Molecular Ecology (2008) 17, 2937–2947 doi: 10.1111/j.1365-294X.2008.03808.x

Cryptic diversity of the symbiotic cyanobacterium *Synechococcus spongiarum* among sponge hosts

PATRICK M. ERWIN* and ROBERT W. THACKER
Department of Biology, University of Alabama at Birmingham, Birmingham, AL 35294-1170, USA

Abstract

Cyanobacteria are common members of sponge-associated bacterial communities and are particularly abundant symbionts of coral reef sponges. The unicellular cyanobacterium *Synechococcus spongiarum* is the most prevalent photosynthetic symbiont in marine sponges and inhabits taxonomically diverse hosts from tropical and temperate reefs worldwide. Despite the global distribution of *S. spongiarum*, molecular analyses report low levels of genetic divergence among 16S ribosomal RNA (rRNA) gene sequences from diverse sponge hosts, resulting either from the widespread dispersal ability of these symbionts or the low phylogenetic resolution of a conserved molecular marker. Partial 16S rRNA and entire 16S–23S rRNA internal transcribed spacer (ITS) genes were sequenced from cyanobacteria inhabiting 32 sponges (representing 18 species, six families and four orders) from six geographical regions. ITS phylogenies revealed 12 distinct clades of *S. spongiarum* that displayed 9% mean sequence divergence among clades and less than 1% sequence divergence within clades. Symbiont clades ranged in specificity from generalists to specialists, with most (10 of 12) clades detected in one or several closely related hosts. Although multiple symbiont clades inhabited some host sponges, symbiont communities appear to be structured by both geography and host phylogeny. In contrast, 16S rRNA sequences were highly conserved, exhibiting less than 1% sequence divergence among symbiont clades. ITS gene sequences displayed much higher variability than 16S rRNA sequences, highlighting the utility of ITS sequences in determining the genetic diversity and host specificity of *S. spongiarum* populations among reef sponges. The genetic diversity of *S. spongiarum* revealed by ITS sequences may be correlated with different physiological capabilities and environmental preferences that may generate variable host–symbiont interactions.

Keywords: coral reef, cyanobacteria, ITS, phylogeny, Porifera, symbiosis

Received 23 December 2007; revision received 8 April 2008; accepted 16 April 2008

Introduction

Marine sponges harbour diverse and abundant microbial assemblages that include most major evolutionary lineages of bacteria and archaea (Wilkinson 1978; Preston *et al*. 1996; Webster *et al*. 2001; Hentschel *et al*. 2002; Webster *et al*. 2004; Taylor *et al*. 2007b). Microbial communities can comprise over 50% of total holobiont volume and have been reported from nearly all sponges investigated (Taylor *et al*. 2007b). Symbiotic bacteria can benefit host sponges by providing supplemental nutrition (Wilkinson 1983; Arillo *et al*. 1993; Yahel *et al*. 2003), producing secondary metabolites (Flatt *et al*. 2005), enhancing the rigidity of the sponge skeleton (Wilkinson *et al*. 1981) and providing ultraviolet protection (Regoli *et al*. 2000). Physiological and ecological costs of harbouring microbial symbionts may include intercellular nutrient competition (Smith 1991) and the attraction of specialist grazers (Becerro *et al*. 2003; Becerro *et al*. 2006). Mounting evidence suggests that sponges host evolutionarily divergent bacterial lineages that are quite distinct from the bacterial communities found in ambient seawater (Hentschel *et al*. 2002; Olson & McCarthy 2005; Hill *et al*. 2006; Taylor *et al*. 2007b). These ancient symbioses may provide

Correspondence: Robert W. Thacker, Department of Biology, University of Alabama at Birmingham, 109 Campbell Hall, 1300 University Boulevard, Birmingham, AL 35294-1170, USA. Fax: (205) 975-6097; E-mail: thacker@uab.edu
*Present address: Center for Marine Science, University of North Carolina Wilmington, Wilmington, NC 28403-3297, USA

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

fundamental insights into the evolution and maintenance of animal–microbe interactions (Taylor *et al*. 2007a, c).

Cyanobacteria are common members of sponge-associated bacterial communities and are especially abundant within coral reef sponges (Rützler 1990; Erwin & Thacker 2007). Sponge-associated cyanobacteria span at least six genera; however, a sponge-specific lineage of *Synechococcus*, formally described as *Candidatus Synechococcus spongiarum* (Usher *et al*. 2004b), represents the most prevalent symbiont group (Rützler 1990; Usher *et al*. 2004a; Steindler *et al*. 2005; Thacker 2005; Erwin & Thacker 2007). *Synechococcus spongiarum* has been identified in tropical and temperate reef sponges from the Indo-Pacific Ocean, the Caribbean Sea, the Mediterranean Sea and the Red Sea (Diaz 1996; Hentschel *et al*. 2002; Usher *et al*. 2004a; Oren *et al*. 2005; Steindler *et al*. 2005; Thacker 2005; Erwin & Thacker 2007). Despite the widespread distribution of these symbionts, previous molecular analyses reveal little genetic differentiation among populations based on host species or geographical region (Steindler *et al*. 2005; Thacker 2005), suggesting widespread dispersal and horizontal transmission of *S. spongiarum*. In contrast, several studies have used electron and fluorescence microscopy to describe the vertical transmission of these symbionts from parents to offspring (Usher *et al*. 2001; Oren *et al*. 2005; Usher *et al*. 2005). If *S. spongiarum* are primarily acquired through vertical transmission, different sponge lineages may be expected to host genetically distinct symbionts.

Traditionally, 16S ribosomal RNA (rRNA) gene sequences are the most frequently targeted molecular marker for microbial characterization, yielding a large database for comparative analyses. Caveats associated with the use of 16S rRNA gene sequences include the presence of multiple copies of ribosomal operons in some species (Stewart & Cavanaugh 2007) and low levels of sequence divergence among closely related bacteria. For example, two marine *Prochlorococcus* species that exhibit > 97% 16S rRNA sequence identity differ considerably in genome size, gene number and gene identity (Rocap *et al*. 2003). These genetic differences correspond to ecological differences, as these species inhabit distinct ecological niches and display variable physiological capabilities (Moore *et al*. 1998). Other molecular markers can be used to characterize closely related cyanobacteria and increase fine-scale phylogenetic resolution, including the 16S–23S rRNA internal transcribed spacer (ITS) region (Iteman *et al*. 2000; Boyer *et al*. 2001; Rocap *et al*. 2002; Stewart & Cavanaugh 2007).

