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Authors

Sipkema, Detmer
Holmes, Bradley
Nichols, Scott A.
[et al.](#)

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Biological Characterisation of *Haliclona* (?gellius) sp.: Sponge and Associated Microorganisms

Detmer Sipkema · Bradley Holmes · Scott A. Nichols · Harvey W. Blanch

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Abstract We have characterised the northern Pacific undescribed sponge *Haliclona* (?gellius) sp. based on rDNA of the sponge and its associated microorganisms. The sponge is closely related to *Amphimedon queenslandica* from the Great Barrier Reef as the near-complete 18S rDNA sequences of both sponges were identical. The microbial fingerprint of three specimens harvested at different times and of a transplanted specimen was compared to identify stably associated microorganisms. Most bacterial phyla were detected in each sample, but only a few bacterial species were determined to be stably associated with the sponge. A sponge-specific β - and γ -*Proteobacterium* were abundant clones and both of them were present in three of the four specimens analysed. In addition, a *Planctomycete* and a *Crenarchaea* were detected in all sponge individuals. Both were closely related to operational taxonomic units that have been found in other sponges, but not exclusively in sponges. Interestingly, also a number of clones that are closely related to intracellular symbionts from insects and amoeba were detected.

Introduction

Marine sponges have proven to be a rich reservoir for bioactive metabolites (e.g. [8, 47]). An early, conservative estimate based upon thousands of assayed sponge species suggested that as many as 11% produce cytotoxic compounds [10]. This percentage is high compared to other organisms and possibly may result from the intimate association between sponges and diverse microbial communities. Marine sponges provide a home to species from most bacterial phyla [15, 54], but in most cases it is unclear whether the cytotoxic compounds are produced by the sponges or by their associated bacteria. The issue is difficult to resolve, as neither sponges nor their associated bacteria can be readily cultured in the laboratory [2, 11, 15, 39] and to produce secondary metabolites from sponges it is necessary to identify their source. In a few instances, the producer of the secondary metabolite has been identified. For example, the sesquiterpenoid hydroquinone avarol, isolated from the marine sponge *Dysidea avara*, was localised inside choanocytes of the sponge [57], while the cytotoxic macrolide swinholide isolated from the sponge *Theonella swinhoei* was produced by unicellular bacteria inhabiting the sponge [3]. Other important sponge-derived secondary metabolites, such as polyketides, are generally associated with bacterial metabolism [42], but their putative production by bacterial sponge symbionts can only be confirmed if a polyketide-producing strain is found in stable association with the sponge.

The current paradigm is that certain bacterial clades are found only in marine sponges [14] and should be regarded as an integral part of the sponge. Sponges that harbour microorganisms of sponge-specific clades are generally high-microbial-density sponges, whereas low-microbial-density sponges are thought to have less specific associated

D. Sipkema (✉) · B. Holmes · H. W. Blanch
Department of Chemical Engineering,
University of California Berkeley,
201 Gilman Hall,
Berkeley, CA 94720, USA
e-mail: detmer.sipkema@wur.nl

S. A. Nichols
Department of Molecular and Cell Biology,
University of California Berkeley,
142 Life Sciences Addition,
Berkeley, CA 94720, USA

microorganisms [16]. The existence of sponge-specific clades suggests a certain dependency of these bacteria on the sponge (e.g. providing an appropriate growth environment). It has been documented that sponges provide a habitat that is richer in nutrients than seawater and sediments (reviewed in [29]) and could prevent the inhabitants from drifting away to less favourable environments. While this may be the case, it does not explain species specificity of the symbionts that reside in marine sponges. High levels of specificity suggest that the interaction is more likely to be based on metabolite exchange. Such relationships were predicted by Reisinger [41] and a number of examples support this hypothesis, including the translocation of glucose from symbiotic green algae to their sponge host [65] and translocation of nitrogen from bacteria to the sponge [63]. Of many other bacterial metabolic processes that take place in marine sponges, it is yet unclear whether they are part of a commensal or a mutualistic relationship, such as sulphate reduction [18], methane oxidation [59] and putative ammonia oxidation by symbiotic crenarchaeota [12].

The hypothesis that bacteria from certain clades are only found in marine sponges does not imply that they need to be present in every individual of a certain sponge species. To date, studies of the intraspecific variation of sponge-associated bacteria have precluded generalisation. While the sponge species *Chondrilla nucula* [56], *Ircinia felix*, *Aplysina cauliformis*, *Niphates erecta* [63] and *Cymbastela concentrica* [53] have low intraspecific bacterial variation, the sponge species *Tethya aurantium* [55], *Halichondria panicea* [1, 64] and *Ircinia felix* larvae [44] exhibit considerable intraspecific variation.

In the present study, we have analysed the sponge-microbe consortium of *Haliclona* (?gellius) sp. from western North America, a region that has been characterised as an underrepresented location with regard to 16S

rRNA gene libraries from sponges [54]. It is our hypothesis that if certain associated microbes are present in the sponge at different times of the year and if they persist in the sponge when it is translocated to a different environment, they are potentially stable associates of the sponge.

Materials and Methods

Sample Collection

Sponges of the genus *Haliclona* (Fig. 1a) were collected near the Monterey harbour at a depth of approximately 12 m on April 15, 2006 (Hg1 and Hg9), May 24, 2006 (Hg5), January 23, 2007 (Hg6) and October 15, 2007 (for electron microscopy). The sponges were rinsed three times with sterile artificial seawater (natural sea salt mix, Oceanic Systems, Dallas, TX, USA) before grinding the tissue with a sterilised mortar and pestle. Two tissue volumes of sterile artificial seawater (ASW) were used to obtain a homogeneous cell suspension. The cell suspension was divided in aliquots of 1.2 ml and mixed with 0.6 ml 50% glycerol in ASW. The samples were gently frozen until -20°C before they were stored at -80°C . One specimen (Hg9) was maintained in an aquarium with $0.22\ \mu\text{m}$ filtered natural seawater at 10°C for 1 month before macerating the tissue. The identity of the specimens was confirmed by 18S rRNA gene analysis.

DNA Library Construction

Genomic DNA was extracted from cryopreserved cells from four specimens (Hg1, Hg5, Hg6 and Hg9) using the Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). Cryopreserved cell suspension ($400\ \mu\text{l}$) was used for DNA isolation. The cell suspension was centrifuged at

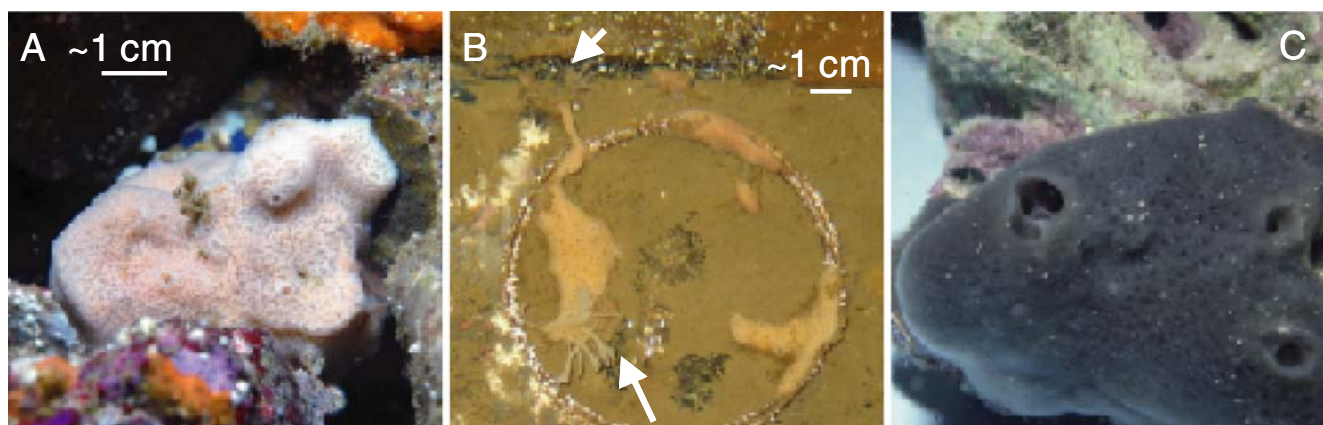


Figure 1 *Haliclona* (?gellius) sp. in the sea (a) and in the lab (b). Formation of new tissue at the tip of an extension is marked with the white arrow. *Amphimedon queenslandica* (c) is closely related to

Haliclona (?gellius) based on 18S rDNA comparison [*A. queenslandica* picture courtesy of John Hooper]

17,900×g and the supernatant was decanted. DNA was isolated according to manufacturer's instructions.

