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Spatial distribution of bacteria associated with the marine sponge *Tethya californiana*

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Abstract Microbial diversity and spatial distribution of the diversity within tissue of the marine sponge *Tethya californiana* was analyzed based on 16S rRNA gene sequences. One candidate division and nine bacterial phyla were detected, including members of all five subdivisions of *Proteobacteria*. Moreover, chloroplast-derived *Stramenopiles*- and *Rhodophyta*-affiliated 16S rRNA gene sequences were found and *Stramenopiles* represented the most abundant clones (30%) in the clone library. On the phylum-level, the microbial fingerprint of *T. californiana* showed a similar pattern as its Mediterranean relative *T. aurantium*. An interesting difference was that *Cyanobacteria* that were abundantly present in *T. aurantium* were not found in *T. californiana*, but that the latter sponges harbored phototrophic *Stramenopiles* instead. Surprisingly, the phototrophic microorganisms were evenly distributed over the inner and outer parts of the sponge tissue, which implies that they also reside in regions without direct light exposure. The other phyla were also present in both the outer cortex and the mesohyl of the sponges. These results were confirmed by analysis on the operational taxonomic unit level. This leads to the conclusion that from a qualitative point of view, spatial distribution of microorganisms in *T. californiana* tissue is quite homogeneous. Thirty-two percent of the operational taxonomic units shared less than 95% similarity with any other known sequence. This indicates that marine sponges are a rich source of previously undetected microbial life.

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Introduction

Marine sponges are reservoirs of many unknown, and certainly of many uncultured, microbial species. The microbial inhabitants of a growing number of sponge species have been characterized with molecular tools (Taylor et al. 2007 and references cited therein), and it has been suggested that sponges harbor many specific bacterial species and clades that are not found in other environments (Hentschel et al. 2002). Initially, these sponge-specific clades could still be the result of insufficient sampling, but huge sequencing efforts during the last decade have supported this hypothesis. However, very little information exists about the actual relationship between the host and its specifically associated bacteria and the distribution of these bacteria throughout the sponge. Although many potential beneficial roles have been ascribed to the microorganisms, it has been difficult to find in situ evidence for these hypotheses due to the complexity of the host–bacteria network. For that reason, scientists have been hesitant to call the associated bacteria 'symbionts'. However, sponges are considered to be the oldest metazoans still present, and their presumably consistent association with microorganism through time and space strongly suggests a functional relationship between the sponge and its bacteria. Sponges provide a niche that is richer in nutrients than seawater and sediments (reviewed in Lee et al. 2001) and could prevent the inhabitants from drifting away to less favorable environments. In addition, the primary and secondary metabolism of the microorganisms and the host may be interrelated in such a way that bacteria utilize sponge's metabolic waste products (ammonia, nitrate, carbon dioxide), while sponge cells could potentially make use of, e.g., oxygen and small organic molecules excreted by associated phototrophic microbes (Wilkinson and Garrone 1980;

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Hallam et al. 2006; Bayer et al. 2008; Hoffmann et al. 2009). Moreover, it has become clear that bioactive secondary metabolites produced by associated microbes play an important role in reducing predation (Faulkner 2000). If associated bacteria are involved in the chemical defense of the host, their preferable location in the sponge could be near the surface, which is most vulnerable to predation and larval settlement. A substantial number of bacteria isolated from the surface of marine sponges have indeed shown to produce bioactive compounds (Becerro et al. 1994; Osinga et al. 2001; Chelossi et al. 2004; Müller et al. 2004). In addition, photosynthetically active cyanobacteria and microalgae are also generally found in the outer tissue layer of the sponge (reviewed in Hentschel et al. 2003), but exceptions to this general picture are not uncommon. For example, the cyanobacteria *Oscillatoria spongeliae* was found abundantly in the mesohyl of *Lendenfeldia chondrodes* but not in the epithelial sections (Ridley et al. 2005). The inner core of sponges is generally populated by heterotrophic and autotrophic bacteria (reviewed in Hentschel et al. 2003), and it has been shown in times of ceased pumping activity, which frequently occurs for many sponges, this inner core can easily become anoxic (Hoffmann et al. 2005, 2008), creating a perfect niche for facultatively anaerobic species.

A clear idea of spatial distribution of associated bacteria is important to understand the relationship between the sponge and its associated microorganisms, and currently very little detailed information exists on this topic. However, a few studies have indicated that different microbial profiles are likely to exist between exterior and interior tissue samples (Wichels et al. 2006; Thiel et al. 2007). Knowledge of the location of associated species in the sponge may provide information about the preferred microenvironment of a particular strain, which can be used to design cultivation experiments as these organisms are still regarded as mostly uncultivable (e.g., Friedrich et al. 2001).

We have studied the spatial variation of associated microorganisms of *Tethya californiana* (De Laubenfels, 1932). This is an ideal species for this type of studies as it has a clearly defined mesohyl and cortex (Fig. 1). In addition, the sponge is ball-shaped, which results in the largest possible distance from the core of the sponge to the surface and increases the chance of occurrence of gradients in the tissue.

Materials and methods

Sample collection

Two *Tethya californiana* specimens (Tc1 and Tc2) were collected near Monastery Beach (Monterey, CA, USA) at a
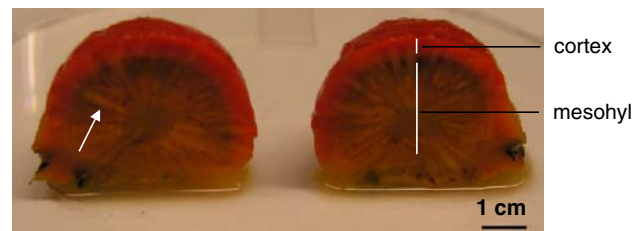
Fig. 1 A *Tethya californiana* specimen cut into halves. The orange outer tissue can easily be distinguished from the inner *brownish core*. In addition, long whitish spicules can be observed that are positioned radially from the core to the periphery (see e.g., *white arrow*)

depth of approximately 8 m on May 24, 2006. They were immediately transported in excess of natural seawater to the laboratory and processed within 2 h. The sponges were rinsed 3 times with sterile artificial seawater (natural sea salt mix, Oceanic Systems, Dallas, TX, USA) before preparing cortex and mesohyl samples. Special care was taken to avoid contact between the two tissue types and a safety margin in the tissue between mesohyl and cortex was used when the samples were cut. The samples were separately ground with a sterilized mortar and pestle. Two tissue volumes of sterile artificial seawater (ASW) were added to the tissue samples to obtain homogeneous cell suspensions. Each cell suspension was divided in aliquots of 1.2 ml and mixed with 0.6 ml 50% glycerol in ASW. The samples were frozen until −20°C before they were stored at −80°C.

DNA library construction

Genomic DNA was extracted from the cryopreserved cells using the Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). Cryopreserved cell suspension with the volume of 400 μl was used for DNA isolation. The cell suspension was centrifuged, and the supernatant was decanted. DNA was isolated according to manufacturer's specifications.