The presence of multiple, divergent copies of the rRNA operon in cyanobacteria can confound phylogenetic analyses of 16S–23S rRNA ITS gene sequences (Boyer *et al*. 2001). However, nonidentical ITS copies are most commonly reported from filamentous cyanobacteria and frequently differ based on the presence or absence of the transfer RNA (tRNA) genes encoding tRNA-Ile and tRNA-Ala (Iteman *et al*. 2000; Stewart & Cavanaugh 2007). In contrast, multiple ITS sequences characterized from cultured and environmental strains of unicellular *Prochlorococcus* and *Synechococcus* contain both tRNA gene regions and few, if any, sequence ambiguities (Laloui *et al*. 2002; Rocap *et al*. 2002; Chen *et al*. 2006). A recent analysis of whole genome sequences found that five species of unicellular cyanobacteria each contain only two copies of the rRNA operon; in each of these five genomes, the two copies of 16S and ITS are identical (Stewart & Cavanaugh 2007). Taken together, these studies suggest that ITS sequences may be a valuable tool for resolving fine-scale phylogenetic differences within *S. spongiarum*.

In this study, we characterized partial 16S rRNA and entire 16S–23S ITS sequences from *S. spongiarum* inhabiting 18 host sponge species to assess the phylogenetic utility of rRNA sequence data in resolving the phylogeny of sponge-associated cyanobacteria. We compared symbiont communities among host species and geographical regions to assess the host-specificity and global distribution of these symbionts.

Materials and methods

Sample collection

Sponge samples were collected by SCUBA at the Smithsonian Tropical Research Institute's Bocas del Toro Research Station, Bocas del Toro, Panamá, in June through August 2005, the Caribbean Marine Research Center, Lee Stocking Island, Bahamas, during June 2001 and October 2003, the Coral Reef Research Foundation, Koror, Palau, during June 2002, La Tixera, Canary Islands, during June 2003, and Gray's Reef National Marine Sanctuary, USA, during May 2004 (Table 1). Samples were preserved separately in RNAlater (Ambion), for genetic analysis, and 70% ethanol, for morphological identification and voucher specimens.

DNA extraction, amplification and sequencing

Whole genomic DNA was extracted from 32 sponge samples (representing 18 species; Table 1) using the Wizard Genomic DNA Purification Kit (Promega); genomic extracts were cleaned using the Wizard DNA Clean-Up Kit (Promega). The cyanobacteria-specific oligonucleotide forward primers CYA359F and CYA781F (Nübel *et al*. 1997) and the reverse primer CYA23S1R (Primer 340; Iteman *et al*. 2000) were used to amplify a segment of rRNA corresponding to the 3′ end of the 16S region (either 737 or 1149 bp, depending on the forward primer used) and the entire 16S–23S ITS region (ranging from 487 to 607 bp). Total polymerase chain reaction (PCR) volume was 50 μL, including 25 pmol of each primer, 10 nmol of each dNTP, 1× Master*Taq* PCR

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

Table 1 Taxonomy, collection location and symbiont clades of sponge species investigated in this study. Letters in the far right column indicate the symbiont clade associated with each host species and numbers in parentheses indicate the number of clones recovered

Host order	Host family	Host species	Region	Symbiont clades (number of clones)
Chondrosida	Chondrillidae	*Chondrilla nucula f. hermatypica*	Bermuda	H (1) + I (2)
			GRNMS*	H (1) + I (2)
			Panama	H (1) + I (2)
		Chondrilla nucula f. mangle	Panama	I (3)
Dictyoceratida	Irciniidae	*Ircinia campana*	Panama	J (3)
		Ircinia felix	Panama	D (3)
		Ircinia sp. FT	Panama	J (3)
		Ircinia sp. GO	Panama	G (3)
		Ircinia sp. RA	Panama	G (1) + J (2)
	Thorectidae	*Smenospongia aurea*	Bahamas	D (3)
Haplosclerida	Petrosiidae	*Neopetrosia subtriangularis*	Panama	B (17) + C (3)
		Xestospongia muta	Panama	B (2) + L (3)
		Xestospongia proxima	Panama	K (3)
		Xestospongia rosariensis	Panama	B (4)
Verongida	Aplysinidae	*Aplysina aerophoba*	Canary Islands	F (7)
		Aplysina cauliformis	Bahamas	A (3)
			Panama	B (3)
		Aplysina fistularis	Panama	A (3)
		Aplysina fulva	Bahamas	A (3)
			Panama	A (15) + B (3)
		Aplysina lacunosa	Panama	A (1) + B (2)
		Verongula rigida	Bahamas	D (3)
	Pseudoceratinidae	*Pseudoceratina arabica*	Palau	E (3)

*Gray's Reef National Marine Sanctuary.

buffer (Eppendorf), and 1× *Taq*Master additive (Eppendorf). Thermocycler reaction conditions included an initial denaturing time of 5 min at 85 °C, followed by the addition of 1.0 U Master*Taq* DNA polymerase (Eppendorf), then 29 cycles of 1.5 min at 94 °C, 2 min at 50 °C, and 3 min at 72 °C, and a final extension time of 10 min at 72 °C. PCR products were gel-purified and cleaned using the Wizard PCR Preps System (Promega) then were ligated into plasmids using the pGEM-T Easy Vector System (Promega); plasmids were harvested using the QIAprep Spin Miniprep Kit (QIAGEN). For each sponge individual, three to eight clones were harvested and sequenced. Forward and reverse sequencing reactions were performed for each clone at the University of Alabama at Birmingham (UAB) Center for AIDS Research DNA Sequencing Core Facility.

Phylogenetic analyses

Forward and reverse sequences were compared to ensure the accuracy of sequencing reactions using SEQUENCHER (GeneCodes), yielding a final consensus for each clone. Clones from the same sponge specimen that exhibited 100% sequence identity were combined into a single consensus sequence. Consensus sequences were aligned using BIOEDIT (Hall 2000) and CLUSTAL_X (Thompson *et al*. 1997), using default alignment parameters, to assess the variability of each gene region. A GenBank BLAST search was used to identify the sequences most closely related to the recovered sequences for outgroup comparisons. Outgroup sequences included the top three BLAST matches (AY033310, Suzuki *et al*. 2001; DQ009327 and DQ900359, Brown *et al*. 2005) and four cultured cyanobacterial sequences (AF397728, AF397713, AF397721, and AF397727, Rocap *et al*. 2002) from *Synechococcus* group 6 (*sensu* Honda *et al*. 1999; Robertson *et al*. 2001). A partition homogeneity test conducted in PAUP* 4.0 (Swofford 1998) yielded identical tree topologies ($P = 1.00$) for phylogenies constructed using either all sequence data (16S and ITS) or only the ITS region; subsequent phylogenetic analyses were based solely on ITS data. Consensus sequences from the ITS region were aligned with outgroup sequences using BIOEDIT and CLUSTAL_X, with a gap opening penalty of 24 and a gap extension penalty of 4.