PCR was performed with several primer sets (Table 1) according to the following profile: initial denaturation for 10 min at 95°C; ten cycles of denaturation for 30 s at 95°C, annealing for 30 s at 58°C (touch down to 49°C) and elongation for 1 min 45 s at 72°C; 25 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and elongation for 1 min 45 s at 72°C; and a final extension step for 10 min at 72°C.

PCR products of successful reactions were cloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Of the bacterial 16S products that were obtained with the primer set 8F–1492R, a total of 837 colonies were picked, cultured and plasmids purified (Hg1/276 colonies; Hg5/184; Hg6/193; Hg9/184). A total of 559 clones with an archaeal 16S PCR product (primers 21F–958R) were picked, cultured and plasmids purified (Hg1/192; Hg5/192; Hg6/96; Hg9/79). Twenty clones containing a eukaryotic 18S rRNA gene segment were picked, cultured and plasmids purified. For Hg1 (four clones), Hg5 (four) and Hg9 (four), the EUKF–EUKR primer set was used, while for Hg6 (eight) only the 82FE–690RE primer set was successful.

Plasmids containing a PCR product were sequenced using the primers that were used for the PCR. Sequence chromatograms were manually inspected and assembled using the Contig Express software (Invitrogen). Sequences with similarities >99% were considered as one operational taxonomic unit (OTU) [49]. In addition, they were checked for possible chimeric origins by using the program Bellerophon (version 3) from the Greengenes website [22]. Sequences were deposited in the NCBI Genbank under accession numbers: EU095523 (18S rRNA gene *H. (?gellius)* sp.), EU236274–EU236437 and EU817104–EU817119 (16S rRNA bacteria) and EU251478–EU251483 (16S rRNA archaea).

Phylogenetic Analysis

The DNA sequences of the OTUs and nearest neighbours (as determined using the Greengenes database <http://greengenes.>

ibl.gov) were imported in the ARB software package [32]. DNA sequences were aligned using the FastAlign function of the alignment editor implemented in the ARB program and refined manually. Ambiguous regions of the alignment were systematically removed using the program Gblocks v.0.91b [4]. The default program parameters were used, exclusive of allowing a minimum block length of 5 and gaps in 50% of positions. Bayesian (MB), maximum likelihood (ML) and maximum parsimony (MP) phylogenetic methods were employed to estimate phylogenetic relationships in the data sets. MP trees were generated with PAUP* [52]. Heuristic searches consisted of 1,000 random step-wise-addition-sequence replicates using TBR branch swapping. ML trees were calculated with the GTR model and estimated proportion of invariable sites and gamma distribution in GARLI v0.951 [66]. Runs were automatically terminated when tree topology did not change for 10,000 generations. Five separate runs were performed and the tree with the best likelihood was selected. Bayesian analyses were performed using MrBayes v3.0b4 [23]. All parameters were treated as unknown variables with uniform prior-probability densities at the beginning of each run and their values were estimated from the data during the analysis [43]. All Bayesian analyses were initiated with random starting trees and were run for 1×10^7 generations.

Rarefaction Analysis

The extent of diversity examined in the clone libraries was determined by rarefaction analysis. Rarefaction curves were obtained using the algorithm described by Hurlbert [24] with the freeware program aRarefactWin (<http://www.uga.edu/~strata/software.html>). Rarefaction curves were plotted and regressions performed using two different regression equations [55]:

$$y = a(1 - e^{-bx}) \quad (1)$$

$$y = a(1 - e^{-bx^c}) \quad (2)$$

Table 1 Primers used for this study

Primer	Sequence 5'–3'	Target	Reference
8F	aga gtt tga tcc tgg ctc ag	Eubacteria 16S rRNA gene	[9]
1492R	ggt tac ctt gtt acg act t	Eubacteria 16S rRNA gene	[28]
21F	ttc cgg ttg atc cyg ccg ga	Archaea 16S rRNA gene	[6]
958R	ycc ggc gtt gam tcc aat t	Archaea 16S rRNA gene	[6]
EUKF	aac ctg gtt gat cct gcc agt	Eukaryotes 18S rRNA gene	[34]
EUKR	tga tcc ttc tgc agg ttc acc tac	Eukaryotes 18S rRNA gene	[34]
82FE	gaa dct gyg aay ggc tc	Eukaryotes 18S rRNA gene	[5]
690RE	tcc aag aat ttc acc	Eukaryotes 18S rRNA gene	[19]

The combinations used were 8F–1492R for bacterial 16S rDNA amplification, 21F–958R for archaeal 16S rDNA amplification and EUKF–EUKR and 82FE–690RE for eukaryotic 18S rDNA amplification

where x is the sample size, y the observed number of OTUs, a the expected number of OTUs with infinite sample size and b and c are regression variables.

Transmission Electron Microscopy

A fresh sponge was cut into fragments of 1 mm³. The fragments were prepared for transmission electron microscopy (TEM) by overnight fixation with 2.5% glutaraldehyde in autoclaved natural seawater. After rinsing the sample 3×15 min with autoclaved natural seawater, the sample was postfixed in autoclaved natural seawater containing 1% osmium tetroxide for 2 h. After rinsing 3×15 min with autoclaved natural seawater and 3×10 min with distilled water the sample was incubated overnight in 0.5% uracyl acetate in the dark at 4°C. After the overnight incubation, the sample was rinsed 3×15 min with distilled water and dehydrated in an acetone series (35, 50, 70, 80, 95, 100 v/v). The dehydrated sample was infiltrated with resin (epon–araldite mixture) by 1 h incubation steps with 2:1, 1:1 and 1:2 proportions of acetone:resin. After overnight incubation in pure resin, the resin was polymerised in moulds and the sample was incubated at 60°C for 48 h. The sample was sectioned by using a microtome (RMC MTX, Reichert Ultracut E, RMC MT6000) with a diamond blade and the tissue was observed with a FEI Tecnai 12 transmission electron microscope.

Results

Sponge

Based upon outer appearance, spicule size and shape (oxeas 147–178–198 µm; sigmas 29–36–41 µm C shaped), colour (beige with purple tinge to rose–lavender) and habitat, the sponge species was identified as *Haliclona* (?gellius) sp. (personal communication R.W.M. van Soest and W.L. Lee; [7]). The species was first named *Sigmatocia* sp. [13] and further analysed by Lee et al. [30]. A characteristic that was never observed in the sea, but was observed every time when individuals of *H. (?gellius)* sp. were transplanted to an aquarium in the laboratory, was the formation of finger-shaped extensions (Fig. 1b) and the formation of new sponge tissue at the tips of some extensions. The process resembles budding although the extensions never had a well-defined shape at the tips.

18S rDNA data were used to determine the phylogenetic position of *H. (?gellius)* sp. in the phylogeny of the order Haplosclerida recently published by Redmond and colleagues (2007). The maximum likelihood phylogram obtained after including *H. (?gellius)* sp. and its nearest

neighbour, *Amphimedon queenslandica* (which was identical to *H. (?gellius)* sp.), was highly similar to the ML tree published by Redmond et al. [40]. Both species branch deeply in the order Haplosclerida in a clade with *Xestospongia muta*, *Oceanapia* sp. and *Dasychalina fragilis* while they are only distantly related to the other *Haliclona* spp. (Fig. 2). A slightly different topology from Redmond et al. was obtained due to repositioning of *D. fragilis* and *Oceanapia* sp. in a separate clade (posterior probability 97%, maximum parsimony bootstrap 68%) with the two new species in the tree and *X. muta*. Phylogenetic tree topologies generated with parsimony, maximum likelihood and a Bayesian model were highly similar (data not shown).

Bacteria

Bacteria were determined to reside primarily extracellularly in the mesohyl (Fig. 3) in moderate numbers. A total of 796 high-quality clone sequences were obtained and, based on the rule of thumb of Stackebrandt and Ebers [49] that sequences that share <99% similarity are considered to be derived from different operational taxonomic units (OTUs), these represented 170 OTUs. Rarefaction curves showed that actual bacterial diversity is probably well over 250 different OTUs for *H. (?gellius)* sp. (Fig. 4). Regression model 2 gave a slightly better fit, which is to be expected when a three-parameter model is compared to a two-parameter model. Coverage of the bacterial diversity is estimated between 37% and 63%, for regression models 2 and 1, respectively (Table 2). Coverage of bacterial diversity of individual specimens was generally higher than for the combined sponge samples. Despite average coverage of the samples of approximately 55%, the high number of analysed clones implies that the part of bacterial diversity that was not detected probably represents a minor quantitative fraction in the sponge. Clone libraries for sponge individuals typically contained 17–36 OTUs, but 101 OTUs were found in Hg9, which accounted for more than 50% of the total diversity that was observed.