Universal eubacterial primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′ [Felske et al. 1997]) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′ [Lane 1991]) were used to amplify 16S rRNA gene fragments. The PCR conditions were as follows: initial denaturation for 10 min at 95°C; 30 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 for sequencing (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. A total of 384 colonies were picked, and plasmids were purified for each specimen (192 for the cortex and 192 for the mesohyl).

Plasmids containing a 16S rRNA gene PCR product were sequenced using the 8F primer. 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) (Stackebrandt and Ebers 2006). In addition, they were checked for possible chimerical origins by using the program Bellerophon (version 3) from the Greengenes website (Huber et al. 2004). Sequences were deposited in Genbank under accession numbers: EU290173–EU290248, EU290250–EU290266, EU290268–EU290275, EU290277–EU290401 and EU290403–EU290460.

Phylogenetic analysis

The DNA sequences of the OTUs and nearest neighbors (as determined using the greengenes database http://greengenes.lbl.gov) were imported in the ARB software package (Ludwig et al. 2004). 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 (Castresana 2000). 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* (Swofford 2003). Heuristic searches consisted of 1000 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 (Zwickl 2006). 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 (Huelsen-beck and Ronquist 2001) at the freely available Bioportal server (www.bioportal.uio.no). 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 (Ronquist et al. 2005). 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 (1971) with the freeware program aRarefactWin (http://www.uga.edu/~strata/software.html). Rarefaction curves were plotted, and regressions were calculated using two different regression equations (Thiel et al. 2007):

$$y = a\left(1 - e^{-bx}\right) \tag{1}$$

$$y = a\left(1 - e^{-bx^{\wedge}c}\right) \tag{2}$$

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.

Statistical analysis

Mesohyl and cortex samples were compared for the presence or absence of OTUs that were present in at least two samples with the PRIMER 6 Software (Clarke and Warwick 2001). The qualitative data were used to calculate Bray–Curtis similarity to evaluate the importance of two factors: tissue type and specimen. Nonmetric multidimensional scaling (MDS) was used to plot the analysis. Based on the Bray–Curtis similarity index, in MDS plots, the distance between points is proportional to their degree of similarity; so, closer points are more similar than points farther away.

Results and discussion

Phylum-level analysis of *T. californiana*-associated microorganisms

A total of 680 high-quality partial 16S rRNA gene clone sequences (average ~900 bp) were obtained that were quite evenly distributed over the cortex and mesohyl samples of the two *Tethya californiana* specimens (179 for T1cor, 155 for T1mes, 166 for T2cor and 180 for T2mes). Representatives of nine bacterial phyla including the subdivisions α-, β-, γ-, δ- and ε-*Proteobacteria* and one candidate division were present in *T. californiana* (we did not screen for *Archaea*). In addition, phototrophic *Stramenopiles*- and *Rhodophyta*-affiliated sequences were present in the clone libraries (Fig. 2a–e). Clones containing sequences related to *Stramenopiles*-derived chloroplasts were actually the most abundant ones in the combined clone libraries (30%). Other major groups were α-*Proteobacteria* (23%), γ-*Proteobacteria* (15%), *Bacteroidetes* (9%) and β-*Proteobacteria* (8%), while δ-*Proteobacteria*, *Firmicutes* and *Verrucomicrobia* (all ~3%) were represented by lower numbers of clones (Fig. 2a). *Actinobacteria* (0.7%) and ε-*Proteobacteria* (0.3%) were present in very low numbers and were only detected in T1 (Fig. 2b, c), while *Rhodophyta* (0.3%) were only detected in T2 (Fig. 2d, e). *Planctomycete* clones were more abundantly obtained from mesohyl samples (3.4 ± 2.5%) than from cortex samples (0.6 ± 0.03%), but in general, the distribution of clones over the microbial phyla was roughly comparable for both specimens and both locations within the specimens.

Fig. 2 a–e Distribution of 16S rRNA gene PCR products over the bacterial phyla and *Stramenopiles* and *Rhodophyta* for separate cortex and mesohyl samples from two *T. californiana* specimens and for the total of the clones. The numbers of clones are in *parentheses*. The phyla as listed in the legend are depicted clockwise starting at 12 O'clock. *Acidobacteria* is only present in T1mes, T2cor and T ALL. *Actinobacteria*, *ε-Proteobacteria* and *Nitrospirae* are only present in T1 and T ALL. *Rhodophyta* was found only in T2cor
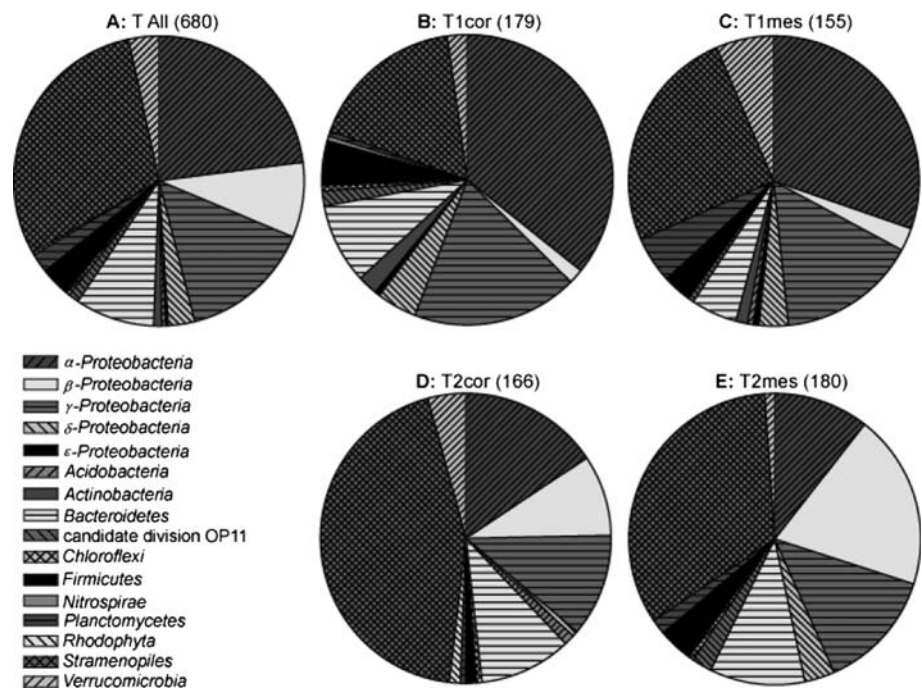
The major phylogenetic distribution of *T. californiana* 16S rRNA gene clones is comparable to the distribution in *Tethya aurantium* (Thiel et al. 2007). The bulk of 16S rRNA gene clone sequences from *T. aurantium* was derived from *Proteobacteria* (38%), followed by *Cyanobacteria* (27%) and *Bacteroidetes* (25%). We did not find any cyanobacterial sequence, but phototrophic *Stramenopile* chloroplast-affiliated sequences represented 30% of all clones. Phototrophic organisms (green algae) associated with the sponges *Ephydatia fluviatilis* and *Haliclona cymiformis* translocate metabolites to their hosts (Wilkinson 1980; Grant et al. 2006), and it could be speculated that the presence of *Cyanobacteria* in *T. aurantium* and phototrophic *Stramenopiles* in *T. californiana* are different solutions with a similar outcome with respect to interactions in the sponge–microbe network. A difference between *T. californiana* and *T. aurantium* is that for the latter sponge phototrophic associates were mostly confined to the mesohyl (Thiel et al. 2007). Microbial profiles of other, more distantly related, sponges are considerably different from the *Tethya* species, especially with respect to the fraction of phototrophic microorganisms (Hill et al. 2006).