Neighbour-joining (NJ) phylogenetic analyses of aligned DNA sequences were performed in MEGA 3.1 (Kumar *et al*. 2004), with the Kimura 2-parameter model of nucleotide substitution. Data were resampled using 1000 bootstrap replicates. Maximum-likelihood (ML) phylogenetic analyses were performed in PAUP* 4.0 (Swofford 1998) using a heuristic search; data were resampled using 100 bootstrap replicates. MODELTEST 3.7 (Posada & Crandall 1998) was used to select the best model of DNA substitution, the Hasegawa–Kishino–Yano model with a gamma distribution

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

Table 2 Comparison of the variability and phylogenetic utility of cyanobacterial 16S rRNA and 16S–23S rRNA internal transcribed spacer (ITS) gene sequences from sponge-associated *Synechococcus spongiarum* symbionts, based on pairwise percentage sequence divergence

			Distance between clones		Parsimony-informative sites	
Gene	Length (bp)	Segment	Maximum	Average	Number	Percentage
16S rRNA	737 or 1149	Partial	4.8%	1.1%	73	6.4%
16S–23S ITS	487–607	Complete	21.5%	6.7%	153	24.8%

of variable substitution rates among sites (HKY + G). For Bayesian analyses, MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003) was used to calculate the posterior probabilities of branch nodes, implementing the HKY + G model. The Monte Carlo Markov chain length was set at 7 million generations with sampling every 100th generation and a burn-in value of 17 500 cycles. After 6 852 000 generations, the average standard deviation of split frequencies between two independent chains reached less than 0.01.

Synechococcus spongiarum clades were defined as those that showed both reciprocal monophyly and greater than 3% sequence divergence from sister clades. The distribution of *S. spongiarum* clades was compared among host sponge species and higher taxonomic groupings and among geographical locations using analysis of molecular variance (AMOVA) and a phylogenetic lineage sorting test (*P* test; Martin 2002). AMOVA calculations were completed using ARLEQUIN 2.000 (Schneider *et al*. 2000), calculating distances by the Tajima–Nei algorithm and setting $\alpha = 0.05$. Among-group comparisons were calculated as F_{CT} and statistical significance was based on 1000 permutations. The *P* test determined whether the distribution of symbiont sequences among hosts and geographical regions covaried with symbiont phylogeny and was calculated using MACCLADE (Maddison & Maddison 1993) and the Bayesian tree topology.

Results

16S rRNA and 16S–23S ITS sequences

Recovered gene sequences are archived in GenBank under accession nos EF121775–EF121812 and EU307440–EU307509. The 16S rRNA regions of all recovered sequences were most similar (99% BLASTN similarity) to the sponge symbiont *Candidatus Synechococcus spongiarum* (Usher *et al*. 2004a) and related cyanobacteria belonging to a sponge-specific clade of *Synechococcus* (Steindler *et al*. 2005; Thacker 2005). *Synechococcus spongiarum* ITS sequences were most similar to those of uncultured, marine *Synechococcus* and *Prochlorococcus* bacteria. Extensive gaps were evident in these comparisons and sequences overlapped only in two tRNA coding regions (tRNA-Ile and tRNA-Ala) located within the 16S–23S ITS region. When comparing among host sponges, *S. spongiarum* ITS sequences exhibited over four times the variability of the partial 16S sequences, with higher maximum and average distances between clones (Table 2). The ITS region also had a higher number and percentage of parsimony-informative sites than the 16S region (Table 2). Since previous studies found no intragenomic variation between the two *Synechococcus* rRNA operons, including ITS sequences (Stewart & Cavanaugh 2007), we interpreted the presence of multiple ITS sequences from a single sponge host as the presence of multiple, genetically distinct symbionts.

ITS phylogenies

Phylogenetic analyses of the recovered ITS gene sequences revealed 12 distinct symbiont clades (Fig. 1), defined by reciprocal monophyly and greater than 3% sequence divergence among sister clades. Average pairwise *p*-distances (proportion of divergent nucleotide sites) were lower within symbiont clades (mean ± SE: 0.83 ± 0.19%) than among clades (9.32 ± 0.56%; Table 3). Symbiont clades were well-supported by all phylogenetic analyses, with the single exception of clade A forming a polyphyletic clade in the maximum-likelihood phylogeny. Relationships among clades were not well resolved, showing no consistent groupings across all analyses. Neighbour-joining analyses supported clade L as basal to all other symbiont clades (Fig. 1). Clades A, D and E formed a moderately supported monophyletic group in the Bayesian and neighbour-joining phylogenies. Clades G and J formed a monophyletic group in the Bayesian and likelihood phylogenies (Fig. 1).

Host specificity and geographical distribution of symbionts

The 12 distinct symbiont clades exhibited a wide range of host specificity (Table 1). Two symbiont clades (B, D) were detected in taxonomically unrelated host sponges, indicating that these clades are likely to be host generalists. Clade B symbionts inhabited six host species from three genera, two families and two sponge orders. Clade D symbionts inhabited three host species from three genera, three families and two sponge orders. Ten symbiont clades inhabited one or several closely related host species,

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

indicating that these clades may be host specialists. Clade A symbionts were recovered exclusively from four species within the genus *Aplysina*, clades G and J were recovered from three species within the genus *Ircinia*, clades H and I were recovered from two forms of the species-complex *Chondrilla nucula* Schmidt 1862 and the remaining seven symbiont clades were detected in a single host sponge and a single geographical region (Table 1).

Several sponges hosted multiple symbiont clades, occurring in six of the 18 species investigated (Table 1). Four of these species associated with generalist clade B symbionts in addition to a host-specific clade: B + A in *Aplysina fulva* (Pallas 1766), B + A in *Aplysina lacunosa* (Pallas 1766), B + C in *Neopetrosia subtriangularis* (Duchassaing 1850) and B + L in *Xestospongia muta* (Schmidt 1870). The remaining two species hosted two distinct, host-specific clades: H + I in *Chondrilla nucula f. hermatypica* Duran & Rützler 2006 and G + J in *Ircinia* sp. RA. In no case were more than two clades recovered from a single individual or host species; the majority of host sponges harboured a single symbiont clade (Table 1).