Representatives of most bacterial phyla including α -, β -, γ -, δ - and ϵ -Proteobacteria, Cyanobacteria, Planctomycetes, Firmicutes, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Fusobacteria, Nitrospirae, Verrucomicrobia and candidate division TM6 were present in *H. (?gellius)* sp. (Fig. 5). γ -Proteobacteria (54.8%) formed the dominant group based on PCR product frequency, followed by Firmicutes (12.1%), Planctomycetes (9.4%), β -Proteobacteria (7.3%), α -Proteobacteria (6.9%) and Bacteroidetes (3.0%). For each sponge individual, the γ -Proteobacteria dominated the cloned 16S PCR products. α -, β -, δ -Proteobacteria and a significant proportion of Planctomycetes were also present in all individuals. Acidobacteria, Actinobacteria, Chloroflexi, ϵ -Proteobacteria, Fusobacteria,

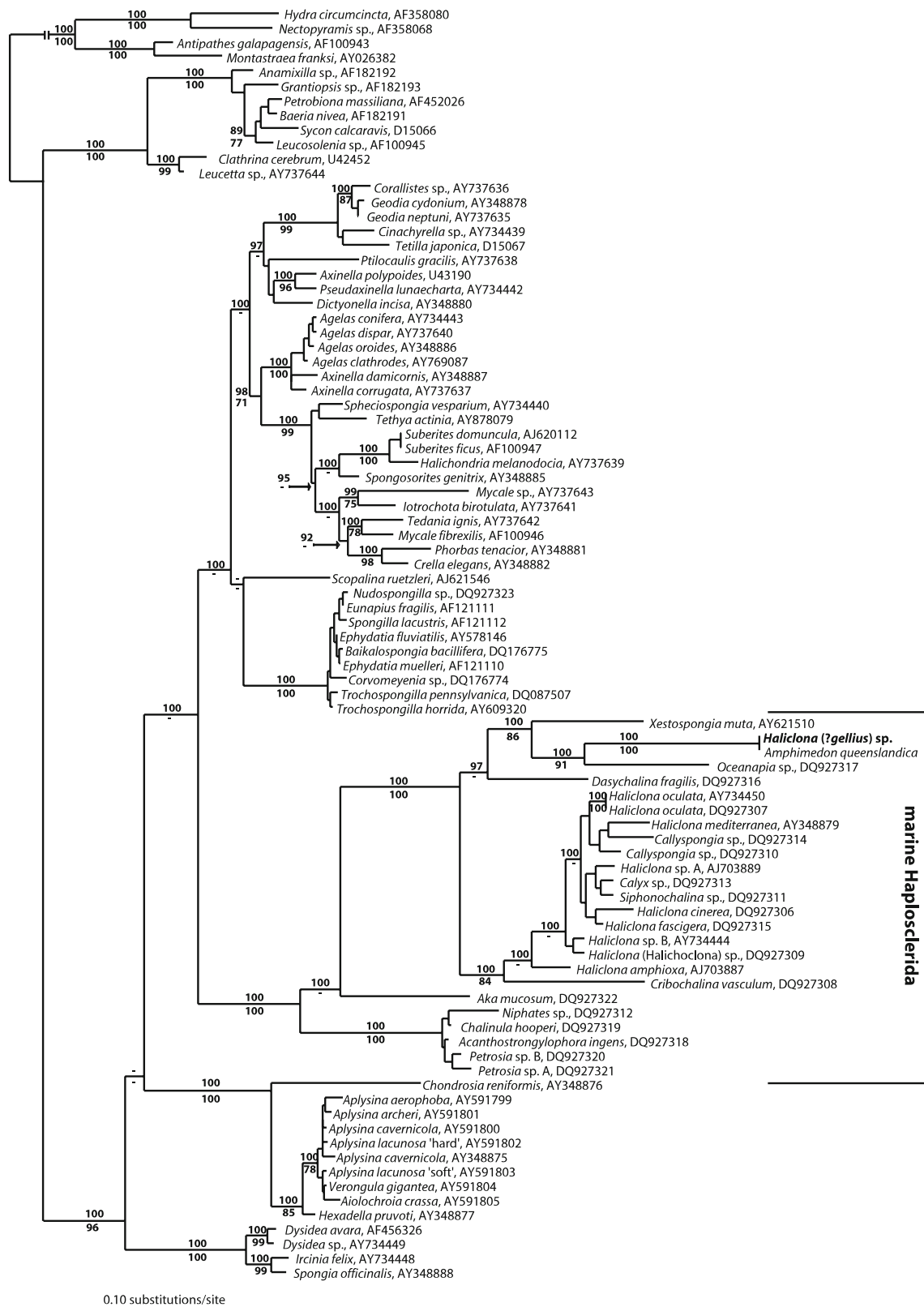


Figure 2 Maximum likelihood phylogram of 18S rRNA gene sequences. The numbers above the branches correspond to posterior probability (PP) values of the Bayesian analysis. The numbers shown below the branches are MP bootstrap values. Nodes with PP or bootstrap values of <70% are indicated with a '-'. Some PP and

bootstrap values were not included due to clarity (only those not relevant to the manuscript). The species in the tree are based on [40] with the addition of *Amphimedon queenslandica* (EF654521) and *Haliclona* (?gellius) sp. (EU095523)

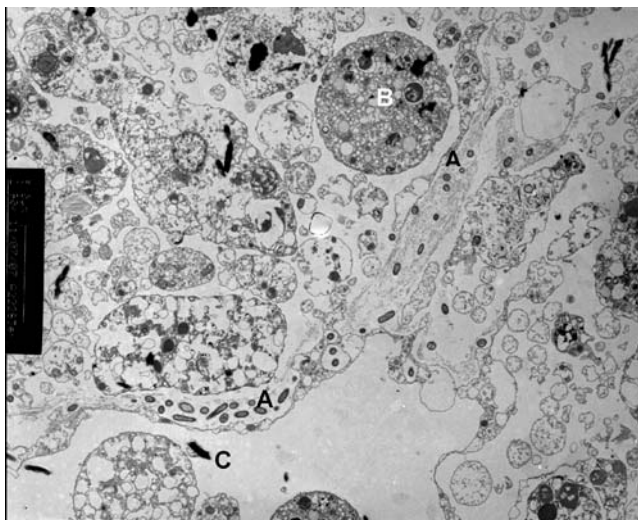


Figure 3 Transmission electron micrograph of a section of *H. (?gellius)* sp. Bacteria (A) are embedded in the mesohyl outside of sponge cells (B). The dark fragment (C) is part of a fragmented spicule. The diameter of cell B is approximately 9 μm

Nitrospirae and *Verrucomicrobia*-derived sequences were observed in only one of the individuals (this was also the case for *Stramenopile* and *Viridiplantae* PCR products). Similarities and differences between individuals on the OTU-level are summarised in Fig. 6. Only OTU 2 was present in all individuals and a BLAST search showed it is closely related to an uncultured *Pirellula* species (*Planctomycetes*) from the seawater close to a coral reef at the Palmyra Atoll. OTUs 1, 24 and 26 were observed in three of four

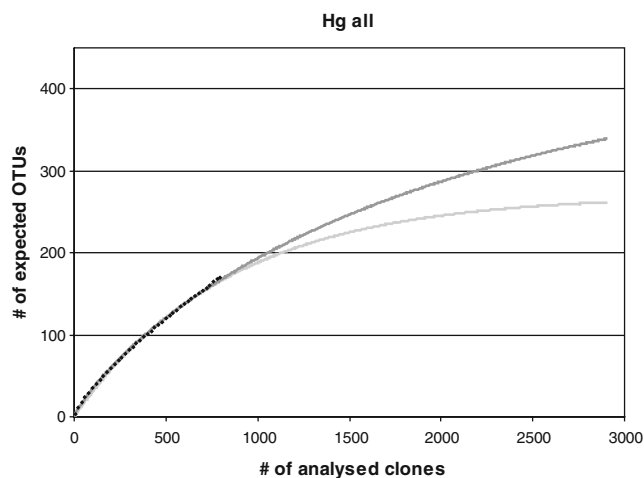


Figure 4 Example of a rarefaction curve plotted for the total bacterial diversity that was discovered in the four *Haliclona (?gellius)* sp. specimens. The expected number of OTUs as determined by Hurlbert's algorithm [24] is plotted against the number of analysed 16S clones (black diamonds). Extrapolated regression curves for equations 1 and 2 are depicted as the light and dark solid line, respectively

individuals and represent different β - and γ -*Proteobacteria*. Seven OTUs were found in half of the individuals and each individual contained approximately 15–25 singles (OTUs not found in any of the other individuals). Hg9 was an outlier in that respect with 95 singles.