The preferred habitat of the *T. californiana*, on top of exposed rocks in relatively shallow water (5–10 m), guarantees the availability of sufficient light for its phototrophic microorganisms in the cortex. However, members of the genus *Tethya* also harbor phototrophic microorganisms in their interior: *Cyanobacteria* in *T. aurantium* (Thiel et al. 2007) and green alga in *T. seychellinsis* (Gaino and Sará 1994). It has recently been shown that the thick radially

positioned spicules conduct enough light into the core of *Tethya aurantium* to sustain the growth of phototrophic microorganisms (Brümmer et al. 2008). Thus, phototrophic *Stramenopiles* could obtain their energy from light inside *T. californiana*.

Estimation of microbial diversity

Based on the rule of thumb of Stackebrandt and Ebers (2006) that sequences that share less than 99% similarity are considered to be derived from a different operational taxonomic unit (OTU), the 680 clones represented 263 different OTUs. Rarefaction curves showed that the actual diversity is probably well over 400 OTUs for *T. californiana* and that especially for the cortex samples diversity was not completely covered (Table 1). However, the high number of analyzed clones suggests that the part of bacterial diversity that was not detected probably represents a minor quantitative fraction in the sponge. The number of detected OTUs was higher in the cortex samples (101 and 87 for Tc1 and Tc2, respectively) than in the mesohyl samples (77 and 62 for Tc1 and Tc2, respectively). In addition, the number of detected OTUs in Tc1 was slightly higher than in Tc2.

A similar general pattern for diversity in cortex and mesohyl was observed by Thiel et al. (2007) for the Mediterranean sponge *Tethya aurantium*. The number of OTUs detected in *T. californiana* (cortex 92.5 ± 9.2; mesohyl 67 ± 8.5 OTUs) was significantly higher than in *T. aurantium* tissue (cortex 16 ± 14.1; mesohyl 11 ± 2.8 OTUs), but this is probably caused by a lower clone

Table 1 Observed and expected bacterial diversity in *Tethya californiana*

Clone library	n	OTU	Regression (1)		Regression (2)	
			a	Coverage (%)	a	Coverage (%)
Tc1ect	179	101	170	60	179	56
Tc1mes	155	77	115	67	142	54
Tc2ect	166	87	145	60	200	43
Tc2mes	180	62	76	81	82	76
Tc all	680	263	405	65	496	53

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 × 100%

All R^2 of the regression models and the rarefaction points were larger than 0.99

number per sample for the latter sponge (on average: 32.8 ± 3.3 clones).

OTU-level analysis of *T. californiana*-associated microorganisms

In order to obtain more detailed insight in the microbial populations associated with *T. californiana*, phylogenetic trees containing the detected OTUs and their nearest neighbors were constructed (Figs. 3, 4, 5, 6). Many near neighbors were derived from other sponges, but only a few OTUs from *T. californiana* seem to be really sponge-specific, because for many OTUs sponge-derived neighbors (if present) were accompanied by nonsponge-derived neighbors. In order to bring to light microorganisms that were potentially derived from the surrounding seawater, we compared the sponge-derived sequences with 94 16S rRNA gene sequences that were obtained from Monterey Bay bacterioplankton samples in other studies (data not shown: e.g., Suzuki et al. 2001, 2004). This analysis revealed that four *T. californiana*-derived OTUs (OTU08Tcor, OTU10T1, OTU79Tmes and T1cor2D6) probably originated from seawater (Figs. 3, 5).

Among the α-*Proteobacteria*-affiliated OTUs, one OTU, which was retrieved from both cortex and mesohyl from Tc1 (OTU15T1), seems to be sponge- or even *Tethya*-specific (Fig. 3). It is only distantly related to other known α-*Proteobacteria*. Its nearest neighbor is a clone that was obtained from a *T. aurantium* cortex sample (97% identity), and it shares less than 90% identity with any other sequence in Genbank. The most abundant α-*Proteobacteria*-affiliated clone sequences OTU17T1 (6.3% of all clone sequences) and OTU47T2 (3.7%) were present in both mesohyl and cortex of Tethya1 and Tethya2, respectively.

Only one β-*Proteobacteria*-affiliated OTU (OTU53T) was present in *T. californiana*. It was detected in both cortex and mesohyl samples of both specimens. Based on phylogenetic analysis, the OTU belongs to a clade with only sponge-derived sequences (Fig. 4). Its nearest neighbor is an isolate from the Pacific sponge *Haliclona* (?*gellius*) sp., and other related sequences were obtained from sponges from a wide range of geographical regions. For the *Tethya aurantium*-derived neighbors, it was found that clone sequences in this cluster represented 19% of all clones sequences (Thiel et al. 2007) and also for *T. californiana* they represented a large fraction (8.7%) of all clone sequences. The fact that it was isolated from all tissue samples implies that this bacterial species is potentially an important partner for sponges, which role still needs to be identified.

A wide variety of γ-*Proteobacteria*-affiliated OTUs were found in *T. californiana*. OTU60T was the most abundant γ-proteobacterial sequence in the clone library with 3.5% of all clones and was detected in all mesohyl and cortex samples. Although it is distantly related to an *Aplysina aerophoba* clone, nearest neighbors were derived from nonsponge sources (Fig. 4). With two exceptions, no sponge-specific γ-*Proteobacteria* were detected in *T. californiana*. In addition, only for OTU60T and OTU78Tmes, overlap between sponge specimens was found. This indicates that most associated γ-*Proteobacteria* are most likely rather temporary dwellers than permanently associated microbes with *T. californiana*. Only T2mes2D12 and T1mes2G2 grouped in a cluster with sponge-derived clones and isolates. In the sponge *Haliclona simulans*, clones belonging to the latter cluster represented 44% of the total sponge library (Kennedy et al. 2008). However, both γ-proteobacterial clones belonging to putative sponge-related clusters were detected only as a single clone in the mesohyl sample of one of the specimens.