AMOVA supported a hypothesis of significant genetic differentiation of symbionts among hosts when grouped by host species (F_{CT} = 0.651, P < 0.001), family (F_{CT} = 0.312, P < 0.001), or order (F_{CT} = 0.296, P < 0.001). The P test demonstrated that hosts sample significantly different

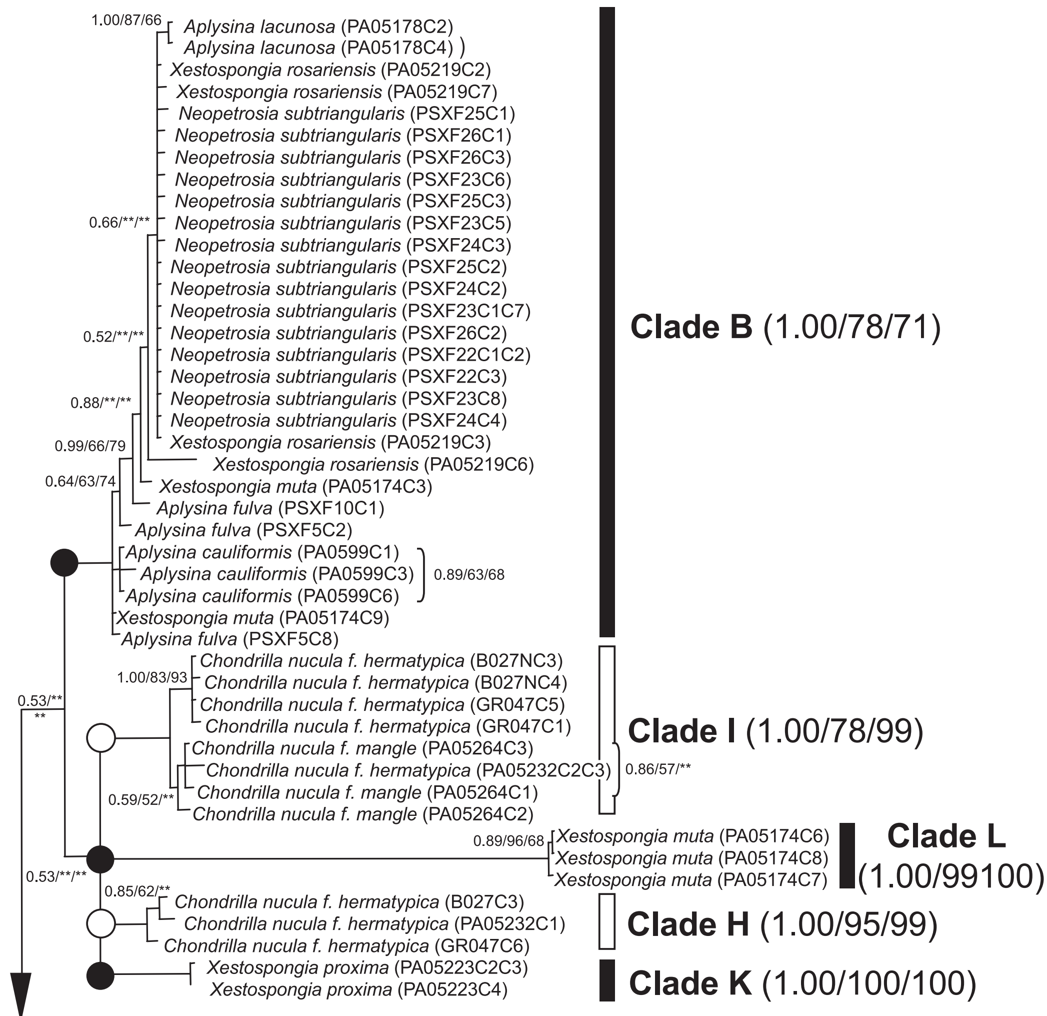

Fig. 1 Phylogeny of 16S–23S rRNA ITS gene sequences from the symbiotic cyanobacterium *Synechococcus spongiarum*, revealing 12 distinct symbiont clades. Labels on terminal nodes refer to the host sponge species of each symbiont clone, with individual sequence labels in parentheses corresponding to GenBank Accession nos EF121775–EF121812 and EU307440–EU307509. Dark and light circles indicate monophyletic symbiont clades (A–L) and correspond to dark and light bars on the right. Tree topology was constructed using Bayesian inference. Posterior probability (*PP*), maximum likelihood (ML) bootstrap and neighbour-joining (NJ) bootstrap support values are shown on the far right (in parentheses), on internal nodes and next to brackets. *PP* values less than 0.50 and ML/NJ values less than 50 are denoted by asterisks (**). Outgroup sequences include the top three GenBank matches and four congeneric, cultured *Synechococcus* strains, with GenBank Accession nos in parentheses. Scale bar represents 0.1 substitutions per site.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