In order to obtain more detailed insight in the bacterial populations associated with *H. (?gellius)* sp., phylogenetic trees of these bacteria and their nearest neighbours were constructed (Figs. 7, 8, 9 and 10). In the α -*Proteobacteria* tree, two *Roseobacter*-affiliated OTUs were detected in both Hg5 and Hg9 (OTUHg15 and OTUHg19), while 38 other OTUs were found in only one of the sponge individuals (Fig. 7).

Two β -*Proteobacteria*-affiliated OTUs, OTUHg1 and OTUHg24, were found in three (of four) individuals. Including the singles, a total of ten β -*Proteobacteria* OTUs were detected in *H. (?gellius)* specimens (Fig. 8b). The nearest neighbour of OTUHg24 is a clone from the marine sponge *Tethya aurantium*, but it is only distantly related (identity=83%). OTUHg1 was found in Hg1, Hg5 and Hg9. The nearest neighbours are a *Cupriavidus* species isolated from cystic fibrosis patients and a DNA fragment detected on old Antarctic ice.

One γ -*Proteobacteria* OTU, OTU26, was found in three individuals and represented the most abundant sequence in the clone library with more than 30% of the clones. The nearest neighbours are bacteria from other marine sponges: *Axinella verrucosa*, *Chondrilla nucula* and *Tethya aurantium* (Fig. 8a). Three other γ -*Proteobacteria*-related OTUs were found in two individuals, while 43 OTUs were singles.

Twenty-three OTUs grouped within the *Bacteroidetes* (Fig. 9). All of them were singles detected in Hg9, except for one *Bacteroidetes*-affiliated clone in Hg1 and in Hg5.

In the other phyla, three OTUs were found in more than one individual (Fig. 10). In addition to the *Planctomycetes* species that was found in all four individuals (OTUHg2), another *Planctomycete* species (OTUHg29) was found in Hg5 and Hg6. Moreover, 16 single *Planctomycete*-related OTUs were found (Fig. 10b). The Cyanobacteria-affiliated OTUHg9 was detected in both Hg1 and Hg5 and has a *Synechococcus* species isolated from Californian seawater as its nearest neighbour (Fig. 10a). All OTUs that were discovered in other phyla were singles. Seven δ -*Proteobacteria* OTUs were discovered and each sponge individual harboured different δ -*Proteobacteria*. In most individuals (not in Hg1), members of candidate division TM6 were found. All but one *Firmicutes* OTU was found in Hg1. They represented a large proportion of the clones from this individual (37%) but were negligible in the other individuals. A similar situation was observed for *Nitrospirae*-related clones in Hg9, although not to the same extent as

Table 2 Observed and expected bacterial diversity in *Haliclona* (?gellius) sp.

Clone library	n	OTU	Regression (1)		Regression (2)	
			a	Coverage	a	Coverage
Hg1	256	30	35.7	84%	62.8	48%
Hg5	177	36	46.7	77%	75.4	48%
Hg6	188	17	27.0	63%	53.2	32%
Hg9	175	101	176	57%	244	41%
Hg all	796	170	270	63%	458	37%

a is the expected number of OTUs according to regression models 1 and 2 of the rarefaction curves. The coverage is calculated by dividing the number of observed OTUs by the expected number of OTUs: $OTU/a \times 100\%$. All R^2 of the regression models and the rarefaction points were larger than 0.99 except for regression model 1 for Hg1 (0.986)

they represented 4% of the Hg9 clones. Only one or two clones belonging to *Verrucomicrobia*, *Actinobacteria*, *Chloroflexi*, ϵ -*Proteobacteria* and *Fusobacteria* were detected.

Archaea

Five archaeal OTUs were found in *H. (?gellius)* sp. (Fig. 11). One of them was found in all individuals (OTUHgAr2) and belongs to the C1a- α group. Its closest relative is an archaeal clone from the marine sponge *Astrosclera willeyana*. The other OTUs, also belonging to the C1a- α group, were only detected in Hg5 or Hg6.

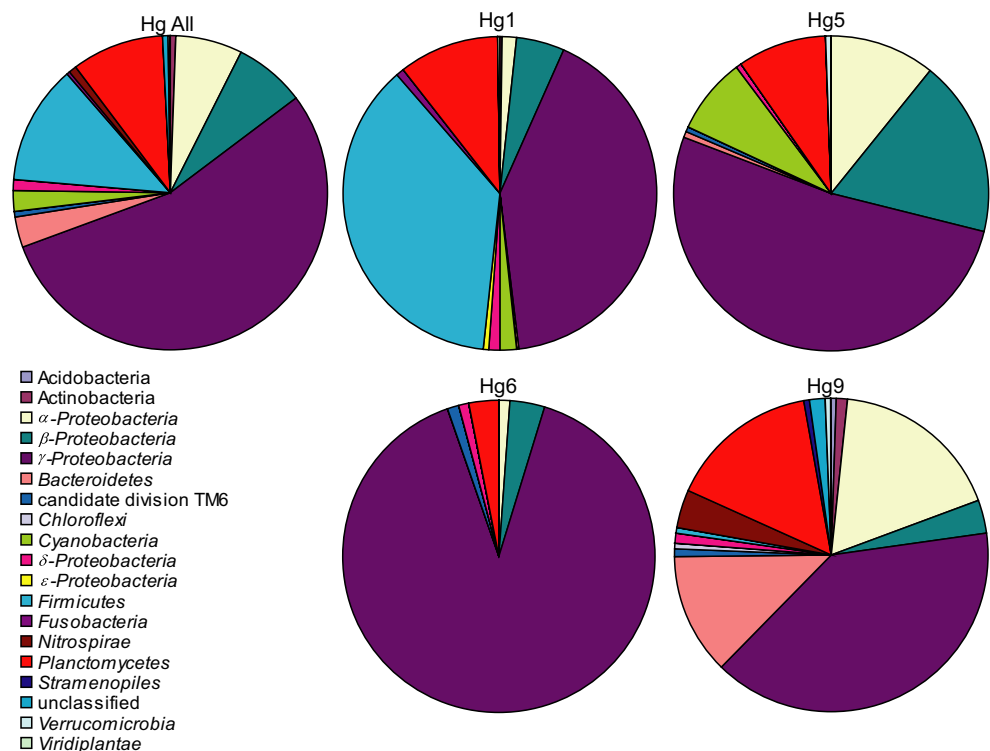
Discussion

Sponge

Haliclona (?gellius) sp. has been called an uncommon species by Hartman [13], but that may be because it generally resides in cold water under rocks and is not easily detectable. In addition, the species has not been studied in subsequent scientific publications except as part of systematic surveys [7, 30].

This is a sharp contrast to *Amphimedon queenslandica*, which is being developed as a model experimental system and is subject of a genome project (Joint Genome Institute). *A. queenslandica* is only found in the warm waters around the

Figure 5 Distribution of 16S PCR products over the bacterial phyla (including *Stramenopiles* and *Viridiplantae*) for sponge individuals Hg1 (256 clones), Hg5 (177), Hg6 (188), Hg9 (175) and the total (796). The phyla as listed in the legend are depicted clockwise starting at 12'o clock. The first listed phyla, *Acidobacteria* and *Actinobacteria*, are only present in Hg9 and Hg ALL. α -*Proteobacteria* is the first phylum that is present in all individuals. Hg6 is least diverse and only yielded α -, β -, γ - and δ -*Proteobacteria*, candidate division TM6 and *Planctomycetes* related clones



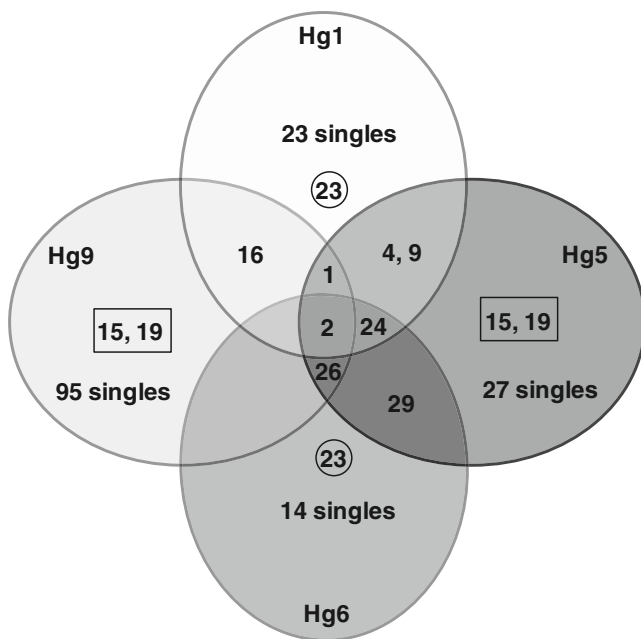


Figure 6 OTUs found in *H. (?gellius)* sp. individuals. Each number represents an OTU that was observed in more than one individual. Their position on the map shows which sponge individuals harboured the OTUs. OTUs that were found in only one individual are included in the category ‘singles’

Great Barrier Reef [21]. Despite these differences and their superficially different appearance (compare Fig. 1a to 1c), the *A. queenslandica* 18S rRNA gene sequence is identical to the near-complete sequence of *H. (?gellius)* sp. This is of particular interest, because of its similarity on the 18S level to *A. queenslandica*, *H. (?gellius)* sp. may present easily accessible genetic targets. Some preliminary results suggest that EST sequences from *H. (?gellius)* sp. of randomly chosen protein coding sequences share high identity at the nucleotide level with *A. queenslandica* (unpublished data).