Only a few *T. californiana Bacteroidetes*-affiliated sequences have sponge-derived nearest neighbors (Fig. 5) and most of their sponge-derived neighbors were obtained from *Haliclona* (?*gellius*) sp., a species that is found in the vicinity of *T. californiana* (Sipkema et al. 2009), which could indicate that they are related to the environment rather than specifically to sponges. Despite the presence of significant numbers of *Bacteroidetes*-affiliated clone sequences in *T. aurantium* (25%) (Thiel et al. 2007) and *T. californiana* (9%), no closely related OTUs were observed for the two *Tethya* species. This is in agreement with the observation that sponge-specific clusters are not prevalent for *Bacteroidetes* (Taylor et al. 2007). OTU28T was the most abundant *Bacteroidetes*-affiliated sequence in *T. californiana* and represented 12% of *Bacteroidetes*-affiliated clones (1% of all clones) and was present in both cortex and mesohyl samples from the two *T. californiana* specimens. Its nearest neighbors were isolated from different environments, and it probably represents a cosmopolitan bacterium.
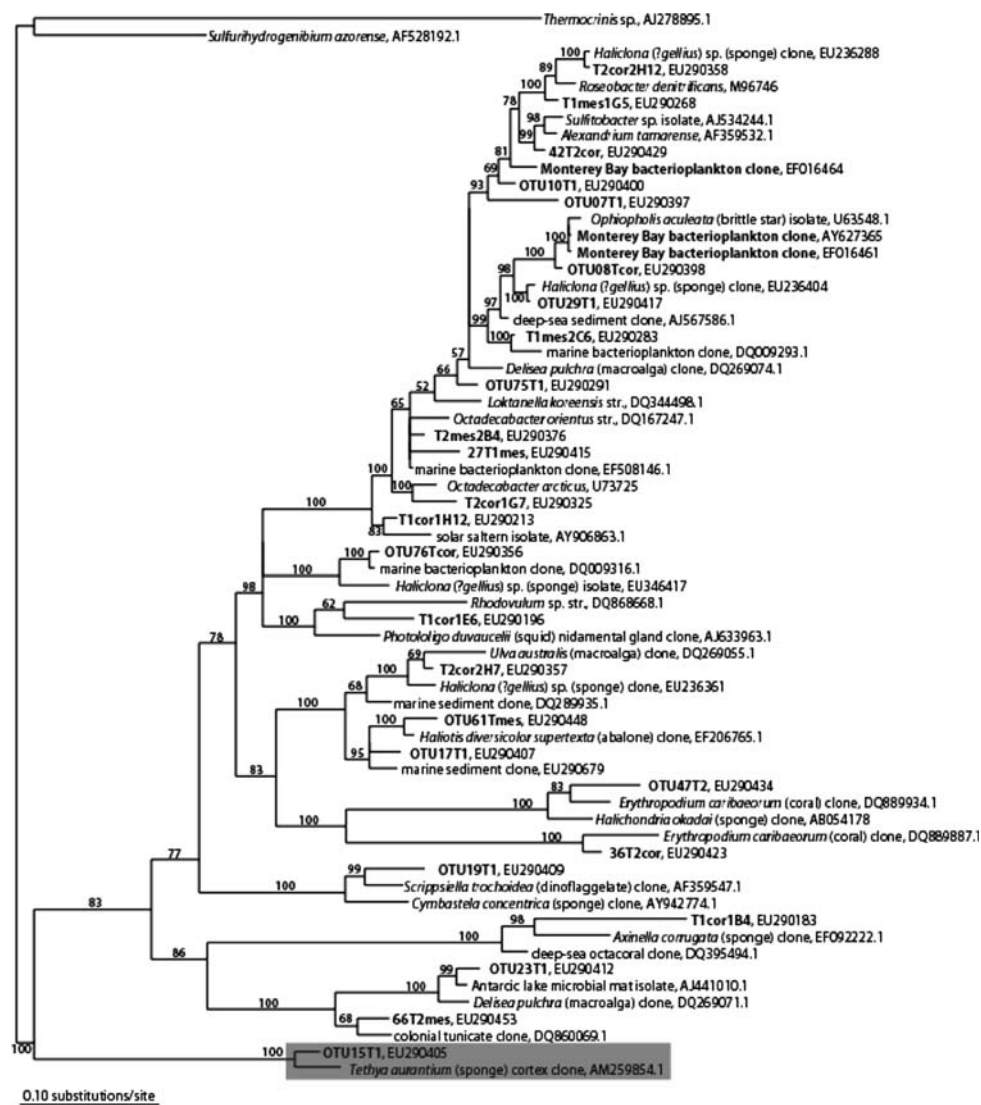
Fig. 3 Bayesian phylogram of α-*Proteobacteria* 16S rRNA gene sequences from *T. californiana* bacteria (*in bold*) and their nearest neighbors (greengenes database August 18, 2007). The numbers above or below the branches correspond to posterior probability (PP) values of the Baysian analysis. OTUs that were found in more than one sample start with the letters 'OTU' followed by a code indicating the samples in which it was detected: T1 = *T. californiana* specimen 1, T2 = *T. californiana* specimen 2, T = both specimens, cor = cortex, mes = mesohyl, no addition of 'cor' or 'mes' = found in both locations. OT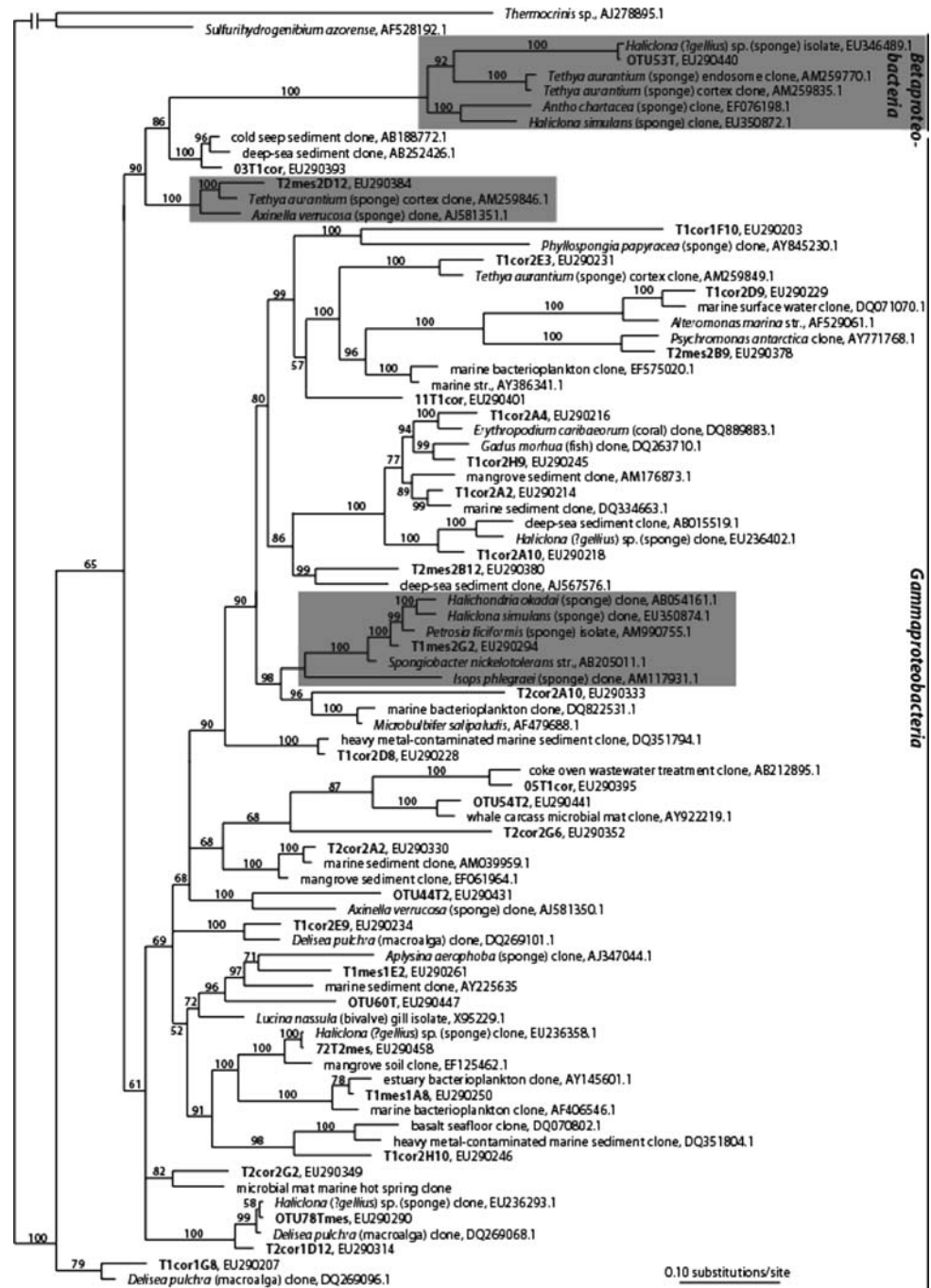Us that were found multiple times in only one individual are encoded with a number followed by the individual and location where it was found. If *T. californiana* OTUs were more closely related to each other than to any other sequence, only one of the OTUs was used to construct the tree. The *gray box* indicates a *Tethya*-specific cluster. *Thermocrinis* sp. and *Sulfurihydrogenibium azorense* were used as outgroup. Seawater-derived sequences obtained from Monterey Bay (the sampling site) are in *bold*. Phylogenetic tree topologies generated with a parsimony and maximum likelihood model were highly similar (data not shown)

