showed P:R ratios greater than 1.0, indicating that cyanobacterial photosynthesis can compensate for sponge respiration. In addition, Wilkinson (1987) defined phototrophic sponges as having a P:R ratio greater than 1.5; all four of these Caribbean sponges can be considered phototrophic under this definition.

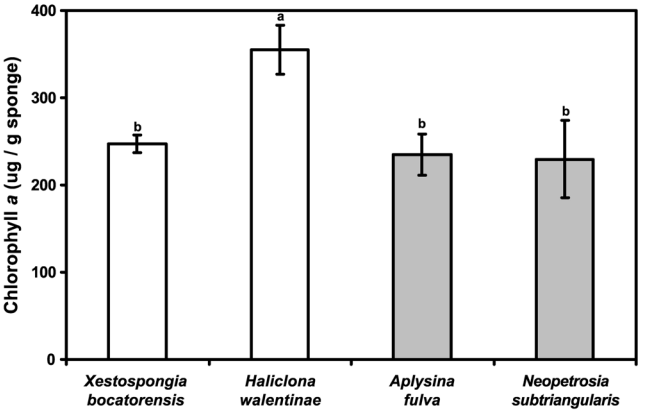

Fig. 2: Chlorophyll *a* concentrations (mean ± SE) measured in Caribbean sponges that host filamentous cyanobacterial symbionts (*Xestospongia bocatorensis* and *Haliclona walentinae*; open bars) and unicellular symbionts (*Aplysina fulva* and *Neopetrosia subtriangularis*; shaded bars). Five individuals were sampled per species; different letters above bars indicate significantly different means.

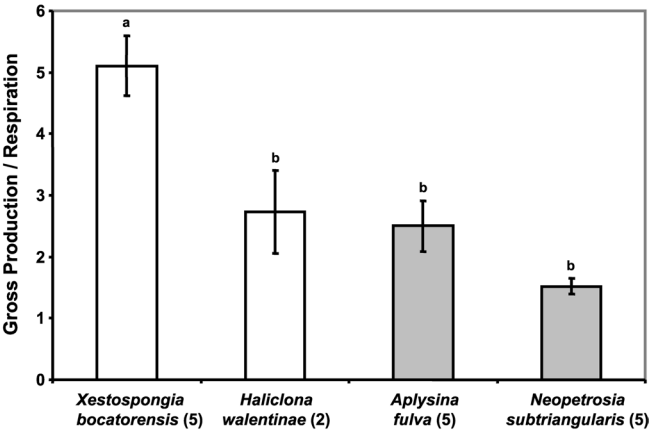

Fig. 3: Gross photosynthetic production to respiration ratios (mean ± SE) measured in Caribbean sponges that host filamentous cyanobacterial symbionts (*Xestospongia bocatorensis* and *Haliclona walentinae*; open bars) and unicellular symbionts (*Aplysina fulva* and *Neopetrosia subtriangularis*; shaded bars). Numbers in parentheses indicate the number of individuals sampled; different letters above bars indicate significantly different means.

Discussion

Microscopic examination of the Caribbean sponges *Hyrtios violaceus*, *Haliclona walentinae*, and *Xestospongia bocatorensis* revealed that all three of these species host abundant filamentous cyanobacterial symbionts (Rützler 1990, Diaz *et al.* 2007). These symbionts are morphologically similar to the symbionts classified as *Oscillatoria spongeliae* that are hosted by Indo-Pacific sponges in the genera *Dysidea*, *Lamellodysidea*, *Lendenfeldia*, and *Phyllospongia* (Thacker and Starnes 2003, Ridley *et al.* 2005). The molecular phylogenetic analyses described in this study confirm that the cyanobacterial symbionts of *H. violaceus*, *H. walentinae*, and *X. bocatorensis* are members of the *Oscillatoria spongeliae* clade. Moreover, each of these sponge species hosts a unique subclade or ribotype of the cyanobacterium, as has been demonstrated for the Indo-Pacific hosts (Thacker and Starnes 2003, Ridley *et al.* 2005).

Previous molecular phylogenies of the Indo-Pacific hosts and their symbionts supported the hypothesis of cospeciation between sponges and *O. spongeliae* (Thacker and Starnes 2003, Ridley *et al.* 2005). Ridley *et al.* (2005) found qualitative evidence of only one host-switching event among the Indo-Pacific hosts; however, this single event was not supported by statistical analyses. The addition of the Caribbean taxa to the phylogenetic tree of *O. spongeliae* raises questions about the

hypothesis of cospeciation. If a thorectid ancestor was initially colonized by *O. spongeliae*, two independent colonizations of Dysideidae and two independent colonizations of two haplosclerid taxa are needed to generate the observed phylogenetic pattern (Fig. 1). In addition the symbionts of the Caribbean sponges are derived from two different lineages of *O. spongeliae*. Data on the molecular systematics of the Caribbean host sponges are clearly needed to quantify the contribution of sponge hosts to these patterns. However, the current data set provides qualitative support for a hypothesis of independent colonization of these hosts by the symbionts, and suggests that cospeciation events might only occur within genera.