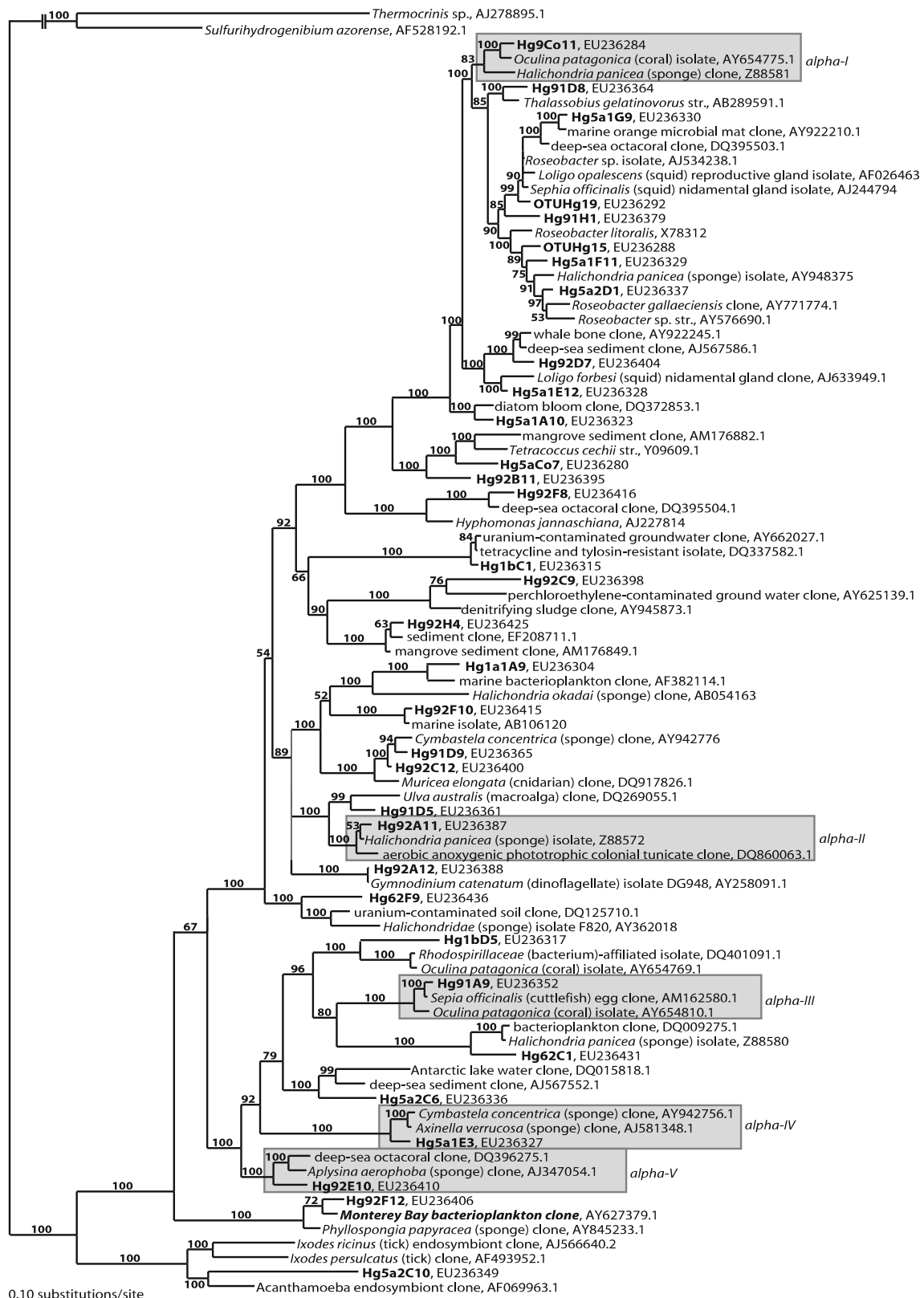
Bacteria

The bacterial diversity in *H. (?gellius)* is larger than what has been observed for most other sponge species investigated. One hundred and seventy OTUs (within 796 clone sequences) were found, while in many other 16S rRNA clone-based studies, typically 20–40 OTUs are discerned (Table 3). However, this difference may be attributed to the number of clones analysed in most studies, because if more clones were analysed, as in the cases for *Ircinia felix* and a *Corticium* sp., approximately 100 OTUs were obtained [44, 46]. We cannot compare coverage of bacterial diversity between the sponges, because not for all species rarefaction data have been published, but the number of OTUs/clone can be used as an indicator for sampling density. A high number of OTUs/clone implies that the number of OTUs that will be discovered by increasing the sample size will be

relatively high. It is shown that, except for *K. variolosa* and *C. australiensis*, which have comparative values to *H. (?gellius)* sp., all other sponge species have a considerable lower sampling density than *H. (?gellius)* sp. At the same time, we have reasons to believe that the numbers of detected and expected OTUs are overestimated for *H. (?gellius)* sp. The 99% shared identity standard to distinguish between OTUs [49] requires completely unambiguous sequences, which is not the case for most DNA fragments in our analysis. This will lead to a conservative estimation of overlap between sponge specimens while overestimating diversity. For the expected number of OTUs, as determined by rarefaction analysis, it has been noted that if many OTUs of low abundance are present in the data set (as is the case for *H. (?gellius)* sp.) the rarefaction curve tends to converge to a line without an apparent limit [55], which limits the usefulness of rarefaction analysis to obtain a reliable estimate of expected diversity. The latter issue is especially true for Hg9 (and therefore also Hg all) with 95 clone sequences that were obtained only once at a total of 175 clones. The experimental difference between Hg9 and the other specimens was that Hg9 was kept in an aquarium for 1 month before DNA was extracted and microorganisms present in the aquarium may have invaded the sponge.

A part of the bacterial diversity detected can probably be attributed to the environment rather than to the sponge due to the presence of bacteria that serve as food, sediment particles and ambient seawater in the sponge tissue. Although tissue with visible particles was discarded before DNA extraction and sponge tissue was rinsed three times with artificial seawater, it is impossible to remove all environmental DNA. When sponge-derived clones are compared to their nearest neighbours in a phylogeny (Figs. 7, 8, 9 and 10), three categories of neighbours can be discerned: invertebrate-derived clones, seawater clones and sediment clones. Twenty-five percent of the OTUs from Hg9 were related to sediment clones, while for the other individuals only $9.5 \pm 0.8\%$ (data not shown) had sediment-derived nearest neighbours. It is possible that the water circulation in the aquarium whirled the sediment in the aquarium and thus Hg9 was colonised with a large

Figure 7 Bayesian phylogram of α -Proteobacteria 16S rRNA gene sequences from *H. (?gellius)* sp. bacteria and their nearest neighbours (Greengenes database May 31, 2007). The numbers above or below the branches correspond to posterior probability (PP) values of the Bayesian analysis. OTUs that were found in more than one individual start with the letters ‘OTU’, OTUs that were found in only one individual are encoded as a combination of the name of the individual (e.g. Hg9)+the clone name. The grey boxes indicate OTUs with invertebrate-derived neighbours. *Thermocrinis* sp. and *Sulfurihydrogenibium azorense* were used as outgroup. If *H. (?gellius)* sp. OTUs were more closely related to each other than to any other sequence, only one of the OTUs was used to construct the tree