Stramenopiles chloroplast-affiliated OTUs were among the most abundantly obtained clone sequences. They represent closely related phylotypes that are not confined to sponges but are also detected in phytoplankton and soil samples (Fig. 6a). This is consistent with the finding that all photosynthetic *Stramenopiles* are monophyletic based on 18S rDNA sequences (Massana et al., 2004). Although very few microbial eukaryote sequences have been isolated from marine sponges (Taylor et al. 2007), photosynthetic *Stramenopile*-affiliated clones were also abundant and stably associated with the sponge *Cymbastela concentrica* (Taylor et al. 2004).

For the other phyla, no sponge-specific clusters were observed (Fig. 6a, b), except for perhaps one *Acidobacterium* (T2cor2F4). This clone sequence is related to *Acidobacteria* derived from the sponges *Mycale laxissima* and *Axinella corrugata* and has no other near relatives. However, the clone was obtained as a singleton from only one tissue sample and is, therefore, probably only a minor constituent of the *T. californiana* microflora. OTUs

Fig. 4 Bayesian phylogram of β- and γ-*Proteobacteria* 16S rRNA gene sequences from *T. californiana* bacteria (*in bold*) and their nearest neighbors (greengenes database August 18, 2007). The *gray boxes* indicate putative sponge-specific clusters. For further explanation, see Fig. 3

Thermocrinis sp., AJ278895.1
Sulfurihydrogenibium azorense, AF528192.1

Haliclona (?gellius) sp. (sponge) isolate, EU346489.1
OTU53T, EU290440
Tethya aurantium (sponge) endosome clone, AM259770.1
Tethya aurantium (sponge) cortex clone, AM259835.1
Antho chartacea (sponge) clone, EF076198.1
Haliclona simulans (sponge) clone, AM350872.1
Betaproteobacteria

cold seep sediment clone, AB188772.1
deep-sea sediment clone, AB252426.1
03T1cor, EU290393
T2mes2D12, EU290384
Tethya aurantium (sponge) cortex clone, AM259846.1
Axinella verrucosa (sponge) clone, AJ581351.1

T1cor1F10, EU290203
Phyllospongia papyracea (sponge) clone, AY845230.1
T1cor2E3, EU290231
Tethya aurantium (sponge) cortex clone, AM259849.1
T1cor2D9, EU290229
marine surface water clone, DQ071070.1
Alteromonas marina str., AF529061.1
Psychromonas antarctica clone, AY771768.1
T2mes2B9, EU290378
marine bacterioplankton clone, EF575020.1
marine str., AY386341.1
11T1cor, EU290401
T1cor2A4, EU290216
Erythropodium caribaeorum (coral) clone, DQ889883.1
Gadus morhua (fish) clone, DQ263710.1
T1cor2H9, EU290245
mangrove sediment clone, AM176873.1
T1cor2A2, EU290214
marine sediment clone, DQ334663.1
deep-sea sediment clone, AB015519.1
Haliclona (?gellius) sp. (sponge) clone, EU236402.1
T1cor2A10, EU290218
T2mes2B12, EU290380
deep-sea sediment clone, AJ567576.1
Halichondria okadai (sponge) clone, AB054161.1
Haliclona simulans (sponge) clone, EU350874.1
Petrosia ficiformis (sponge) isolate, AM990755.1
T1mes2G2, EU290294
Spongiobacter nickelotolerans str., AB205011.1
Isops phlegraei (sponge) clone, AM117931.1
T2cor2A10, EU290333
marine bacterioplankton clone, DQ822531.1
Microbulbifer salipaludis, AF479688.1
heavy metal-contaminated marine sediment clone, DQ351794.1
T1cor2D8, EU290228
coke oven wastewater treatment clone, AB212895.1
05T1cor, EU290395
OTU54T2, EU290441
whale carcass microbial mat clone, AY922219.1
T2cor2G6, EU290352
T2cor2A2, EU290330
marine sediment clone, AM039959.1
mangrove sediment clone, EF061964.1
OTU44T2, EU290431
Axinella verrucosa (sponge) clone, AJ581350.1
T1cor2E9, EU290234
Delisea pulchra (macroalga) clone, DQ269101.1
Aplysina aerophoba (sponge) clone, AJ347044.1
T1mes1E2, EU290261
marine sediment clone, AY225635
OTU60T, EU290447
Lucina nassula (bivalve) gill isolate, X95229.1
Haliclona (?gellius) sp. (sponge) clone, EU236358.1
72T2mes, EU290458
mangrove soil clone, EF125462.1
estuary bacterioplankton clone, AY145601.1
T1mes1A8, EU290250
marine bacterioplankton clone, AF406546.1
basalt seafloor clone, DQ070802.1
heavy metal-contaminated marine sediment clone, DQ351804.1
T1cor2H10, EU290246
T2cor2G2, EU290349
microbial mat marine hot spring clone
Haliclona (?gellius) sp. (sponge) clone, EU236293.1
OTU78Tmes, EU290290
Delisea pulchra (macroalga) clone, DQ269068.1
T2cor1D12, EU290314
T1cor1G8, EU290207
Delisea pulchra (macroalga) clone, DQ269096.1