Cyanobacterial abundance within a host sponge is directly correlated with chlorophyll *a* concentrations (Wilkinson 1983, Rai 1990). Since photoacclimation can also influence chlorophyll *a* concentrations within cyanobacterial cells (MacIntyre *et al.* 2002), all sponges used in this study were collected from similar light environments between 2 and 5 m depth. Chlorophyll *a* concentrations varied significantly among *H. walentinae*, *X. bocatorensis*, and two sympatric sponges that host the unicellular cyanobacterial symbionts classified as *Candidatus Synechococcus spongiarum* (Usher *et al.* 2004, Steindler *et al.* 2005), *Aplysina fulva* and *Neopetrosia subtriangularis*. *H. walentinae* yielded a higher concentration of chlorophyll *a* than the other three sponges, indicating that it may host a higher density of symbiotic cyanobacteria. On average, sponges hosting filamentous cyanobacteria contained a higher concentration of chlorophyll *a* than sponges hosting unicellular cyanobacteria. However, since our comparisons only included two species hosting each symbiont type, and since this pattern was driven by the extremely high concentration of chlorophyll *a* in a single species, this hypothesis clearly needs to be tested with a wider range of host species. The observed variation in chlorophyll *a* concentrations within and among host species and between symbiont types may reflect variation in the abundance of cyanobacteria, which may reflect variation in the costs and benefits associated with these symbioses (Thacker 2005).

Our current data can also be compared to chlorophyll *a* concentrations measured in other sponges (e.g., Wilkinson 1983, Thacker 2005); however, there is a large degree of variability in chlorophyll extraction and analysis methods among studies. For example, in Palau, *Lamellodysidea chlorea* (which hosts *O. spongeliae*) contained 685 ± 84 (mean ± SE) µg chlorophyll *a* / g sponge, while *Neopetrosia exigua* (Kirkpatrick, 1900) (which hosts *S. spongiarum*) contained 610 ± 40 µg chlorophyll *a* / g sponge (Thacker 2005). These values are greater than twice the average of the Caribbean sponges sampled in this study, indicating a large amount of variation that could be due not only to differences in sample processing (the Palauan sponges were freeze-dried prior to chlorophyll *a* extraction) but also to biogeographic factors that influence chlorophyll *a* concentrations and/or cyanobacterial abundance. These sources of variation can be larger than the variation observed between particular hosts and symbionts within a single study.

Previous experiments used shading to manipulate the interactions between *L. chlorea* and *O. spongeliae* and between *N. exigua* and *S. spongiarum* (Thacker 2005). *L. chlorea* was dependent on photosynthesis for survival and growth, rapidly dying when shaded, while *N. exigua* did not suffer any reduction in survival or growth when shaded, indicating that filamentous symbionts may make larger contributions to their hosts than unicellular symbionts. Based on these results, we hypothesized that sponges hosting filamentous symbionts would have higher gross production to respiration (P:R) ratios than those hosting unicellular symbionts. On average, sponges hosting filamentous symbionts did display higher P:R ratios than those hosting unicellular symbionts; however, this pattern was driven by a single species, *X. bocatorensis*. Thus, this hypothesis should also be tested with a larger selection of host species. The large variability in P:R ratios, also observed by Wilkinson (1983), may be biologically significant, potentially illustrating the changing balance of costs and benefits involved in these symbioses. All four sponges showed that cyanobacterial photosynthesis can more than compensate for sponge respiration. Furthermore, according to Wilkinson's (1987) definition, all four of these species can be considered phototrophic, suggesting that more phototrophic sponges exist in the Caribbean than previously acknowledged. Rützler (unpublished) measured P:R ratios for *H. violaceus* in Belize, and found values of 4.6 (full sunlight), 3.4 (lightly overcast), and 2.2 (overcast), concluding that this species is also phototrophic.

The phylogenetic data obtained in this study confirms that the filamentous cyanobacteria found within three species of Caribbean sponges are members of the *Oscillatoria spongeliae* clade, with each sponge hosting a unique subclade or ribotype of symbiont. The current phylogeny emphasizes multiple colonizations or host-switches over cospeciation; however, there is a clear need to obtain the host phylogeny to quantify these patterns. In general, filamentous symbionts may provide a larger contribution to sponge metabolism than unicellular symbionts (Thacker 2005); however, since we found very similar P:R ratios for *A. fulva* and *H. walentinae*, such generalizations may ignore the substantial variation observed among host sponges. This variability may reflect a dynamic relationship between sponge hosts and their cyanobacterial symbionts, which may be strongly influenced by the type of symbiont, the host sponge species, and environmental conditions.

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