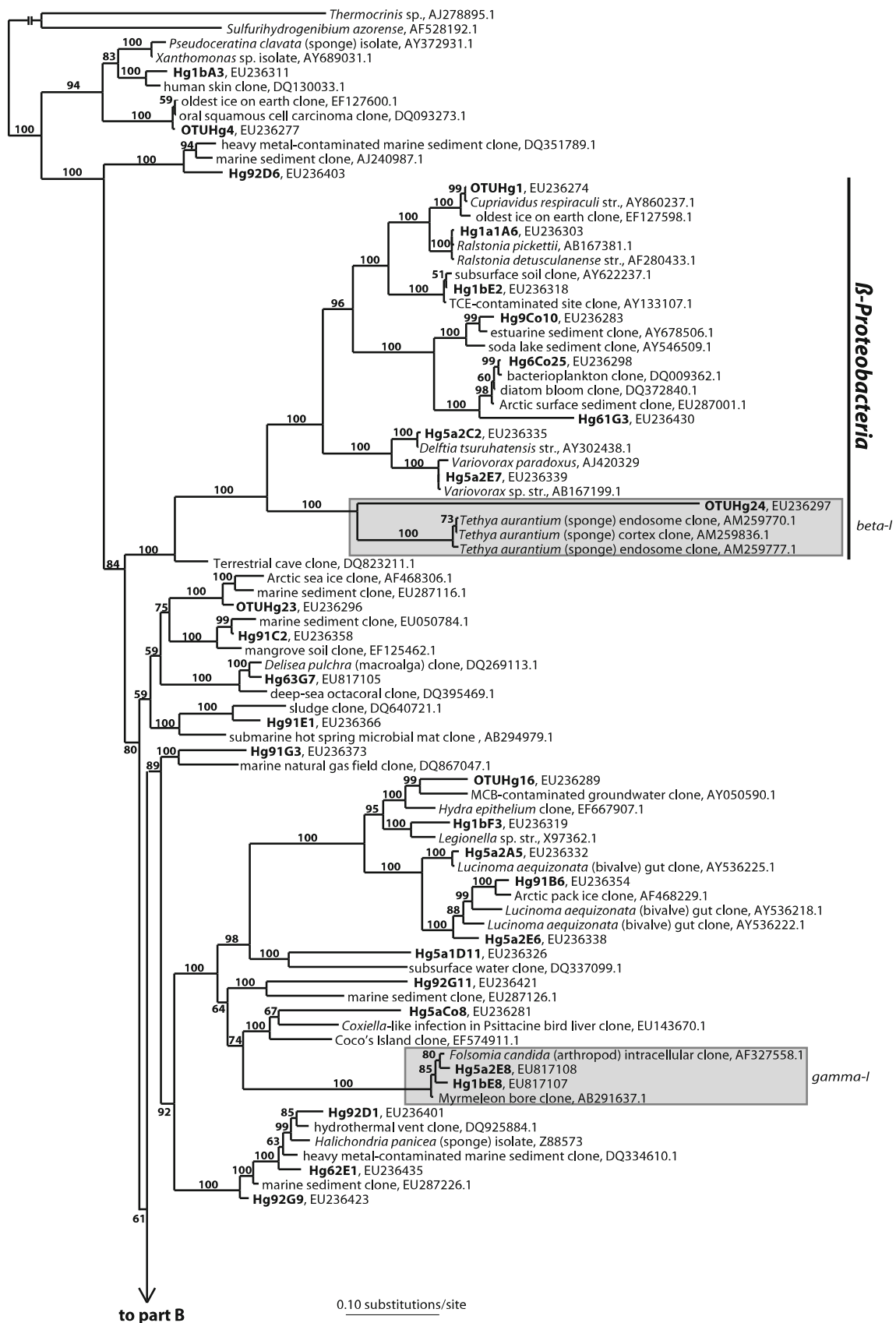


Figure 8 a, b Bayesian phylogram of β - and γ -Proteobacteria 16S rRNA gene sequences from *H. (?gellius)* sp. bacteria and their nearest neighbours (for details, see Fig. 7)

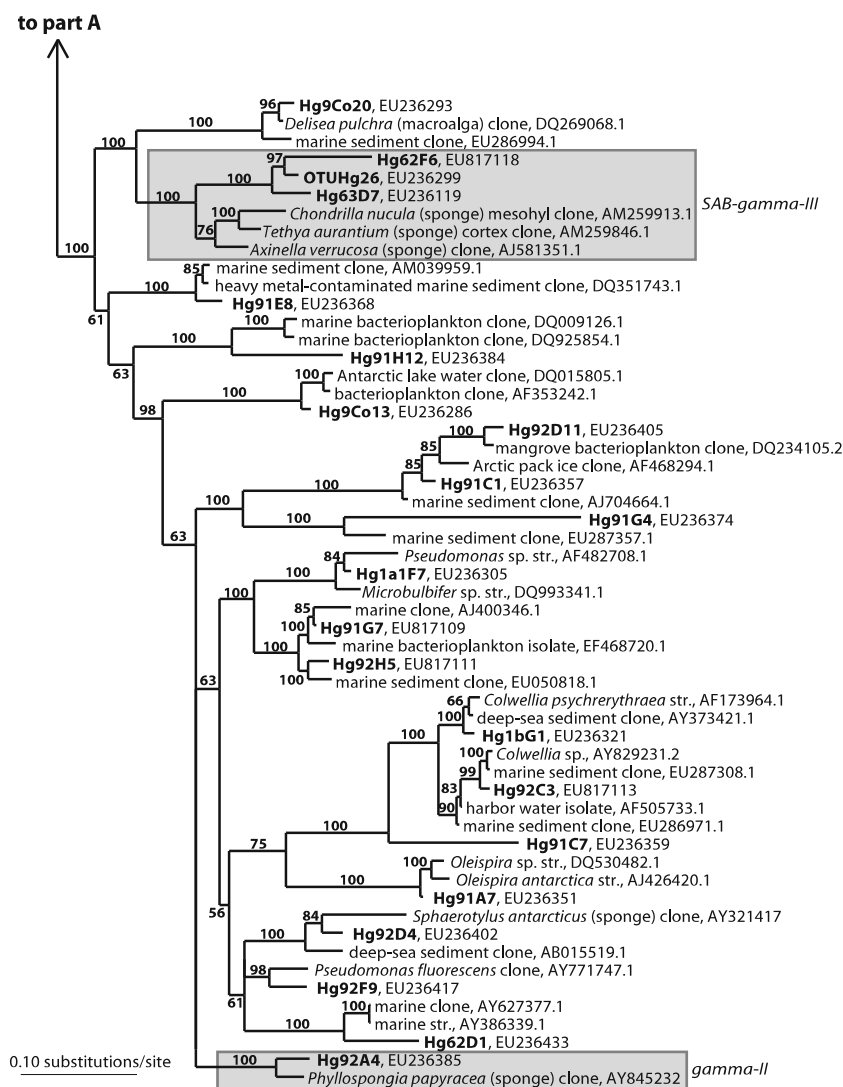


Figure 8 (continued)

number of sediment bacteria (the sample looked ‘dirty’ in the aquarium). This could explain a large part of the differences found between Hg9 and the other individuals. In addition, we compared the sponge-derived sequences with 94 16S rDNA sequences that were obtained from Monterey Bay bacterioplankton samples in other studies (data not shown; e.g. [50, 51]). This analysis revealed that three *H. (?gellius) sp.*-derived OTUs (Hg92F12, Hg92F3 and Hg5a2D8) were closely related to Monterey Bay bacterioplankton samples (Figs. 7, 9 and 10a, respectively).

Most OTUs had high sequence similarity with bacterial clones from different environments and do not belong to sponge-specific clades that were proposed by Hentschel et al. [14]. However, this does not exclude the possibility that they are stably associated with the sponge. For example, many clones of OTUHg2 were derived from all *H. (?gellius) sp.* individuals and was found in bacterio-

plankton, but also in the marine sponge *Tethya aurantium* (AM259827). Nevertheless, based on our results, we cannot make a firm conclusion for OTUs with neighbours from different groups, because we cannot distinguish horizontally acquired associated bacteria from ‘contaminants’ from the seawater. In addition, other sponge-derived sequences, such as the one from *T. aurantium*, may also have been the result of a seawater ‘contamination’. However, the fact that OTUHg2 persisted in Hg9 when it was kept in an aquarium with filtered seawater for 1 month suggests it could be a stable associate of the sponge.