Gammaproteobacteria

0.10 substitutions/site

affiliated to *Chloroflexi*, *Nitrospirae*, *Actinobacteria*, δ-*Proteobacteria*, ε-*Proteobacteria*, candidate division OP11, *Verrucomicrobia*, *Planctomycetes* and *Firmicutes* represented only a small fraction of the clone library. The only exception is *Firmicutes*-affiliated OTU57T that was present in both cortex and mesohyl samples from the two *T. californiana* specimens and represented 2.8% of all clones. *Firmicutes* quite commonly represent a significant percentage of bacterial 16S rRNA gene clone sequences from polar and cold-temperate marine sponges (Hill et al. 2006);

however, sponge-specific clusters are uncommon (Taylor et al. 2007).

A remarkably high fraction of the *T. californiana*-derived OTUs shared less than 95% with any other sequence in Genbank. Twenty percent of all sequences in the 16S rRNA gene clone library (32% of all OTUs) belong to this category (BLAST search April 29, 2008). Although most of these OTUs were detected in only one tissue sample and are unlikely to be sponge-specific or important partners for the sponge, they demonstrate the aptness of

Fig. 5 Bayesian phylogram of *Bacteroidetes* 16S rRNA gene sequences from *T. californiana* bacteria (*in bold*) and their nearest neighbors (greengenes database August 18, 2007). Seawater-derived sequences obtained from Monterey Bay (the sampling site) are also in *bold*. For further explanation, see Fig. 3
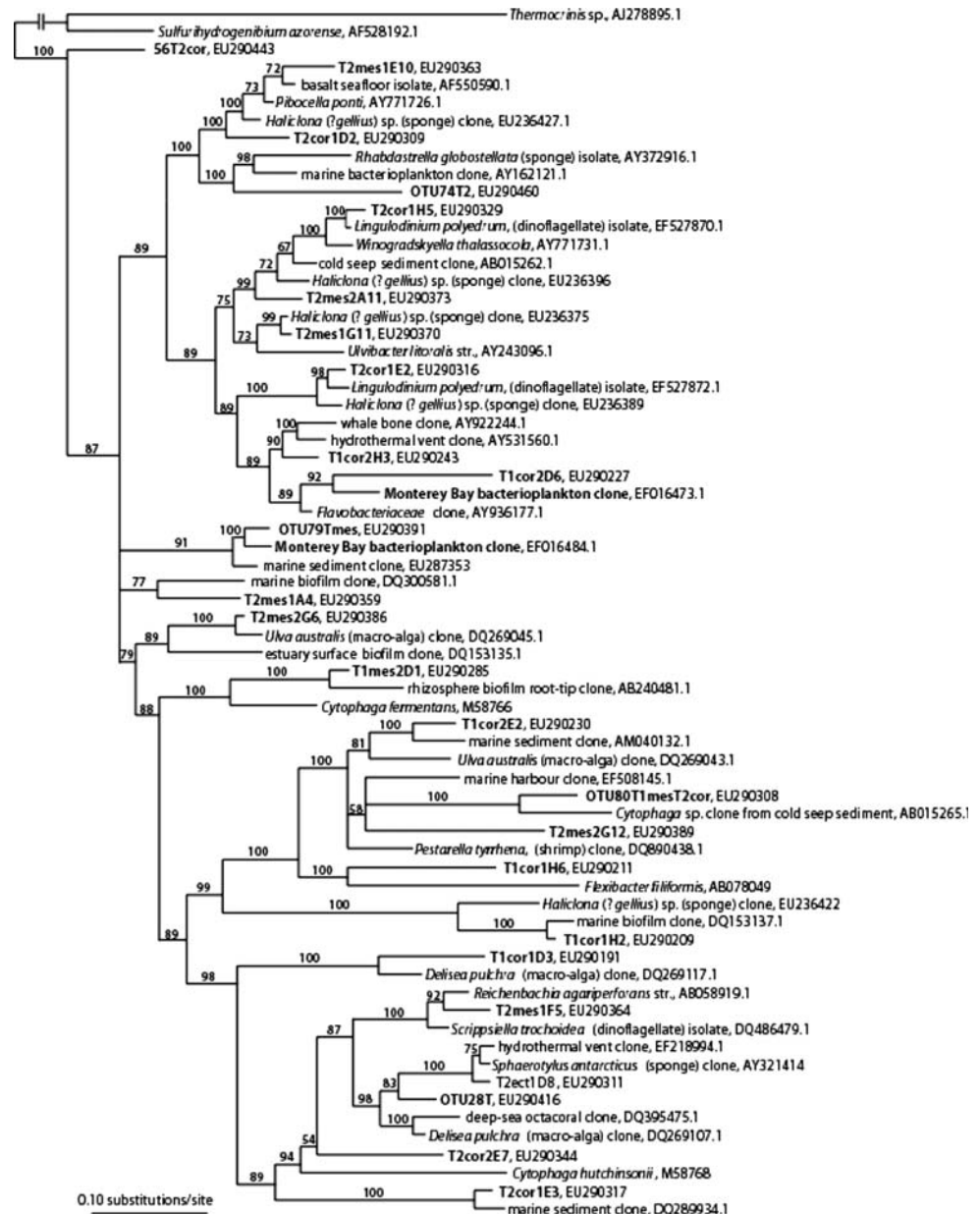
sponges as a source to discover 'new' microorganisms. Sponges from under-represented locations with regard to 16S rRNA gene libraries (Taylor et al. 2007), such as *T. californiana*, may be particularly rich in previously undetected microbes.