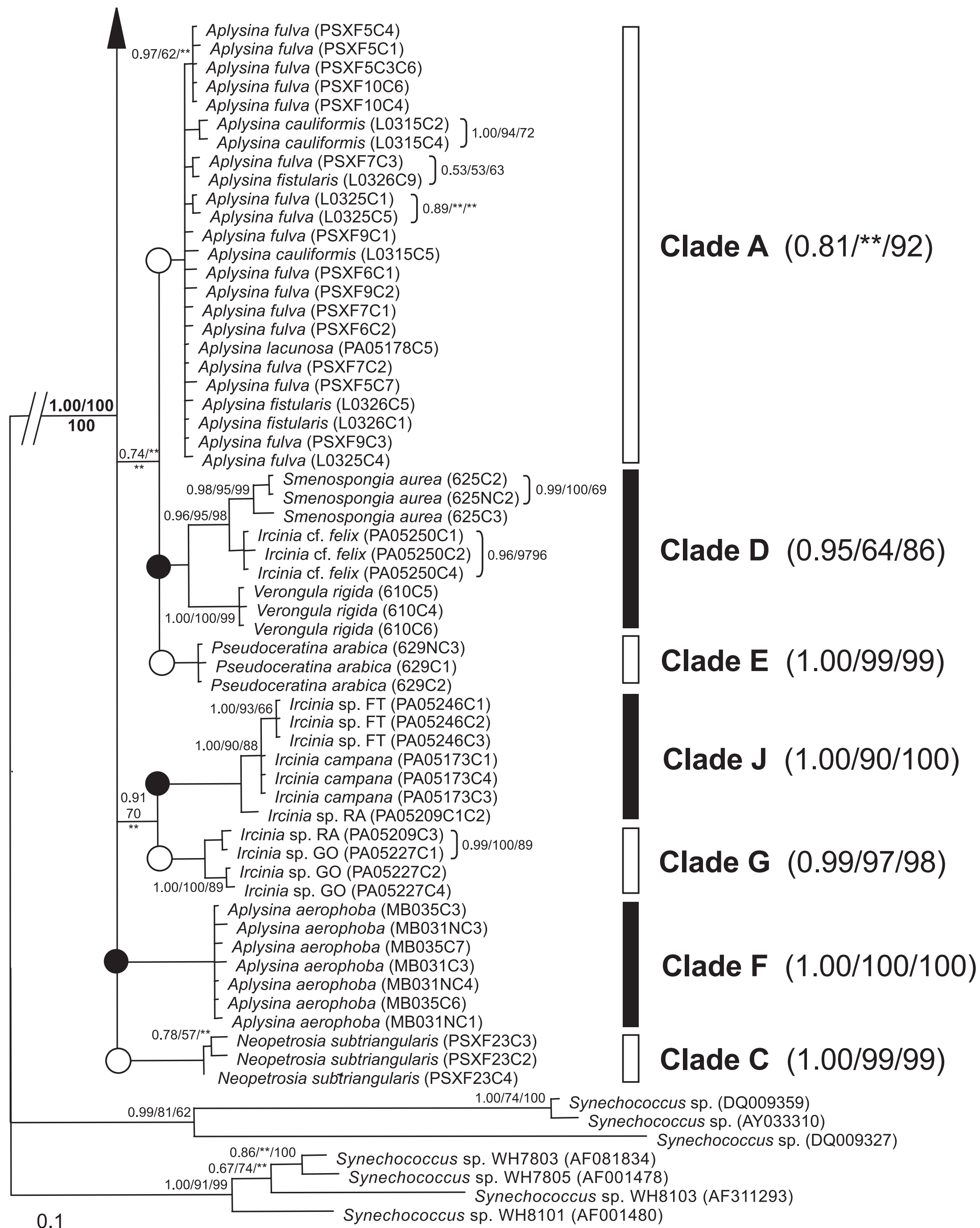

Fig. 1 *Continued*

phylogenetic lineages of symbionts when grouped by host species ($P < 0.001$), family ($P < 0.001$), or order ($P < 0.001$). Together, these tests indicate that different sponge species host significantly different subsets of *S. spongiarum* genetic diversity and distinct phylogenetic lineages of these symbionts (Martin 2002).

The composition of symbiont populations in sponge species sampled from multiple locations showed both geographical variability and stability (Table 1). *Aplysina cauliformis* (Carter 1882) and *A. fulva* specimens from the Bahamas harboured clade A symbionts, while conspecific specimens from Panamá harboured clade B or clade A and B symbionts. *Chondrilla nucula f. hermatypica* was sampled from three regions, spanning 1500 miles from the southern Caribbean to Bermuda; however, no differences in symbiont communities (clade H and I) were observed across regions.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

Table 3 Distance matrix comparing the genetic divergence within and among *Synechococcus spongiarum* clades based on 16S–23S rRNA ITS gene sequences. Numbers below the diagonal represent average pairwise *p*-distances (proportion of divergent nucleotide sites) between clades; bold values on the diagonal indicate average pairwise *p*-distances within each clade

Symbiont clade	A	B	C	D	E	F	G	H	I	J	K	L
A	**0.71**	–	–	–	–	–	–	–	–	–	–	–
B	6.35	**1.06**	–	–	–	–	–	–	–	–	–	–
C	6.48	7.62	**0.93**	–	–	–	–	–	–	–	–	–
D	4.61	7.05	8.26	**2.58**	–	–	–	–	–	–	–	–
E	3.66	5.11	7.32	4.88	**0.16**	–	–	–	–	–	–	–
F	6.71	5.84	8.74	7.52	6.02	**0.20**	–	–	–	–	–	–
G	6.51	6.50	7.50	8.90	6.80	7.99	**1.05**	–	–	–	–	–
H	6.90	6.09	7.42	8.07	6.46	6.72	6.64	**1.09**	–	–	–	–
I	8.07	7.43	8.75	8.42	7.79	8.72	7.07	5.40	**1.08**	–	–	–
J	8.29	7.95	8.68	9.11	8.36	7.28	6.65	7.33	8.32	**0.67**	–	–
K	8.53	7.93	8.06	9.25	8.14	9.33	8.00	6.97	8.29	8.63	**0.23**	–
L	18.41	19.56	18.15	20.30	19.31	19.30	18.24	18.61	18.51	18.07	21.22	**0.16**

Interestingly, the mangrove form *Chondrilla nucula f. mangle* Duran & Rützler 2006 only hosted clade I symbionts.

AMOVA did not support a hypothesis of genetic differentiation of symbionts among geographical regions ($F_{CT} = 0.163$, $P = 0.077$), but the *P* test indicated that different geographical regions contain significantly different phylogenetic lineages ($P < 0.001$). Together, these results imply that there is relatively high genetic diversity of *S. spongiarum* within geographical regions compared to total diversity and indicate the presence of distinct phylogenetic lineages within each region (Martin 2002).

Discussion

Phylogenetic analyses of 16S–23S rRNA ITS gene sequences revealed 12 distinct clades of the sponge-associated cyanobacterium *Synechococcus spongiarum*. Although intragenomic variation can complicate the analysis of ITS sequences for some cyanobacteria (Boyer *et al*. 2001), *Synechococcus* and other unicellular cyanobacteria possess only two identical copies of the rRNA operon (Chen *et al*. 2006; Stewart & Cavanaugh 2007). On average, we found < 1% ITS sequence divergence within *S. spongiarum* clades and over 9% ITS sequence divergence among clades. When comparing ITS sequences across all bacteria, grouped by tRNA composition, Stewart & Cavanaugh (2007) found an average intragenomic variation of 0.94% ITS sequence divergence. The relative similarity of our sequences within symbiont clades, even when from distinct host species, suggests that the observed differences are not simply a result of intragenomic variation. Likewise, the presence of multiple, divergent ITS sequences from a single sponge host most likely corresponds to the presence of multiple, genetically distinct symbionts within that host.

Analyses of molecular variance and phylogenetic lineage sorting tests confirmed the existence of significant host specificity among *S. spongiarum* clades. Symbiont clades ranged in specificity from two generalists (B, D) found in multiple orders of sponge hosts to 10 specialists (A, C, E–L) associated with a single family. Multiple symbionts were able to co-inhabit a single host individual, yet in no case were more than two distinct clades co-existing within a single host sponge. In contrast to ITS sequence data, cyanobacterial 16S rRNA gene sequences exhibited low levels of variability among symbiont populations (< 1% sequence divergence), providing additional evidence that this molecular marker offers minimal phylogenetic resolution at lower taxonomic scales. Investigations that rely solely on 16S rRNA sequence data may greatly underestimate the genetic diversity of sponge-associated cyanobacteria.