A number of α -, β - and γ -*Proteobacteria* OTUs (Figs. 7 and 8) have only invertebrate-derived neighbours (checked with BLAST search; Jan 27, 2008). The α -*Proteobacteria* from *H. (?gellius) sp.* with invertebrate neighbours will not be considered, because they are all singles and it is therefore unlikely that they represent important partners

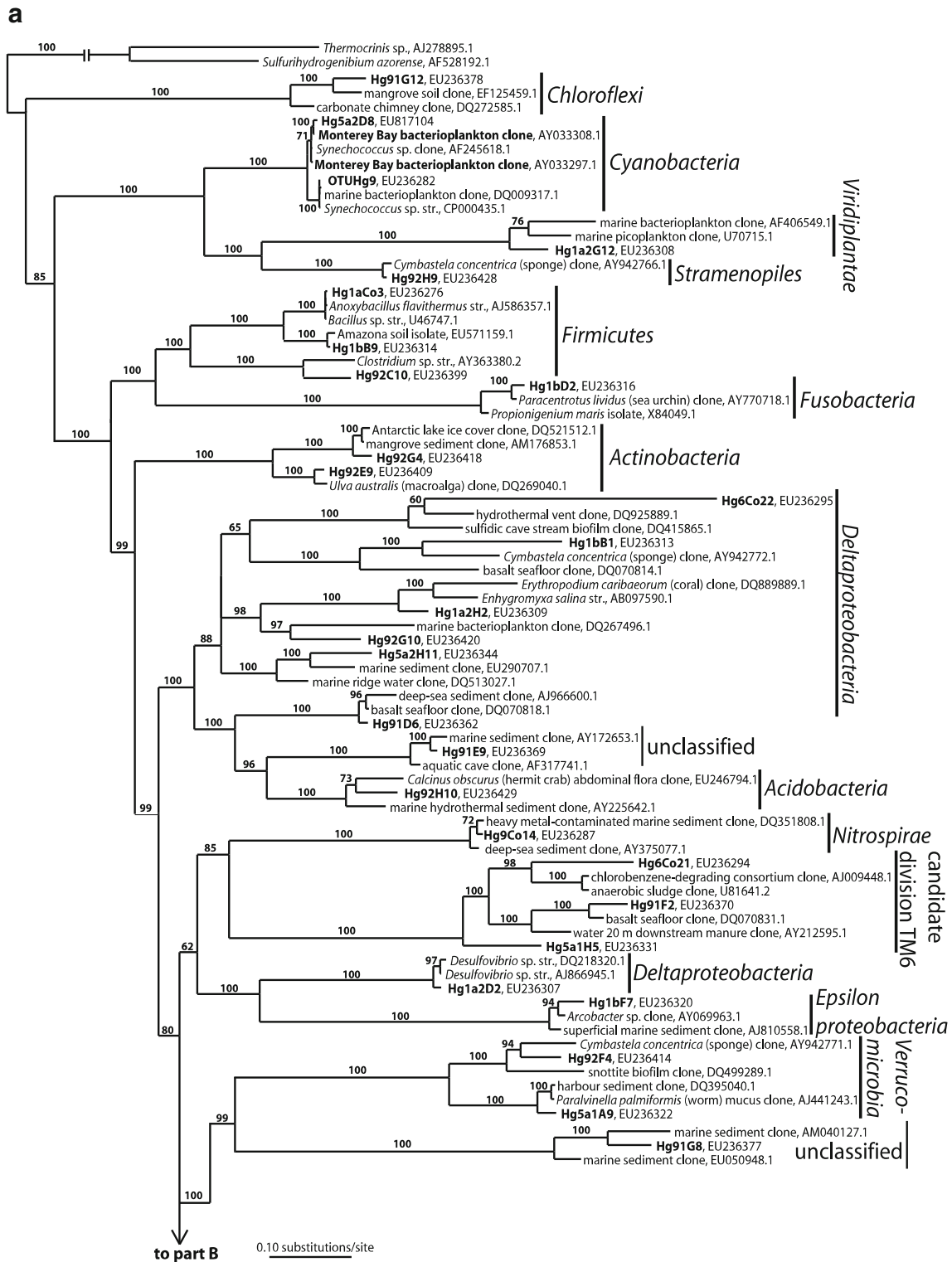


Figure 10 a, b Bayesian phylogram of 16S rRNA gene sequences from *H. (?gellius)* sp. and their nearest neighbours belonging to other phyla (for details, see Fig. 7)

derived from the Mediterranean and Adriatic sponges *Axinella verrucosa*, *Chondrilla nucula* and *Tethya aurantium* and belong to the sponge-specific SAB-Gamma-III cluster [56]. Maximum identity is only 92%,

which indicates that it is only a distantly related species. BLAST search revealed that OTUHg26 has 90% identity with *Thiohalomonas denitrificans* (EF117911.1), which is a halophilic chemolithoautotrophic bacterium that

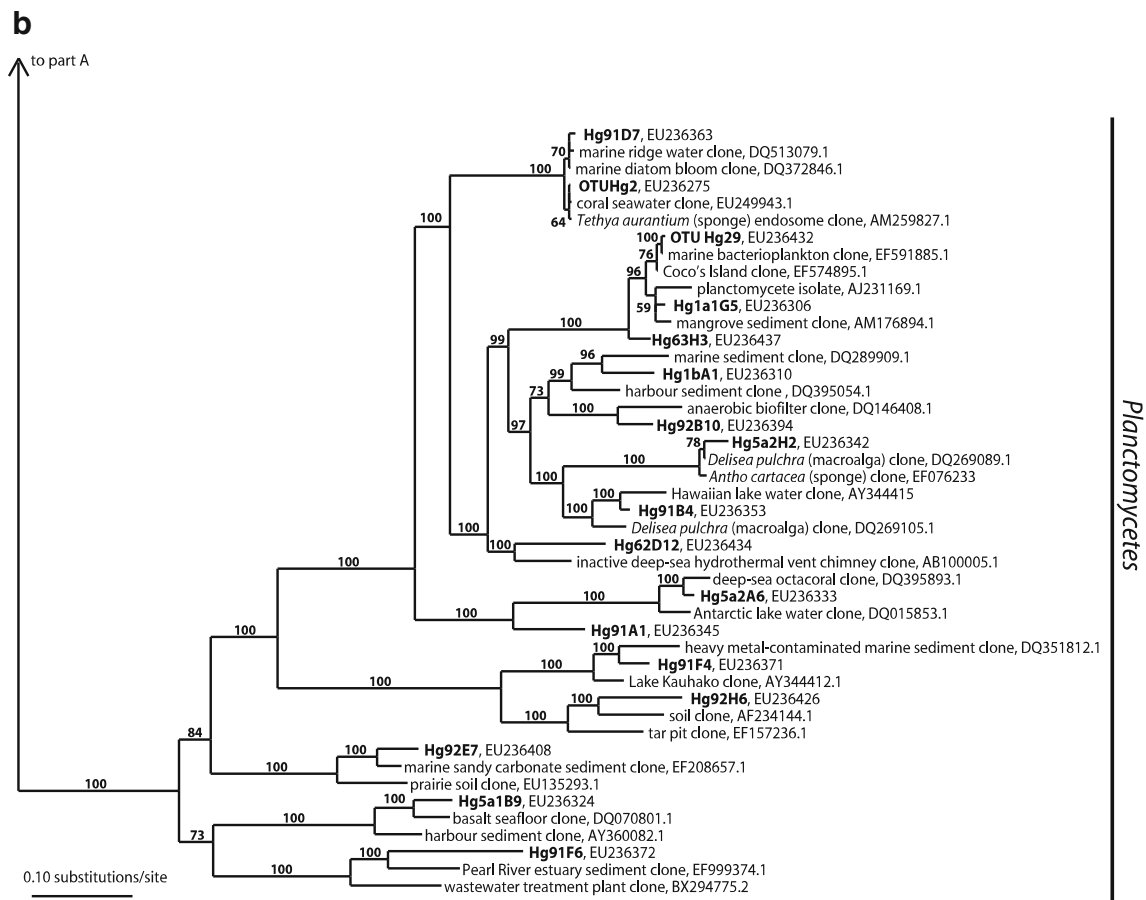


Figure 10 (continued)

utilises sulphide and thiosulphate as electron donor for the reduction of nitrate to N_2 [48]. A sponge-specific symbiotic sulphur cycle with sulphate reducers and -oxidisers was hypothesised to exist in the marine sponge *Polymastia* cf. *corticata* [35] and thus OTUHg26 could be responsible for the oxidation of sulphide in the sponge tissue. The chimera-check earmarked OTUHg26 (and also Hg62F6 and Hg63D7) as putative chimeras, but OTUHg26 was obtained from different samples, which suggests that the sequences are not likely to be chimeras.

The β -*Proteobacterium* OTUHg24 has no near relatives, but it is most similar to clones obtained from *Tethya aurantium* (AM259836.1, 83% identity), which are part of a sponge-specific cluster with sequences from globally distributed sponges [55]. The lack of similarity to other sequences makes it difficult to speculate about the role of the bacteria in the sponge tissue.

The lack of overlap of *Bacteroidetes*-affiliated OTUs between *H. (?gellius)* sp. specimens and the absence of sponge-specific OTUs confirms the idea that *Bacteroidetes* are not specific partners for sponges [54]. *Bacteroidetes*

were found to represent a large part of the necrotic tissue of the sponge *Rhopaloeides odorabile* after it was exposed for 24 h to elevated temperatures, while they were nearly absent in control specimens [62]. If we had based our analysis on DGGE bands, we would probably not have detected *Bacteroidetes* because they would have remained below the detection limit. The presence of low numbers of *Bacteroidetes* in the tissue of the sponge could be the inoculum for rapid proliferation when conditions change.

Cytotoxicity tests of a tissue extract of *H. (?gellius)* sp. showed that it was void of cytotoxic metabolites. No activity of a methanol extract was found against cancer cells and only a slight antimicrobial activity at 64 μ g/ml, the highest concentration tested (unpublished data, W. Fenical). This finding corresponds with the absence of consistent populations of *Actinobacteria* and *Chloroflexi* (only two and one clones, respectively, and only in Hg9) and the low frequency of *Cyanobacteria* (two OTUs and only present in Hg1 and Hg5) that are generally associated with the production of bioactive compounds [26, 38]. It could be speculated that the preferred habitat of the sponge (i.e. under rocks) is shielded from predators and direct light,

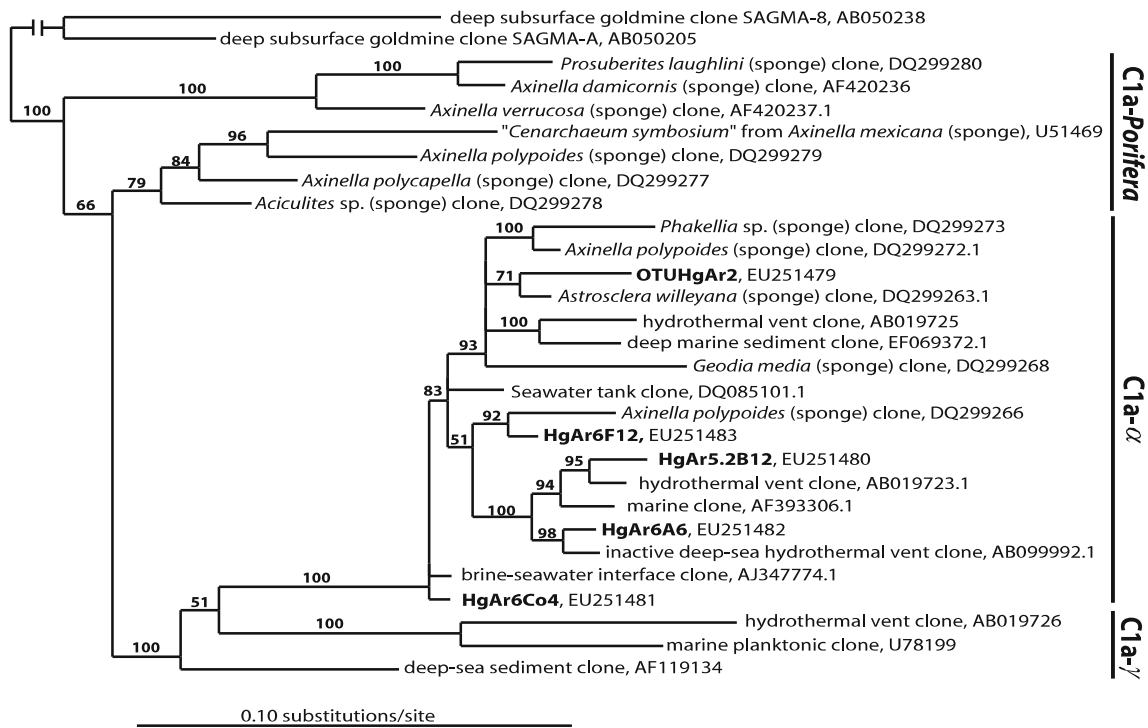


Figure 11 Bayesian phylogram of archaea 16S rRNA gene sequences from *H. (?gellius)* sp. and their near neighbours. The goldmine clones SAGMA-A and SAGMA-8 were used as an outgroup (for details, see Fig. 7)

and therefore it does not rely on chemical protection. When the bacterial composition of *H. (?gellius)* sp. is compared to the symbionts of the multi-cytotoxin-producing high-bacterial-density sponge *Aplysina aerophoba*, it can be noted that the compositions are remarkably different. In *A. aerophoba*, the *Acidobacteria*, *Actinobacteria* and *Chloroflexi* together make up more than 50% of the 16S rRNA

gene sequences [16], while for *H. (?gellius)* sp. they represented less than 1% of the clone sequences. Despite the presence of regions with increased concentrations of bacteria, *H. (?gellius)* sp. also has regions that are virtually bacteria-free. This is a sharp contrast to the high-bacterial-density sponge *A. aerophoba* and *H. (?gellius)* sp. is more likely to belong to the low-bacterial-density sponges based

Table 3 An overview of the number of bacterial OTUs that have been obtained from different marine sponges in relation to the number of clones that were used

Sponge species	Number of clones	Number of OTUs	OTUs/clone	Reference
<i>Chondrilla nucula</i>	36	21	0.58	[17]
<i>Sphaerotylus antarcticus</i>	50	38	0.76	[61]
<i>Homaxinella balfourensis</i>	50	33	0.66	[61]
<i>Kirkpatrickia varialosa</i>	50	12	0.24	[61]
<i>Latrunculia apicalis</i>	50	21	0.42	[61]
<i>Haliclona simulans</i>	51	19	0.37	[27]
<i>Chondrilla nucula</i>	52	22	0.42	[56]
<i>Tethya aurantium</i> mesohyl	65	21	0.32	[55]
<i>Tethya aurantium</i> cortex	66	30	0.45	[55]
<i>Haliclona simulans</i> ^a	81.5	27	0.33	[27]
<i>Craniella australiensis</i>	91	21	0.23	[31]
<i>Ircinia strobilina</i> (wild)	100	35	0.35	[37]
<i>Mycale laxissima</i> (wild)	119	67	0.56	[36]
<i>Corticium</i> sp.	200	90	0.45	[46]
<i>Ircinia felix</i>	218	112	0.51	[44]
<i>Haliclona (?gellius)</i> sp.	796	170	0.21	This paper

^a For *H. simulans*, the average number of clones and OTUs of two clone libraries were used

on TEM micrographs. This corresponds with the theory that in general low-bacterial-density sponges harbour fewer sponge-specific associated bacteria.

Archaea

The only sponge-specific archaea clade, C1a-Porifera, comprises sequences derived from the genus *Axinella* [33]. All other sponge-derived archaea belong to the C1a- α group, but members are not restricted to sponges [20] and the archaea-affiliated sequences from *H. (?gellius)* sp. make part of this group. The nearest neighbour of OTUHgAr2, which was obtained from all four individuals, is a sequence derived from the marine sponge *Astrosclera willeyana*. This OTU is potentially a symbiotic archaea of *H. (?gellius)* sp., but sequence differences on the 16S level are small for archaea [45], which is confirmed by lower posterior probability values when compared to the bacterial phylograms. Therefore, conclusions solely based on 16S phylograms should be regarded carefully.

Conclusion

The cold Pacific sponge *Haliclona (?gellius)* sp. from Northern California is closely related to the Great Barrier Reef sponge *Amphimedon queenslandica* based on 18S rDNA. It harbours bacteria from many bacterial phyla, but only a few are likely to have a stable association with the sponge. Sponge-specific sequences of a β - and a γ -*Proteobacterium* were abundant and most abundant clones in the clone library, respectively. Both of them were present in three of the four specimens analysed. This could imply that *H. (?gellius)* sp. is the restricted environment for growth of these bacteria, but that the sponge is able to proliferate without them. In addition, two OTUs were detected in all specimens: a *Planctomycete*- and a *Crenarchaea*-affiliated microbe. Both were closely related to OTUs that have been found in other sponges, but not exclusively in sponges. The fact that these microbes were also present in Hg9, which was maintained in an aquarium with filtered seawater for 1 month, gives rise to the inclination to include them with the stably associated microorganisms of *H. (?gellius)* sp. Based on TEM micrographs, the sponge is most likely a low-bacterial-density sponge.

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