Distribution of OTUs in *T. californiana* tissue

Overlap of OTUs in tissue samples from different specimens was apparent, but similarity between cortex and mesohyl samples from the same specimen was even more pronounced (Fig. 7). Only six OTUs (08, 50, 61, 76, 78 and 79) were confined to either mesohyl or cortex (singletons excluded), while 37 OTUs were found in both cortex and mesohyl

samples (Fig. 8). OTU08 and OTU79 were discovered to be probably local seawater-derived, while the other OTUs that were confined to either mesohyl or cortex have each near neighbors from various sources (sediment, seawater, marine invertebrates), which does not support spatial specificity in sponge tissue. The singletons represent a large part of microbial diversity in *T. californiana* and by definition they are restricted to only one tissue region and may include spatially selective microorganisms. However, singletons are also restricted to only one of the *T. californiana* specimens, and therefore, they are not included in the analysis for spatial specificity. Pyrosequencing of the samples could yield information whether the singletons are really singletons or whether they represent spatio-selective bacteria.

Fig. 6 a, b Bayesian phylogram of 16S rRNA gene sequences from *T. californiana* and their nearest neighbors (greengenes database August 18, 2007) belonging to other phyla. *Archaeoglobus fulgidus* and *Methanococcus maripaludis* were used as outgroup. For further explanation, see Fig. 3
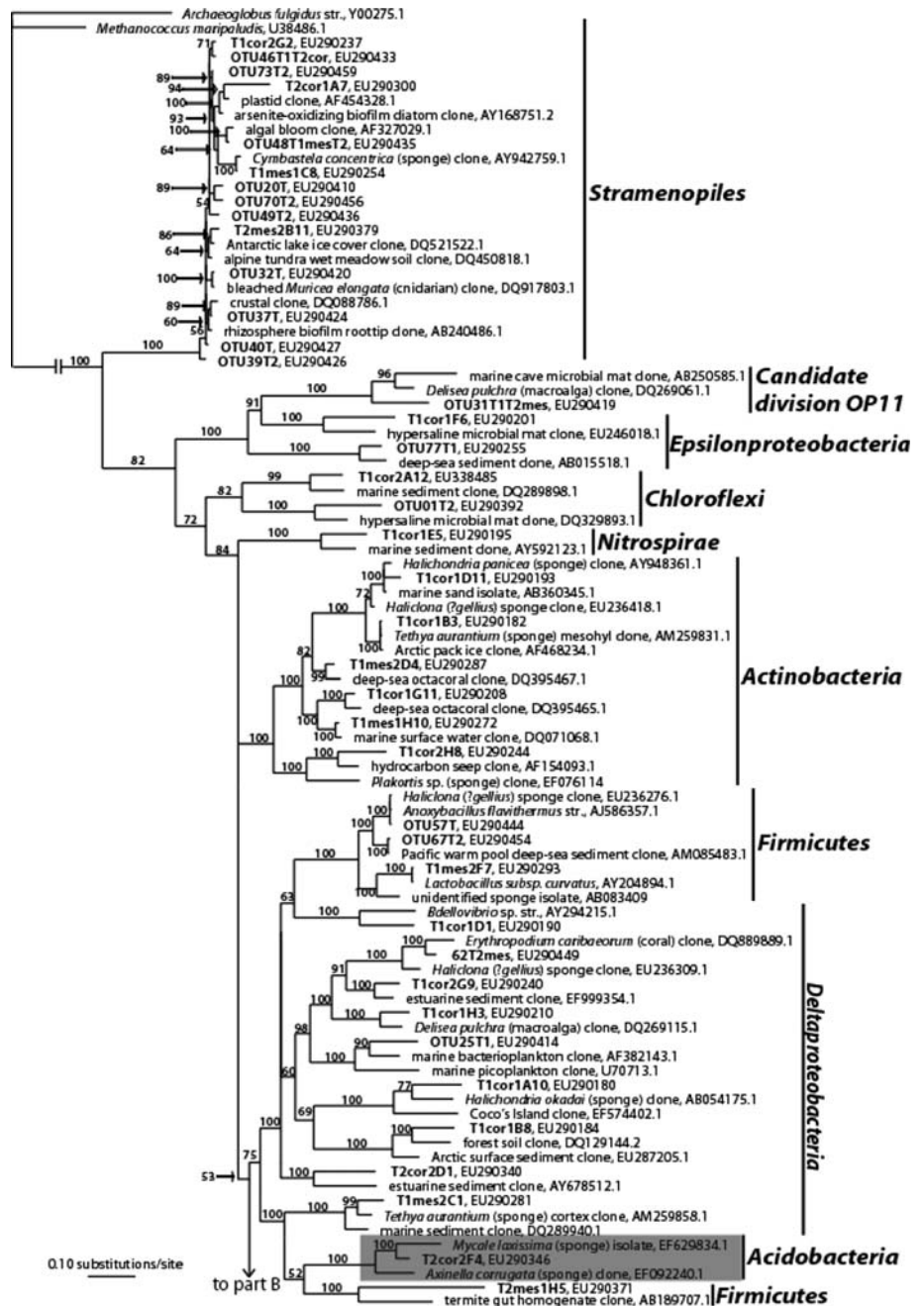
Despite similarities of the microbial communities in *T. californiana* and *T. aurantium* on the phylum-level and, to a lesser extent at the OTU-level, spatio-selectivity of the associated bacteria for *T. aurantium* could not be confirmed for *T. californiana*. Instead, phylogenetic analysis showed that for a number of cases, clones from mesohyl samples from *T. californiana* shared most identity with cortex-derived clones from *T. aurantium* and vice versa (Fig. 4: T2mes2D12, and Fig. 6a: T1cor1B3, T1mes2C1). For *T. aurantium*, a big shift of the OTUs was measured for samples in different years. Overlap between samples of 2003 and 2004 showed that only 3 out of 51 OTUs could be

recovered from samples from both years (Thiel et al. 2007), which could imply that either microbial populations in the sponge are unstable, or that total microbial diversity was not covered. The authors showed with rarefaction analysis that 46 and 58% of microbial diversities were covered for the cortex and mesohyl, respectively. The remaining uncovered diversity leaves room to either confirm or reject a conclusion on regional specificity of associated microorganisms. However, the fact that *Cyanobacteria*-affiliated clones, which represented 27% of all *T. aurantium* clone sequences, were found almost exclusively in the mesohyl suggests that for the *Cyanobacteria*, spatial selectivity exists in the

Fig. 6 continued
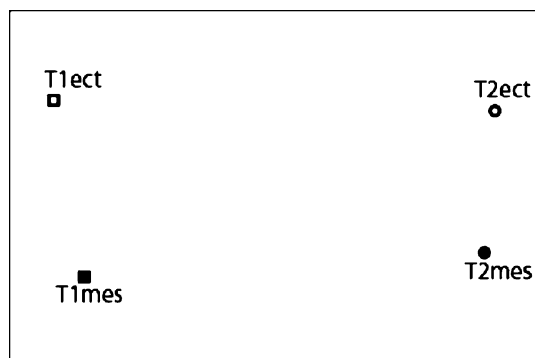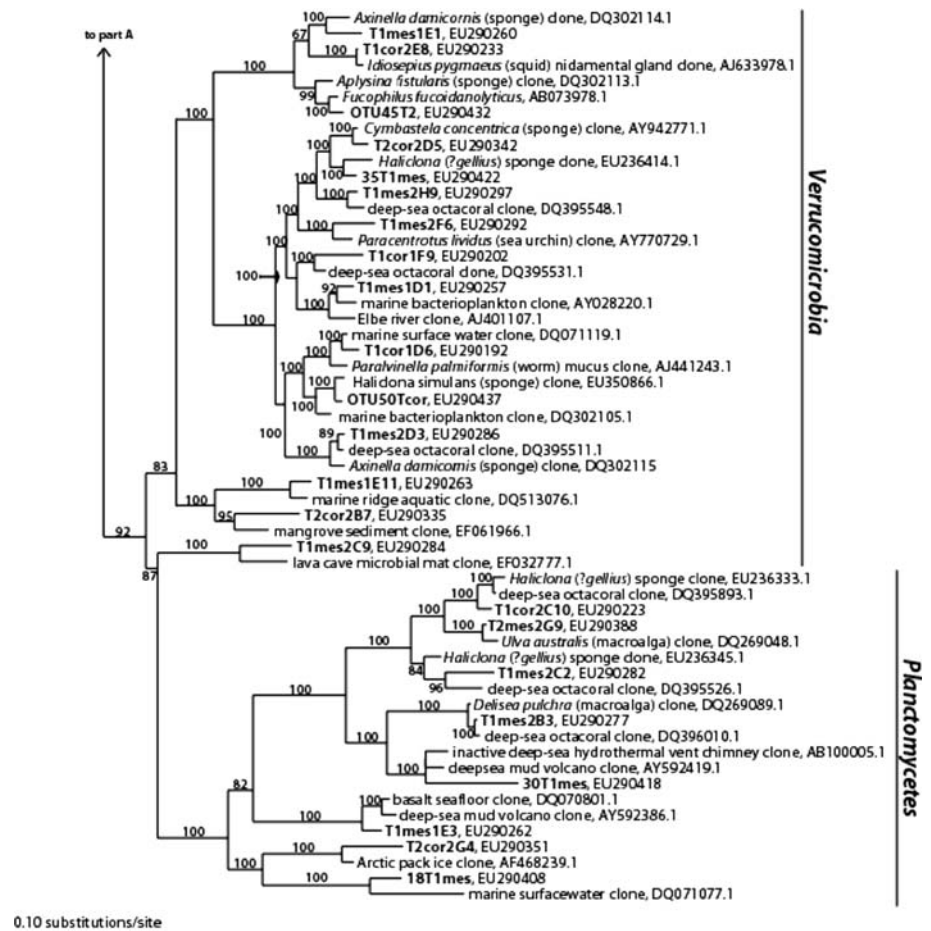
Fig. 7 Bray Curtis analysis of similarity between samples based on OTUs (singletons were not taken into account). The relative distance between the data points is a measure for similarity, i.e., data points that are closer to each other indicate a higher similarity

Mediterranean sponge species. Another study on spatial distribution of bacteria and archaea in the deep-water sponge *Polymastia* cf. *corticata* also suggested spatial selectivity of the microorganisms (Meyer and Kuever, 2008). The authors found that *Acidobacteria*, *Actinobacteria* and *Deltaproteobacteria* were sponge- or species-specific and restricted to the mesohyl. The same was true for sponge-specific archaeal sulfate reducers. This result was not confirmed for *T. californiana*, as we did not find sponge-specific members of *Deltaproteobacteria* and *Actinobacteria* (archaea were not tested), while the only sponge-specific member of the *Acidobacteria* was detected in an cortex sample. In addition, other sponge-specific OTUs in *T. californiana* were not restricted to either cortex or mesohyl.

Differences between mesohyl and cortex in *T. californiana* may exist with respect to the microbial concentration in the different regions, as associated microorganisms are generally more abundant in the mesohyl than in the cortex, which was confirmed for *T. aurantium* (Thiel et al. 2007). The most pronounced (qualitative) difference we found between cortex and mesohyl samples for *T. californiana* was the number of OTUs detected: 92.5 ± 9.2 and 67 ± 8.5 OTUs, respectively. However, this may be an artifact caused by different bacterial densities in mesohyl and cortex samples (if they resemble the concentrations in *T. aurantium*). If the bacterial density is much higher in the latter samples, it would lead to a relative over sampling of the cortex samples compared to the mesohyl samples, because a similar number of clones were used for both

T. californiana1   T. californiana1&2   T. californiana2
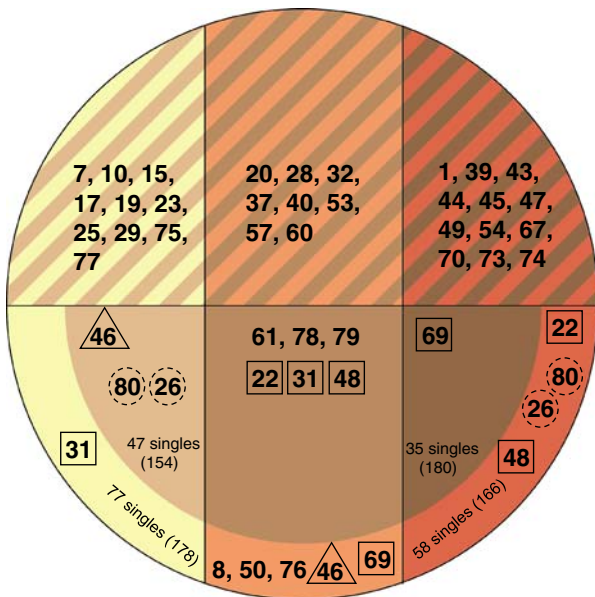
Fig. 8 The distribution of associated microbial species based on OTUs in *T. californiana* tissue samples. Each number refers to the accompanying OTU. The *left part* of the figure represents *T. californiana* specimen 1, the *right part Tethya californiana* specimen 2 and the center overlap between the specimens. The upper half represents OTUs that were detected among cortex AND mesohyl samples, and in the lower half OTUs are depicted that were restricted to one of the zones in the sponge. In addition, OTUs that were present in 2 or 3 of the 4 samples (but in a nonsystematic way) are also depicted in the lower half surrounded by *squares*, *triangles* and *circles*. For example, OTU22 was detected in the cortex of T2 and in the mesohyl of T1 and T2

regions. This could lead to the isolation of relatively more rare or 'accidental' species from the low-density sample (Gotelli and Colwell 2001). This phenomenon might interfere with rarefaction analysis as it has been noted that if many OTUs of low abundance are present in the data set (as is the case especially for the cortex samples), rarefaction curves tend to converge to a line without an apparent limit and not accurately describe the predicted number of OTUs (Thiel et al. 2007). Therefore, based on qualitative data on microbial diversity and distribution of the diversity in the sponge tissue, we do not observe spatial selectivity of *T. californiana* associates.

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