Recent reports describing the vertical transmission of *S. spongiarum* from parent sponges to larvae and gametes (Usher *et al*. 2001; Oren *et al*. 2005; Usher *et al*. 2005) provide a mechanism that can explain the observed host specificity of these symbioses through the physical isolation of symbiont populations within host lineages. Similarly, a filamentous cyanobacterial sponge symbiont, *Oscillatoria spongeliae* (Schulze 1879), also forms specialist associations with sponges, with genetically distinct symbiont clades inhabiting specific sponge hosts (Thacker & Starnes 2003; Ridley *et al*. 2005; Thacker *et al*. 2007). Vertical transmission and physical isolation of *S. spongiarum* lineages may reduce gene flow and promote the genetic divergence of symbiont lineages. Consistent with this hypothesis, our molecular phylogenies revealed a large polytomy among symbiont clades, which could indicate a relatively rapid divergence of symbiont populations following an initial host-colonization event. Horizontal transmission of *S. spongiarum* has not been reported, but remains plausible given the high exposure of sponges to bacteria in seawater through constant filter feeding. Rare instances of horizontal transmission offer a potential explanation for the generalist

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

nature of symbiont clades found inhabiting unrelated host sponges.

Several biogeographical trends in symbiont distribution are evident from these data, even though only six geographical regions were sampled, with the majority of samples from a single region (Panamá). Although analyses of molecular variance indicated that genetic diversity was not significantly different among regions, the *P* test found significant differences in the distribution of phylogenetic lineages among regions. Indeed, most clades were specific to a single geographical region. Some sponge species exhibited variable symbiont community structure when sampled from different areas. For example, two symbiont clades (A + B) inhabited species in the genus *Aplysina*; however, specimens collected from Bahamian reefs associated solely with clade A while specimens collected from Panamanian reefs associated with one or both symbiont clades. Conversely, other host sponges exhibited identical symbiont communities across broad geographical distances. For example, *Chondrilla nucula f. hermatypica* individuals collected from Panamá, eastern Atlantic, and Bermuda consistently hosted two symbiont clades (H + I).

Our sampling was focused primarily in Caribbean regions, but specimens from eastern Atlantic and Indo-Pacific reefs revealed preliminary evidence for interocean differences in symbiont distribution. Within the genus *Aplysina*, Caribbean species hosted clades A and B, whereas Clade F symbionts were recovered from the Mediterranean species *Aplysina aerophoba* Nardo 1843. Similarly, clade E symbionts inhabited the Indo-Pacific sponge *Pseudoceratina arabica* (Keller 1889) and were absent from the Caribbean sponges investigated. Additional sampling is clearly required to accurately delineate the geographical distribution of these 12 symbiont clades and to disentangle the simultaneous effects of host specificity and geography. For example, the absence of clade E and F symbionts in Caribbean sponge populations could suggest a biogeographical component to symbiont community structure or could result from these symbiont clades being found only in host species that are restricted to eastern Atlantic and Indo-Pacific reefs.

Experimental manipulations of sponge–cyanobacteria symbioses have used reduced irradiance to assess the effects of decreased symbiont load on host metabolism, revealing variable host–symbiont interactions. In the genus *Aplysina* (Order Verongida), the Mediterranean species *A. aerophoba* and the Caribbean species *A. fulva* exhibited significant reductions in growth under shaded conditions, where symbiont populations were reduced (Wilkinson & Vacelet 1979; Erwin & Thacker, in press), suggesting a substantial contribution by cyanobacterial symbionts to host nutrition. The Mediterranean species *Chondrilla nucula* (Order Chondrosida) underwent metabolic collapse when transplanted from high-irradiance reef to low-irradiance cave environments (Arillo *et al*. 1993), suggesting that this species can also be strongly dependent on symbiont photosynthesis. In contrast, the growth rates of several species in the Order Haplosclerida were not affected by short-term decreases in symbiont load, including the Indo-Pacific species *Neopetrosia exigua* (Kirkpatrick 1900) (Thacker 2005) and the Caribbean species *Xestospongia muta* (Gómez *et al*. 2002) and *Neopetrosia subtriangularis* (Erwin & Thacker, in press), suggesting that these hosts either receive little nutritional benefit from their photosynthetic symbionts or can compensate for the loss of their symbionts.

The observed genetic diversity of *S. spongiarum* 16S–23S rRNA ITS gene sequences suggests that the variable host–symbiont interactions demonstrated by field experiments may result from distinct symbiont clades inhabiting specific host species. For example, symbiont clade A is associated specifically with the genus *Aplysina*, whose species exhibit reduced growth rates when symbiont populations are low; thus clade A symbionts are correlated with the provision of supplemental nutrition to their hosts. Further experimentation is needed to test whether these correlations reflect causal relationships. Local environmental conditions may also influence the structure of these symbiont communities. Within the *C. nucula* species complex, the reef subspecies *C. nucula f. hermatypica* harboured symbiont clades H and I across a large geographical range; however, within a single region, the mangrove subspecies *Chondrilla nucula f. mangle* hosted only clade I. Environmental conditions characteristic of mangrove habitats (e.g. low irradiance, high nutrients) may preclude clade H symbionts, which could potentially be adapted to reef conditions (e.g. high irradiance, low nutrients), from colonizing and/or surviving in mangrove sponge hosts.

Our phylogenetic tree also suggests that clade L symbionts represent a transitional lineage from free-living to obligate cyanobacterial symbionts. Clade L was recovered from *X. muta* and formed a basal *S. spongiarum* lineage in neighbour-joining analyses. In addition to experimental evidence suggesting a commensal relationship between *Xestospongia* spp. and their cyanobacterial symbionts (Gómez *et al*. 2002; Thacker 2005), the giant barrel sponge *X. muta* commonly exhibits nonlethal and cyclic bleaching events where symbiont populations are greatly reduced with no apparent negative consequences to host sponge fitness (Cowart *et al*. 2006). These observations suggest that *S. spongiarum* populations in *X. muta* might represent facultative symbionts able to survive both as sponge symbionts and free-living plankton. In contrast, bleaching of the Australian species *Chondrilla australiensis*, also known to harbour *S. spongiarum* (Usher *et al*. 2004a), resulted in high mortality rates when symbiont loss persisted for 3 months (Fromont & Garson 1999), suggesting a more obligate host–symbiont relationship.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

The phylogenetic data presented here and previous field-based experimental manipulations reveal several emerging parallels between sponge–cyanobacteria symbioses and the well-studied coral–zooxanthellae symbioses, with both groups of reef organisms hosting genetically diverse symbionts that display variable host specificity, transmission modes and benefits to their hosts. In both associations, symbionts originally described as single species, *S. spongiarum* in sponges and *Symbiodinium microadriaticum* Freudenthal 1962 in corals, now appear to represent genetically diverse species complexes that span a wide range of host specificity (Rowan & Powers 1991; Rowan 1998; LaJeunesse 2001). Multiple symbiont clades can inhabit a single host and distinct symbiont clades may exhibit different physiological capabilities and host-derived benefits (Coles & Brown 2003; Rowan 2004). Corals may be able to acclimate to changing environmental conditions by expelling current symbiont populations (i.e. bleaching) and establishing new assemblages that are better adapted to ambient conditions (Buddemeier & Fautin 1993; Baker 2001; Sotka & Thacker 2005). The exploration of new molecular markers and additional taxonomic and geographical sampling are needed to further elucidate the biogeographical distribution and genetic diversity of *S. spongiarum*. Future investigations characterizing host–symbiont interactions will reveal how far these parallels with coral–zooxanthellae symbioses extend, in particular whether sponge–cyanobacteria symbioses are flexible enough to allow for fluctuations in symbiont community structure that increase host sponge fitness under changing environmental conditions.

Acknowledgements

We thank C. Diaz, R. Collin, G. Jacome, P. Gondola, A. Castillo, A. Amat, and the staff of the Smithsonian Tropical Research Institute's Bocas del Toro Research Station for field and laboratory support. M. Becerro provided *Aplysina aerophoba* samples, and M. Slattery, D. Gochfeld, D. Gleason, and G. McFall assisted with field collections. Additionally, we thank the Caribbean Marine Research Center (Lee Stocking Island, Bahamas), the Coral Reef Research Foundation (Koror, Palau), and NOAA's Gray's Reef National Marine Sanctuary for field support and assistance in obtaining collection permits. Helpful comments and suggestions from M. Becerro and M. Taylor improved this manuscript. M. Salazar and the UAB CFAR DNA Sequencing Core Facility provided DNA sequencing support; this work has been facilitated by the infrastructure and resources provided by NIH CFAR Core Grant P30 AI27767. Funding was provided by grants from the Society for Integrative and Comparative Biology, the Sigma Xi Research Society, and the STRI Short-Term Fellowship Program awarded to PME. Additional funding was provided by the National Science Foundation under Grant no. 0209329 awarded to R.W.T. and by a grant from the National Institute for Undersea Science and Technology awarded to R.W.T. The H.R. Whiteley Center and the Friday Harbor Laboratories generously provided R.W.T. with space and support for the preparation of this manuscript.

References

Arillo A, Bavestrello G, Burlando B, Sarà M (1993) Metabolic integration between symbiotic cyanobacteria and sponges: a possible mechanism. *Marine Biology*, **117**, 159–162.

Baker AC (2001) Reef corals bleach to survive change. *Nature*, **411**, 765–766.

Becerro MA, Turon X, Uriz MJ, Templado J (2003) Can a sponge feeder be a herbivore? *Tylodina perversa* (Gastropoda) feeding on *Aplysina aerophoba* (Demospongiae). *Biological Journal of the Linnean Society*, **78**, 429–438.

Becerro MA, Starmer JA, Paul VJ (2006) Chemical defenses of cryptic and aposematic gastropterid mollusks feeding on their host sponge *Dysidea granulosa*. *Journal of Chemical Ecology*, **32**, 1491–1500.

Boyer SL, Flechtner VR, Johansen JR (2001) Is the 16S–23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. *Molecular Biology and Evolution*, **18**, 1057–1069.

Brown MV, Schwalbach MS, Hewson I, Fuhrman JA (2005) Coupling 16S-ITS rDNA clone libraries and automated ribosomal intergenic spacer analysis to show marine microbial diversity: development and application to a time series. *Environmental Microbiology*, **7**, 1466–1479.

Buddemeier RW, Fautin DG (1993) Coral bleaching as an adaptive mechanism. *Bioscience*, **43**, 320–326.

Chen F, Wang K, Kan J, Suzuki MT, Wommack KE (2006) Diverse and unique picocyanobacteria in Chesapeake Bay, revealed by 16S–23S rRNA internal transcribed spacer sequences. *Applied and Environmental Microbiology*, **72**, 2239–2243.

Coles SL, Brown BE (2003) Coral bleaching – capacity for acclimatization and adaptation. *Advances in Marine Biology*, **46**, 183–223.

Cowart JD, Henkel TP, McMurray SE, Pawlik JR (2006) Sponge orange band (SOB): a pathogenic-like condition of the giant barrel sponge, *Xestospongia muta*. *Coral Reefs*, **25**, 513.

Diaz MC (1996) *Molecular and ecological studies of sponge–microbial associations*. PhD Dissertation. University of California, Santa Cruz, California.

Erwin PM, Thacker RW (2007) Incidence and identity of photosynthetic symbionts in Caribbean coral reef sponge assemblages. *Journal of the Marine Biological Association of the United Kingdom*, **87**, 1683–1692.

Erwin PM, Thacker RW (in press) Phototrophic nutrition and symbiont diversity of two Caribbean sponge-cyanobacteria symbioses. *Marine Ecology Progress Series*.

Flatt PM, Gautschi JT, Thacker RW *et al*. (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. *Marine Biology*, **147**, 761–774.

Fromont J, Garson M (1999) Sponge bleaching on the West and East coasts of Australia. *Coral Reefs*, **18**, 340.

Gómez R, Erpenbeck D, van Dijk T *et al*. (2002) Identity of cyanobacterial symbiont of *Xestospongia muta*. *Bollettino dei Musei e degli Istituti Biologici dell'Università di Genova*, 66–67, 82–83.

Hall TA (2000) BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Hentschel U, Hopke J, Horn M *et al*. (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied and Environmental Microbiology*, **68**, 4431–4440.

Hill M, Hill A, Lopez N, Harriott O (2006) Sponge-specific bacterial

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

symbionts in the Caribbean sponge, *Chondrilla nucula* (Demospongiae, Chondrosida). *Marine Biology*, **148**, 1221–1230.

Honda D, Yokota A, Sugiyama J (1999) Detection of seven major evolutionary lineages in cyanobacteria based on the 16S rRNA gene sequence analysis with new sequences of five marine *Synechococcus* strains. *Journal of Molecular Evolution*, **48**, 723–739.

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.

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics*, **5**, 150–163.

LaJeunesse TC (2001) Investigating the biodiversity, ecology and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using the ITS region: in search of a 'species' level marker. *Journal of Phycology*, **37**, 866–880.

Laloui W, Palinska KA, Rippka R *et al*. (2002) Genotyping of axenic and non-axenic isolates of the genus *Prochlorococcus* and the OMF-'*Synechococcus*' clade by size, sequence analysis or RFLP of the internal transcribed spacer of the ribosomal operon. *Microbiology*, **148**, 453–465.

Maddison WP, Maddison DR (1993) *MACCLADE, v. 3*. Sinauer & Associates, Sunderland, Massachusetts.

Martin AP (2002) Phylogenetic approaches for describing and comparing the diversity of microbial communities. *Applied and Environmental Microbiology*, **68**, 3673–3682.

Moore LR, Rocap G, Chisholm SW (1998) Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. *Nature*, **393**, 464–467.

Nübel U, Garcia-Pichel F, Muyzer G (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and Environmental Microbiology*, **63**, 3327–3332.

Olson JM, McCarthy PJ (2005) Associated bacterial communities of two deep-water sponges. *Aquatic Microbial Ecology*, **39**, 47–55.

Oren M, Steindler L, Ilan M (2005) Transmission, plasticity and molecular identification of cyanobacterial symbionts in the Red Sea sponge *Diacarnus erythraenus*. *Marine Biology*, **148**, 35–41.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Preston CM, Wu KY, Molinski TF, DeLong EF (1996) A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* General nov., sp. nov. *Proceedings of the National Academy of Sciences, USA*, **93**, 6241–6246.

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. *Marine Biology*, **137**, 453–461.

Ridley CP, Bergquist PR, Harper MK *et al*. (2005) Speciation and biosynthetic variation in four dictyoceratid sponges and their cyanobacterial symbiont, *Oscillatoria spongeliae*. *Chemistry and Biology*, **12**, 397–406.

Robertson BR, Tezuka N, Watanabe MM (2001) Phylogenetic analysis of *Synechococcus* strains (cyanobacteria) using sequences of 16S rDNA and part of the phycocyanin operon reveal multiple evolutionary lines and reflect phycobilin content. *International Journal of Systematic and Evolutionary Microbiology*, **51**, 861–871.

Rocap G, Distel DL, Waterbury JB, Chisholm SW (2002) Resolution of *Prochlorococcus and Synechococcus* ecotypes by using 16S–23S ribosomal DNA internal transcribed spacer sequences. *Applied and Environmental Microbiology*, **68**, 1180–1191.

Rocap G, Larimer FW, Lamerdin J *et al*. (2003) Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. *Nature*, **424**, 1042–1047.

Ronquist F, Huelsenbeck JP (2003) MRBAYES3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Rowan R (1998) Diversity and ecology of zooxanthellae on coral reefs. *Journal of Phycology*, **34**, 407–417.

Rowan R (2004) Thermal adaptation in reef coral symbionts. *Nature*, **430**, 742.

Rowan R, Powers DA (1991) A molecular genetic classification of zooxanthellae and the evolution of animal-algal symbioses. *Science*, **251**, 1348–1351.

Rützler K (1990) Associations between Caribbean sponges and photosynthetic organisms. In: *New Perspectives in Sponge Biology* (ed. Rützler K), pp. 455–465. Smithsonian Institution Press, Washington, DC.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN, Version 2.000: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Smith DC (1991) Why do so few animals form endosymbiotic associations with photosynthetic microbes? *Philosophical Transactions of the Royal Society B: Biological Sciences*, **333**, 225–230.

Sotka EE, Thacker RW (2005) Do some corals like it hot? *Trends in Ecology & Evolution*, **20**, 59–62.

Steindler L, Huchon D, Avni A, Ilan M (2005) 16S rRNA phylogeny of sponge-associated cyanobacteria. *Applied and Environmental Microbiology*, **71**, 4127–4131.

Stewart FJ, Cavanaugh CM (2007) Intragenomic variation and evolution of the internal transcribed spacer of the rRNA operon in bacteria. *Journal of Molecular Evolution*, **65**, 44–67.

Suzuki MT, Béjà O, Taylor LT, DeLong EF (2001) Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. *Environmental Microbiology*, **3**, 323–331.

Swofford DL (1998) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4.0b10, Sinauer & Associates, Sunderland, Massachusetts.

Taylor MW, Hill RT, Piel J *et al*. (2007a) Soaking it up: the complex lives of marine sponges and their microbial associates. *International Society for Microbial Ecology Journal*, **1**, 187–190.

Taylor MW, Radax R, Steger D, Wagner M (2007b) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews*, **71**, 295–347.

Taylor MW, Thacker RW, Hentschel U (2007c) Evolutionary insights from sponges. *Science*, **316**, 1854–1855.

Thacker RW (2005) Impacts of shading on sponge-cyanobacteria symbioses: a comparison between host-specific and generalist associations. *Integrative and Comparative Biology*, **45**, 369–376.

Thacker RW, Starnes S (2003) Host specificity of the symbiotic cyanobacterium *Oscillatoria spongeliae* in marine sponges, *Dysidea* spp. *Marine Biology*, **142**, 643–648.

Thacker RW, Diaz MC, Rützler K *et al*. (2007) Filamentous cyanobacterial symbionts of Caribbean sponges: phylogenetic relationships and photosynthetic production. In: *Porifera Research: Biodiversity, Innovation and Sustainability* (eds Hajdu E, Muricy G), pp. 621–626. Museu Nacional, Rio de Janeiro, Brazil.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**, 4876–4882.

Usher KM, Kuo J, Fromont J, Sutton DC (2001) Vertical transmission

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

of cyanobacterial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Hydrobiologia*, **461**, 15–23.

Usher KM, Fromont J, Sutton DC, Toze S (2004a) The biogeography and phylogeny of unicellular cyanobacterial symbionts in sponges from Australia and the Mediterranean. *Microbial Ecology*, **48**, 167–177.

Usher KM, Sutton DC, Toze S, Kuo J, Fromont J (2004b) A new species of cyanobacterial symbiont from the marine sponge *Chondrilla nucula*. *Symbiosis*, **36**, 183–192.

Usher KM, Sutton DC, Toze S, Kuo J, Fromont J (2005) Inter-generational transmission of microbial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Marine and Freshwater Research*, **56**, 125–131.

Webster NS, Wilson KJ, Blackall LL, Hill RT (2001) Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Applied and Environmental Microbiology*, **67**, 434–444.

Webster NS, Negri AP, Munro MMHG, Battershill CN (2004) Diverse microbial communities inhabit Antarctic sponges. *Environmental Microbiology*, **6**, 288–300.

Wilkinson CR (1978) Microbial associations in sponges. I. Ecology, physiology and microbial populations of coral reef sponges. *Marine Biology*, **4**, 161–167.

Wilkinson CR (1983) Net primary productivity in coral reef sponges. *Science*, **219**, 410–412.

Wilkinson CR, Vacelet J (1979) Transplantation of marine sponges to different conditions of light and current. *Journal of Experimental Marine Biology and Ecology*, **37**, 91–104.

Wilkinson CR, Nowak M, Austin B, Colwell RR (1981) Specificity of bacterial symbionts in Mediterranean and Great Barrier Reef sponges. *Microbial Ecology*, **7**, 13–21.

Yahel G, Sharp JH, Marie D, Häse C, Genin A (2003) In situ feeding and element removal in the symbiont-bearing sponge *Theonella swinhoei*: Bulk DOC is the major source for carbon. *Limnology and Oceanography*, **48**, 141–149.

Patrick M. Erwin has focused his research efforts on using molecular tools to understand the ecological nature of sponge–bacteria symbioses. Robert W. Thacker studies the ecology and evolution of sponges and their symbiotic microbial communities.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd