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Comparisons of diversity of bacterial communities associated with three sessile marine eukaryotes

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ABSTRACT: Host-associated bacterial communities are potentially critical components of marine microbial diversity, yet our understanding of bacterial distribution on living surfaces lags behind that for planktonic communities. We used 16S rRNA gene library analysis to compare within-host (alpha) and between-host (beta) diversity among bacterial communities on 3 co-occurring marine eukaryotes from temperate Australia: the demosponge *Cymbastela concentrica*, the red macroalga *Delisea pulchra* and the green intertidal alga *Ulva australis*. The bacterial community on *C. concentrica* had high phylum-level diversity (7 phyla including 3 proteobacterial classes) but relatively low 'species' richness (estimated at 24 species). Among the algae, *D. pulchra* contained 7 phyla including an estimated 79 species, while the *U. australis* library yielded only 4 phyla with an estimated 36 species. *Alpha-*, *Delta-* and *Gammaproteobacteria* were well represented in all libraries, while *Planctomycetes* and *Bacteroidetes* were relatively common on the 2 algae, but absent or rarely encountered in the sponge. At the phylum level, the community of *C. concentrica* largely mirrored that found in other marine sponges (e.g. *Proteobacteria*, *Actinobacteria*, *Nitrospira*), although large numbers of diatoms and the presence of *Verrucomicrobia* were atypical. Overall, within-host (alpha) diversity was relatively high, at least for *C. concentrica* and *D. pulchra*, while between-host (beta) diversity depended heavily on the phylogenetic level examined. Generally, there was a remarkable lack of overlap at the species level. No species showed universal distribution across hosts, indicating high beta diversity at the species level. At the level of phyla, however, both universal (e.g. *Proteobacteria*) and distinct (e.g. *Nitrospira*) groups existed. This study is among the first to compare patterns of alpha and beta diversity for microbial communities associated with co-occurring marine eukaryotes.

KEY WORDS: Bacterial communities · Diversity · Marine · Host organisms · Sponge · Macroalga

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INTRODUCTION

The study of marine bacterial communities has advanced considerably in the past 2 decades. Advances in molecular technology have enabled the previously elusive non-cultivated fraction of bacteria to be characterised, revealing a remarkable level of diversity in the world's oceans (Venter et al. 2004, Giovannoni & Stingl 2005, Rusch et al. 2007). Although it is likely that still only a small fraction of the total diversity has been sampled (Curtis et al. 2002, DeLong 2004; but see Hagström et al. 2002), similar patterns have nonetheless emerged among planktonic bacterial communities throughout the world. For example, members of the *Proteobacteria* (primarily *Alpha-* and *Gamma-* classes) have a global distribution in oceanic and coastal waters (e.g. Britschgi & Giovannoni 1991, Schmidt et al. 1991, Field et al. 1997, Glöckner et al.

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1999, Rappé et al. 2000, Venter et al. 2004, Rusch et al. 2007), with the alphaproteobacterial SAR 11 clade accounting for up to 50% of all microbial cells in ocean surface waters (Morris et al. 2002). Cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* are also extremely prevalent (Partensky et al. 1999, Scanlan & West 2002), while other frequently encountered marine taxa include the *Bacteroidetes*, *Actinobacteria*, *Planctomycetes* and *Chloroflexi* (Giovannoni et al. 1996, Glöckner et al. 1999, Simon et al. 1999, Cottrell & Kirchman 2000, Rappé et al. 2000, Venter et al. 2004, Giovannoni & Stingl 2005, Rusch et al. 2007). This apparent global distribution of various bacterial phyla (and even particular species, e.g. Mullins et al. 1995, Rappé et al. 2002) suggests a reasonable degree of uniformity to marine bacterioplankton communities. Despite this overall uniformity, however, compositional differences have also been observed, perhaps resulting from rare species being detected in some samples (Kemp & Aller 2004, Rusch et al. 2007).

In contrast to the relatively well-studied planktonic and particle-associated communities, in which we are now starting to recognise emergent patterns of bacterial distribution, the study of living surface-associated biofilms is still in its infancy, and insufficient data exist to make similar large-scale comparisons of epibionts (i.e. bacteria associated with eukaryotic hosts). Given the potentially substantial contribution of host-associated microorganisms to marine microbial diversity (Taylor et al. 2004), and the fact that many bacteria live surface-associated (biofilm) rather than planktonic lifestyles (Hall-Stoodley et al. 2004), it is important to consider how such communities are structured among different host organisms. Living surfaces are typically nutrient-rich environments where inorganic molecules and metabolic by-products accumulate, often exude different chemical deterrents or cues (de Nys et al. 1995, Steinberg et al. 2002), and are generally complex morphologically (e.g. tissue differentiation). Thus living surfaces are likely to provide very different and more highly differentiated habitats compared to pelagic environments. As a result, one might expect different assemblages of bacteria between pelagic and host-associated communities.

Several studies have examined bacterial communities associated with marine living surfaces, and indications for patterns of bacterial diversity for at least some host taxa exist (e.g. Krueger & Cavanaugh 1997, Polz et al. 1999, Ashen & Goff 2000, Friedrich et al. 2001, Rohwer et al. 2001, 2002, Webster et al. 2001, López-García et al. 2002, Taylor et al. 2004). Evidence for shared bacterial taxa among unrelated, geographically disparate marine sponges (Hentschel et al. 2002, 2006, Taylor et al. 2007), coupled with indications of host-specific bacteria in other sponges (Taylor et al. 2004), suggests the presence of some (host- or area-) unique community members against a background of underlying uniformity for sponges, similar in this respect to the patterns found in seawater bacterial communities (e.g. Glöckner et al. 1999, Rappé et al. 2000, Venter et al. 2004). Additional data would verify the strength of these patterns for marine sponges, and further research into other eukaryote-associated biofilms is needed to establish trends, if any, for their epibionts. In the case of vestimentiferan tubeworms, animals from different hydrothermal vent sites share closely related gammaproteobacterial symbionts (McMullin et al. 2003). Similarly, 3 species of coral in Bermuda and Panama each had distinct bacterial communities that were stable in both space and time, suggesting a specific microbe–coral association (Rohwer et al. 2002). Thus there is evidence that taxonomically related hosts from different locations in some instances may have a substantial proportion of their symbionts in common. However, there are relatively few examples of the converse, i.e. comparisons of prokaryotic phylogeny and diversity among co-occurring marine eukaryotes.

In plant and animal ecology, a great deal of attention has been devoted to establishing patterns of diversity across a vast range of terrestrial and marine habitats. While the resulting studies have prompted much debate over what comprises appropriate tiers in a hierarchical classification of diversity (e.g. Whittaker et al. 2001), a standard way of differentiating/characterising levels of diversity is to consider within-habitat (in our context within a single host) diversity as alpha diversity and between-habitat (among hosts) diversity as beta diversity (Whittaker 1972). This distinction has only recently been explicitly considered for microorganisms, but is profound (e.g. Green et al. 2004). For example, the destruction of habitats (hosts) for which alpha diversity is high but beta diversity is low may have relatively little consequence for overall diversity; conversely, when beta diversity is high but alpha diversity is low, destruction of habitats results in substantial loss of biodiversity. Other factors affecting diversity include the level of disturbance or the harshness of the environment. For example, disturbed communities may have higher diversity than undisturbed communities in which competition may result in 1 or a few individual species dominating (Begon et al. 1996). In contrast, harsh environments tend to be species-poor and are typically occupied by species that can withstand the extreme conditions.

Here we present an analysis of bacterial communities associated with 3 co-occurring sessile marine eukaryotes from temperate south-eastern Australia — the sponge *Cymbastela concentrica*, the red macroalga *Delisea pulchra* and the intertidal green alga *Ulva australis* — in order to compare alpha and beta diversity

among these communities. In temperate reef systems, macroalgae and sponges are among the most conspicuous benthic components, and these 3 hosts are common in the shallow sublittoral or intertidal zones in coastal habitats near Sydney, New South Wales, Australia. *C. concentrica* is an abundant, small, bowl-shaped demosponge known to harbour a stable microbial community (Taylor et al. 2004, 2005). *D. pulchra* is a foliose red macroalga from the shallow subtidal zone that produces a range of biologically active secondary metabolites (furanones), capable of inhibiting various fouling organisms from bacteria to algae and invertebrates (de Nys et al. 1995, Steinberg et al. 1997, Manefield et al. 1999). *U. australis* is predominantly found in the mid- to low intertidal zone at the sites used for this study, but also occurs subtidally. It is a thin (only 2 cells thick) green macroalga with a relatively simple flat thallus and lacks the structural complexity of the other 2 hosts examined in this study.

Sponges have been the focus of investigations into microbial assemblages of marine eukaryotes with more than 15 bacterial phyla, both major lineages of *Archaea* and an assortment of eukaryotic microbes identified from sponges (reviewed by Wilkinson 1992, Hentschel et al. 2003, 2006, Taylor et al. 2007). The microbial community of *Cymbastela concentrica* has been previously characterised by denaturing gradient gel electrophoresis (DGGE; Taylor et al. 2004, 2005). Less is known about bacterial epiphytes inhabiting marine macroalgae, with only 1 marine macroalga-derived, general 16S rRNA gene library published (Meusnier et al. 2001), and this is reflected in our limited understanding of the bacterial communities on the 2 species of seaweed investigated in this study (but see Maximilien et al. 1998, Tujula et al. 2006). DGGE analysis of inanimate surfaces and seawater in the vicinity of *D. pulchra* shows that all 3 environments harbour distinct bacterial assemblages with very little overlap in community composition (S. R. Longford et al. unpubl.).

Each of the eukaryote hosts examined in this study bears very different surface conditions for microbial epiphytes. Sponges are chemically rich and have very complex architecture with a system of internal channels that are constantly flushed with seawater. The antimicrobial properties of the furanones exuded by *Delisea pulchra* add to the complexity of the substrate and are likely to influence bacterial settlement on the surface. By comparison, *Ulva australis* experiences extreme environmental fluctuation (e.g. in light, temperature and salinity) owing to its location in the intertidal zone. There is also some evidence for biologically active compounds (against herbivores) from *Ulva* (Van Alstyne et al. 2001). As a consequence of all of these factors, one might expect different bacterial communities on each surface.

MATERIALS AND METHODS

Sampling. The marine sponge *Cymbastela concentrica* and marine red alga *Delisea pulchra* were collected as aseptically as possible by SCUBA from Bare Island, Botany Bay, Sydney, Australia (Fig. 1) in March 2002. At low tide on the same day, the intertidal green alga *Ulva australis* (commonly known as 'sea lettuce') was collected from the rock platform at nearby Shark Point, Sydney (Fig. 1). Three portions were taken from each of 3 *C. concentrica* individuals, with portions combined prior to processing to give 1 (pooled) sample per individual. For *U. australis* 3 entire individuals were sampled for gene library construction, while the distal regions of 3 individual *D. pulchra* plants were sampled (additional *U. australis* and *D. pulchra* samples were taken for DGGE analyses; see below). All samples were rinsed 3 times in filter-sterilised seawater to remove loosely attached bacteria, frozen and later freeze-dried prior to DNA extraction.

DNA extraction and PCR. DNA was extracted from 5 mg of *Cymbastela concentrica* tissue and 30 mg of *Delisea pulchra* tissue by bead-beating in an ammonium acetate buffer, as previously described (Taylor et al. 2004). This method was less successful for *Ulva aus-*

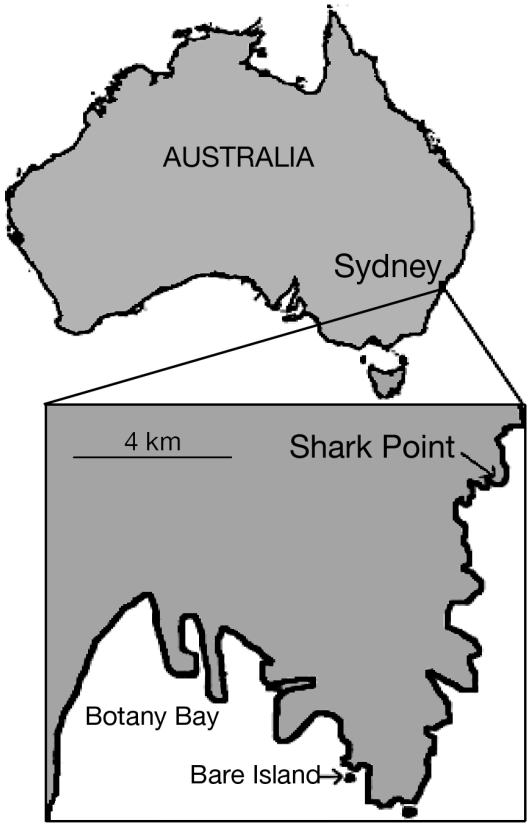

Fig. 1. Sample collection sites at Bare Island and Shark Point (inset), Sydney, Australia

tralis, so for these samples, DNA was extracted from 45 mg of tissue using a DNA Spin Kit for soil (BIO 101 Systems; Q Biogene). The surface area of each alga sampled was held consistent. *D. pulchra* has a thicker, more complex thallus than *U. australis*, and although its surface area is increased by its structural complexity, so too is its mass in relation to the surface area. In contrast, the thallus of *U. australis* is only 2 cells thick and blade-like in form. As such, it has a very high surface area relative to its mass. The porous nature of the sponge *C. concentrica* precludes any comparisons with the algae in relation to surface area. For *C. concentrica*, however, there is no evidence of a species–area relationship for its associated bacteria (M. W. Taylor pers. obs.). 16S rRNA gene fragments were PCR-amplified using the *Bacteria* primers 27F (5′-AGAGTTTGATC MTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTG TTACGACTT-3′; Lane 1991, Marchesi et al. 1998). Reactions were carried out in a volume of 50 µl, which contained 25 pmol of each primer, 50 µM of each dNTP and 1 U RedTaq DNA polymerase (Sigma). PCR was performed in a Hybaid PCR Express thermal cycler as follows: 94°C for 5 min; 80°C hot-start; 25 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; 72°C final extension for 10 min. All reactions yielded correct-sized amplificates with no additional by-products.

16S rRNA gene library construction. For the *Cymbastela concentrica, Ulva australis* and *Delisea pulchra* samples, 3 PCR reactions (each representing an individual sponge/alga) were pooled prior to ligation, and 1 clone library per host species was constructed using the Topo TA cloning system (Invitrogen) according to the manufacturer's instructions. Restriction fragment length polymorphism (RFLP) analysis was performed with the restriction enzyme *Rsa*I (Roche) on randomly selected clones from the *C. concentrica* (n = 155), *D. pulchra* (n = 198) and *U. australis* (n = 200) libraries, and partial sequences were obtained for representatives of each RFLP type. Following preliminary phylogenetic analysis, clones representative of the diversity in each library were selected for sequencing of the entire gene. Bacterial species richness was estimated by the non-parametric estimator Chao1, calculated using EstimateS 7.5 (Colwell 2005), and was based on both RFLP patterns and sequences. Bacterial 'species', or operational taxonomic units (OTUs), were defined based on a 97% sequence similarity threshold (Wayne et al. 1987, Stackebrandt & Goebel 1994, Hagström et al. 2000). We acknowledge that differences in function and phylogeny render this definition controversial (e.g. Cohan 2002, 2006 and references cited therein) but chose to use it in the absence of a current simplified working criterion to delineate bacterial 'species'. Clone library coverage was calculated according to Singleton et al. (2001), and libraries were compared statistically using the parsimony method originally described for microbial communities by Martin (2002) and implemented in TreeClimber (Schloss & Handelsman 2006).

Phylogenetic analysis. To obtain approximate phylogenetic affiliations for the sequenced clones, each sequence was subjected to BLAST analysis against the GenBank database (www.ncbi.nlm.nih.gov/BLAST/). Sequences from this study, and their close relatives derived from GenBank, were aligned in the ARB package (Ludwig et al. 2004) using the FastAligner function, and then all alignments were manually refined. Maximum likelihood-based phylogenetic analysis was conducted in ARB (AxML) using near full-length (>1300 bp) sequences only, and the robustness of tree topologies was tested by parsimony-based bootstrap analysis (1000 resamplings). Chimeric sequences were identified by phylogenetic analysis and by using CHIMERA_CHECK (Cole et al. 2003). Six putative chimeras were detected in the *Delisea pulchra* library, 2 in the *Ulva australis* library and 5 for *Cymbastela concentrica*. All were removed from further analyses. Sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AY942753–AY942781, DQ269035–DQ269125 and DQ309997.

DGGE. The nature of clone libraries (e.g. the amount of work needed to generate each library) precludes their use for replicate sampling. Thus, one difficulty with clone libraries is that variability among samples of the same treatment type (here, each eukaryote host) is not determined. To investigate variability in microbial community composition among individuals of the same host species, we employed 16S rDNA-based DGGE, as described by Taylor et al. (2004). DGGE was performed on 10 *Delisea pulchra* thalli (tips only) and 16 *Ulva australis* individuals, collected at the same time as (and including) the 3 samples of each host that were used for gene library construction. Variability in the microbial community associated with *Cymbastela concentrica* has already been described in detail elsewhere (Taylor et al. 2004, 2005). Band presence/absence was recorded, and banding patterns were compared using cluster diagrams constructed in PRIMER v5.2.2 (PRIMER-E, Plymouth).

RESULTS

Operational taxonomic unit diversity

The alga *Delisea pulchra* contained many more bacterial OTUs (97% 16S rRNA gene similarity threshold; hereafter referred to as bacterial 'species') than did either the alga *Ulva australis* or the sponge *Cymbastela concentrica*.

After removal of 22 mitochondrial and 15 chloroplast sequences from the 236 clones analysed, the *Delisea pulchra*-derived RFLP and sequence types were grouped into 62 bacterial species. The Chao1 estimator predicted an overall richness of 79 species. Library coverage for *D. pulchra* was estimated at 88% (when the 97% similarity threshold was applied).

Twenty-five bacterial species were recovered from the *Ulva australis* library following exclusion of 15 chloroplast sequences from 197 clones analysed. The Chao1 estimator calculated an overall richness of 36 species, and library coverage was estimated at 96%.

Cymbastela concentrica yielded 42 distinct RFLP types, with 7 of these identified by sequencing as diatom plastids. When these non-bacterial clones (77 in total) were removed from the 158 clones analysed, 35 distinct RFLP types were found among the 78 remaining clones. Ultimately, 19 distinct bacterial species were recovered, while Chao1-estimated richness was 24 species. Library coverage was calculated at 92%.

Phylogenetic comparisons of bacteria associated with *Cymbastela concentrica*, *Delisea pulchra* and *Ulva australis*

Phylum-level diversity was highest in the *Cymbastela concentrica* and *Delisea pulchra* libraries, with each containing 7 described bacterial phyla (including, in both cases, 3 classes of *Proteobacteria*; Figs. 2 & 3). In contrast, *U. australis* contained only 4 phyla, although, again, the same 3 proteobacterial classes were represented in this library (Figs. 2 & 3). Additionally, *D. pulchra* and *U. australis* both contained members of a lineage of uncertain affiliation (Fig. 2b).

The phylogenetic compositions of the 3 libraries were compared using the parsimony test (Martin 2002, Schloss & Handelsman 2006). The statistical significance of the overall test (considering all 3 libraries) differed depending on the phylogenetic method used (Table 1). In an attempt to clarify whether significant differences did indeed exist, we therefore performed individual pairwise tests to compare the 3 libraries. Only the *Cymbastela concentrica* and *Ulva australis* libraries differed significantly, and this was independent of the phylogenetic method applied (Table 1). Thus, with at least some evidence for significant differences in bacterial community composition, we sought to determine which taxa were responsible for these differences. Plotting the proportion of bacterial species per phylogenetic group per host (Fig. 3) revealed the following patterns of bacterial distribution. The *Alpha-*, *Delta-* and *Gammaproteobacteria*, the *Actinobacteria* and the *Bacteroidetes* were common to all 3 hosts (Figs. 2 & 3), although in some cases (*Actinobacteria* in *Delisea pulchra* and *C. concentrica*, and *Bacteroidetes* in *C. concentrica*) only a single species from a phylum was detected. *Verrucomicrobia*, *Cyanobacteria* and *Chloroflexi* were restricted to *D. pulchra* and *C. concentrica*, while the *Planctomycetes* and 'uncertain affiliation' lineages were found only in the algal (*D. pulchra* and *U. australis*) libraries. The only phylum to be recovered from a single host was *Nitrospira* from the sponge *C. concentrica*.

Diatom chloroplasts accounted for approximately half of the sequences in the *Cymbastela concentrica* library. The apparent high abundance of diatoms within this sponge was confirmed by fluorescence microscopy (with diatom autofluorescence highest in the illuminated periphery of the sponge) and by transmission electron microscopy (data not shown). Various bacterial types were also visible in 'bacteriocytes' (specialised cells housing endosymbiotic bacteria), as previously observed in other sponges (Ruetzler et al. 2003, Dunlap et al. 2006).

Intra-specific variability in bacterial community composition for *Cymbastela concentrica*, *Delisea pulchra* and *Ulva australis*

To confirm whether the above-mentioned results are truly representative of the respective host species, we examined variability in microbial community composition among individuals of the same host. 16S rDNA-DGGE analyses revealed largely consistent banding patterns (and inferred community composition) for a given host species, with the *least* similar individuals of *Delisea pulchra* still exhibiting >60% overlap, while those of *Ulva australis* were >70% similar. Similarly stable communities (i.e. >70% similarity among individuals) have previously been reported for *Cymbastela concentrica* (Taylor et al. 2004, 2005). This suggests that the microbial diversity as revealed by our clone libraries is representative of different host individuals. Moreover, because the similarity in assemblages as revealed by DGGE was similar across the 3 hosts, there is no apparent bias among hosts in our estimates of diversity from the libraries. The DGGE analyses serve to illustrate the accuracy with which the clone library samples represent the different host communities. The overlap between and among hosts is documented in the phylogenetic trees (Fig. 2) and other analyses outlined above.

DISCUSSION

Marine eukaryotes may constitute a substantial reservoir of bacterial diversity (Taylor et al. 2004), often very different in composition from the planktonic

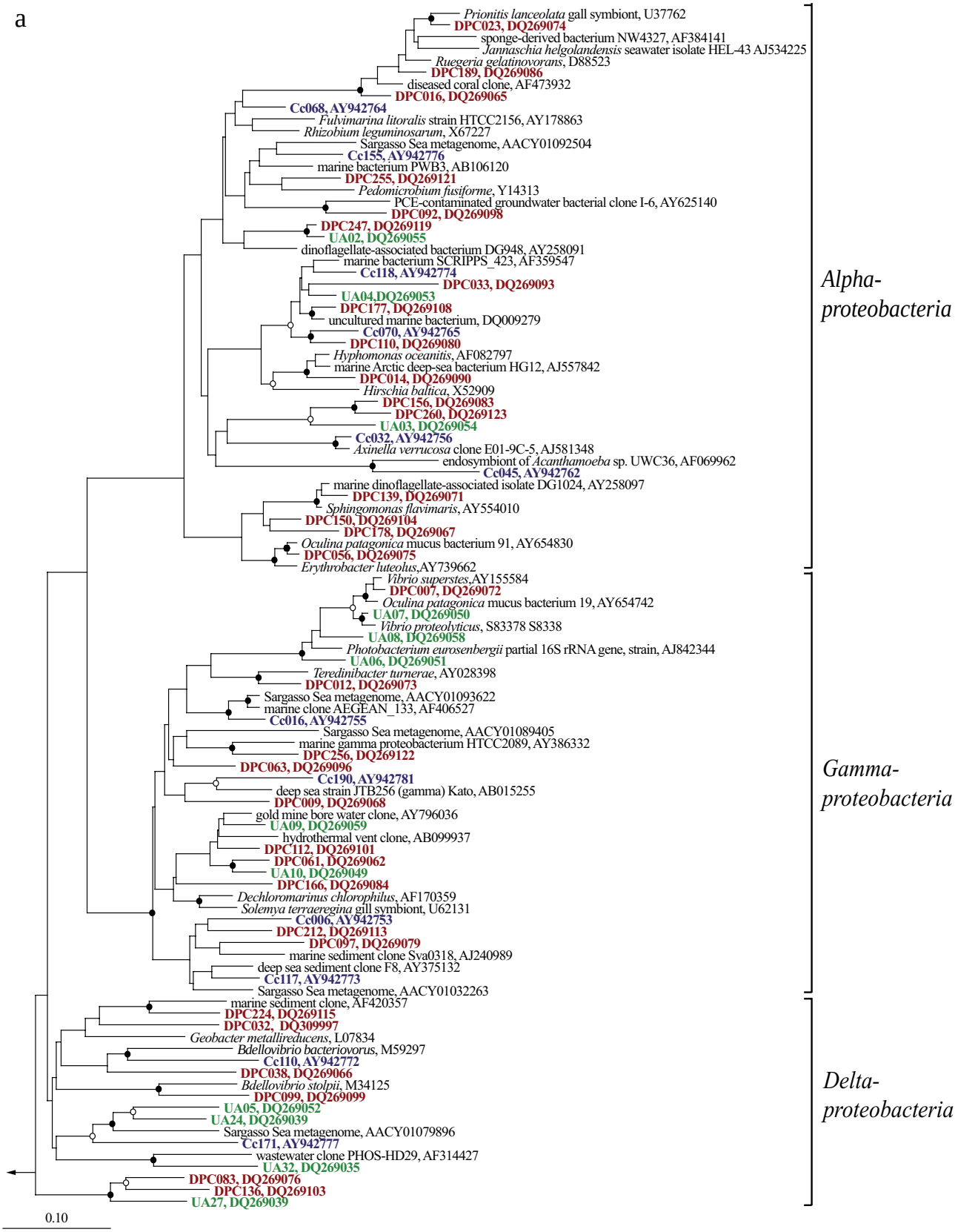

Fig. 2. (Above and facing page.) Maximum likelihood-based 16S rRNA phylogenetic trees of (a) *Proteobacteria* and (b) non-*Proteobacteria* sequences obtained from *Cymbastela concentrica* (Cc), *Delisea pulchra* (DPC) and *Ulva australis* (UA). Maximum parsimony bootstrap values (1000 resamplings) are indicated for well-supported nodes: (●) indicate >90% support; (○) >70% support

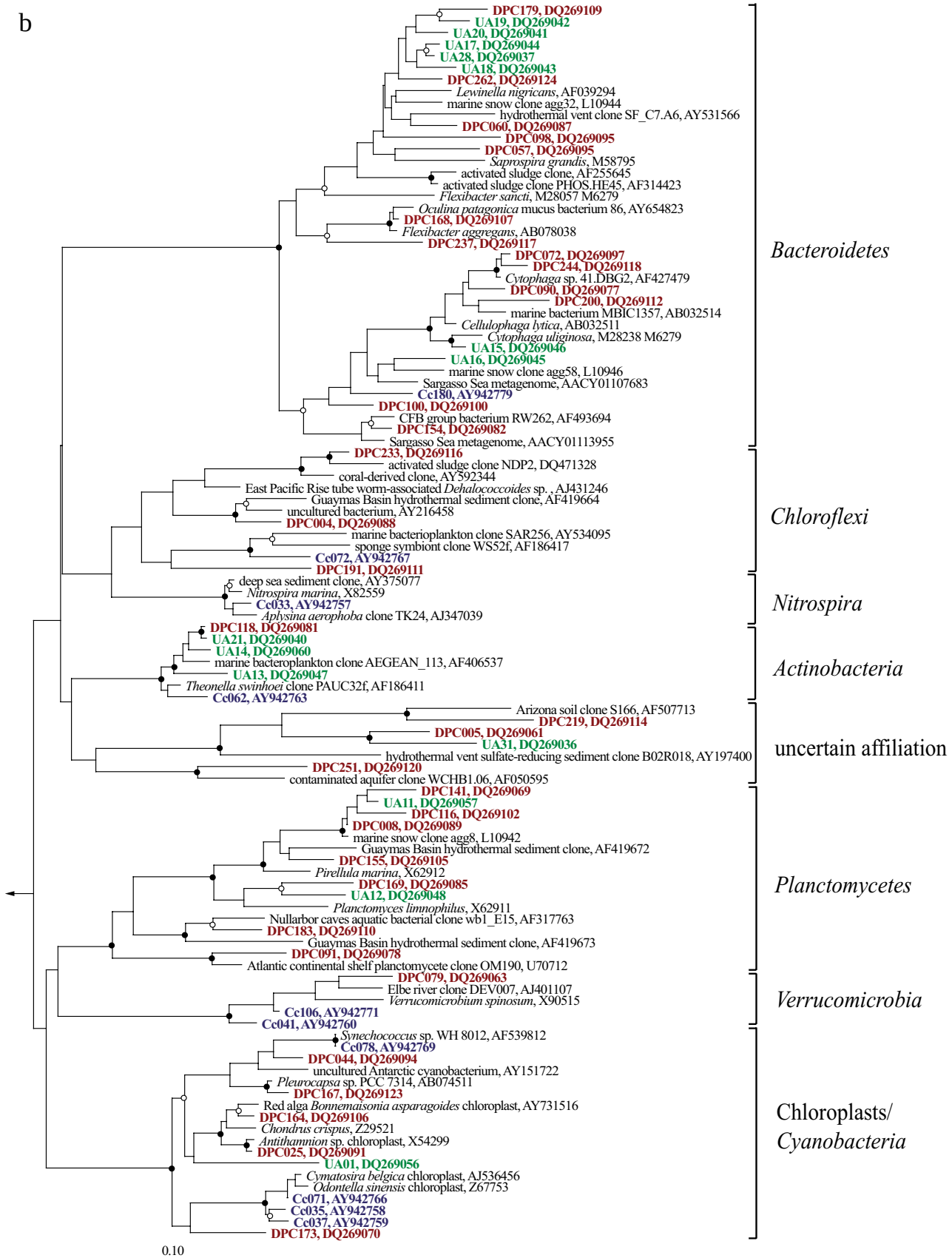

Fig. 2 (continued)

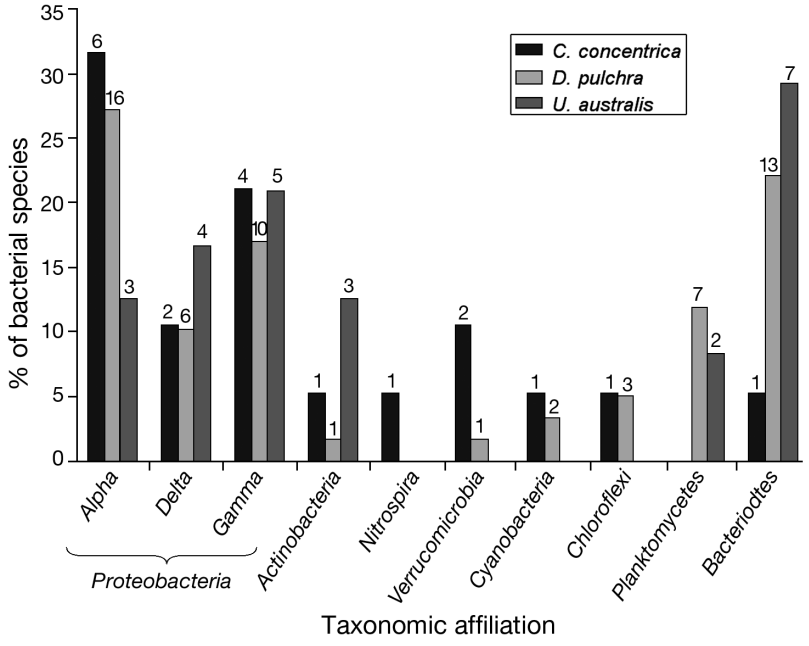

Fig. 3. *Cymbastela concentrica*, *Delisea pulchra* and *Ulva australis*. Representation of various bacterial phyla in 16S rRNA gene libraries. Bars represent the percentage of all 'species' recovered from a given host that belong to each phylum; actual numbers of species for a given taxon in each library are given above each bar

Table 1. Parsimony test results for comparison of the *Cymbastela concentrica* (Cc), *Delisea pulchra* (DPC) and *Ulva australis* (UA) 16S rRNA gene libraries, calculated using different phylogenetic tree methods. Values indicate p-values at the 95th percentile, as calculated by TreeClimber (Schloss & Handelsman 2006). *$p < 0.05$

Libraries compared	Neighbour-joining	Maximum parsimony	Neighbour-joining bootstrap	Maximum parsimony bootstrap	Maximum likelihood
Cc/DPC/UA	0.014*	0.032*	0.165	0.295	0.162
Cc/DPC	0.075	0.074	0.421	0.710	0.196
Cc/UA	0.007*	0.024*	<0.001*	<0.001*	0.030*
DPC/UA	0.222	0.224	0.429	0.425	0.221

communities that dominate discussions of microbial diversity in the oceans. However, even where studies of eukaryote-associated communities have been performed, it is very rare to compare communities associated with co-occurring hosts. This suggests, and has also been implied by studies of the differences between planktonic and benthic communities (DeLong et al. 1993, Bidle & Fletcher 1995), that a fundamental component of diversity, viz. among habitat (= host) or beta diversity, is largely unexplored for marine microbial communities. We have attempted to begin addressing this issue by explicitly comparing microbial diversity among co-occurring marine eukaryotes from different phyla.

Bacterial alpha- and beta-diversity patterns in marine hosts

Using clone libraries and subsequent 16S rRNA gene sequencing, we attempted to characterise alpha (diversity within a single host) and beta (differences among hosts) diversity for 2 marine macroalgae and a marine sponge. With respect to alpha diversity, the bacterial communities associated with the 3 marine eukaryotes studied here exhibited 3 patterns: high phylum-level diversity and high species richness (for the alga *Delisea pulchra*), high phylum-level diversity but low species richness (the sponge *Cymbastela concentrica*) and low phylum diversity and low species richness (the green alga *Ulva australis*; Figs. 2 & 3). The *D. pulchra* library contained 62 species (Chao estimate 79) across 7 phyla, compared to 19 species (Chao estimate 24) across 7 phyla for *C. concentrica* and 25 species (Chao estimate 36) across 4 phyla for *U. australis*.

The between-habitat (or host) or beta diversity was very much a function of the phylogenetic level at which it was assessed. At the bacterial phylum level, there was considerable overlap (indicative of low beta diversity) between the *Delisea pulchra* clone sequences and those from *Ulva australis* and *Cymbastela concentrica*, but there was relatively little overlap between the green alga and the sponge (indicative of high beta diversity; Figs. 2 & 3), which was confirmed by statistical comparison of these 2 libraries (Table 1). Interestingly, analysis of all 3 libraries together did not indicate a consistent significant difference, although this may be due to our pre-screening of clones by RFLP and subsequent representation of each OTU by a single sequence (preventing strict application of the parsimony test; Schloss & Handelsman 2006). Further examination of beta diversity at the phylum level suggests similarities to patterns observed for planktonic communities. In particular, planktonic communities from different locations often share a common group of taxa that represent a substantial fraction of the community as a whole (e.g. Morris et al. 2002), but may also include taxa unique to those particular samples or locations. About 80% of bacterial 16S rRNA gene clones recovered from seawater belong to only 9

marine bacterioplankton phyla (Giovannoni & Rappé 2000), although representatives of most of the 17 well-characterised major divisions can be found in marine habitats (Munn 2004). Similarly, microbial communities from all 3 hosts in this study featured the more common bacterial phyla, containing many representatives of the *Proteobacteria*, with 48 to 63% of the species from each host belonging to the *Alpha-*, *Delta-* or *Gamma-* classes. However, beyond the *Proteobacteria*, phylum-level diversity differed considerably, with (for example) the *Bacteroidetes* and *Planctomycetes* frequently recovered from the algae, but in low frequency or absent from *C. concentrica*, while the converse was true for *Nitrospira*. Although our ability to fully describe the communities on these hosts is limited by the extent to which that diversity is revealed by our techniques, given that these differences are at the phylum level, they would appear to reflect substantial differences among the microbial communities associated with these hosts.

These differences, and thus high beta diversity, are certainly apparent at the bacterial species level. Very few sequence types were identified from 2 or more of the 3 hosts, with many clustering together within a group but maintaining a degree of disparity. The direct overlaps that did occur at the species level were confined to the *Delisea pulchra* and *Ulva australis* communities (*D. pulchra* clone DPC247 and *U. australis* clone UA02 in the *Alphaproteobacteria*, and clones DPC118 and UA21 in the *Actinobacteria*). Thus, of the more than 100 characterised 'species' from these 3 hosts, only 2 were shared. Application of an even stricter species threshold would mean that not a single overlap occurred among the communities, further evidence for remarkably high diversity in these eukaryotic hosts. Whether these bacterial species are found on other, as yet unsampled, species is unknown, and thus it is unknown whether they are globally unique. Irrespective of this, such a high level of distinctiveness among hosts supports previous assertions (Taylor et al. 2004) that host-associated communities have important implications for assessing diversity even at a local level.

Biological interpretation of the diversity patterns described above is not straightforward, and the observed patterns could in part reflect methodological issues. Firstly, clone library construction is inherently laborious, and the analysis of large numbers of replicate samples is generally not feasible. We therefore pooled material from 3 individuals of each host species, to ultimately generate 1 library per host. Such pooling was deemed appropriate due to our DGGE-based finding of largely consistent bacterial communities among individuals of a given host. Low variability among sponge-associated bacterial communities has already been reported (e.g. Taylor et al. 2004, Hentschel et al. 2006), but to our knowledge, the variability data presented here are the first for macroalgae. Ongoing studies in our and other laboratories should reveal whether such patterns are typical for algae. A second point concerns the *Cymbastela concentrica* library. Given that half of it consisted of diatom chloroplast sequences, and the potential PCR biases in favour of diatom sequence amplification (due to the very high 16S rRNA copy number in microalgal plastids; Nübel et al. 2000), it is possible that the apparently low species richness of *C. concentrica* is an underestimate. Chloroplast sequences were also present in the algal libraries, although these represented a much smaller proportion of analysed clones. A final point concerns the use of a somewhat arbitrary bacterial species definition (i.e. 97% 16S rRNA similarity), and it is worth considering how a more evolutionarily sound comparison, for example based on clade structure, may influence our results. If one treats individual clades or lineages as the entity of interest, essentially providing a middle ground between phyla and species considerations, then the story does not change dramatically. There is inevitably a greater degree of overlap among the different hosts than is evident at the bacterial species level, but overall the 3 communities remain relatively dissimilar to each other.

Bacterial communities associated with marine living surfaces: 'signature communities'?

Considering the enormous number of marine eukaryotes (and the fact that many eukaryotic phyla are confined to marine environments; Ormond et al. 1997), the bacterial communities associated with such organisms are clearly dramatically understudied, with examples spread thinly across taxa and habitats (exceptions include, among others, oligochaetes [Dubilier et al. 1995, 2001, Bright & Giere 2005], corals [Rohwer et al. 2001, 2002, Bourne & Munn 2005, Penn et al. 2006], tubeworms and bivalves at hydrothermal vents [Distel 1998, Stewart et al. 2005, Nussbaumer et al. 2006]). One of the better-studied groups of marine eukaryotes in this respect is marine sponges and their associated microorganisms, which have been the subject of investigation for more than 30 yr (e.g. Sara 1971, Wilkinson 1978, Hentschel et al. 2006, Taylor et al. 2007). Although direct comparisons of our *Cymbastela concentrica*-derived diversity estimates to those obtained from other sponges are constrained by differences in (e.g.) OTU definition, sample preparation, and percentage coverage of each library, it appears that bacterial species richness of *C. concentrica* is of a similar magnitude to that of other sponges. For example, 34 'distinct sequences' were obtained from the Great

Barrier Reef sponge *Rhopaloeides odorabile* (Webster et al. 2001), while OTU richness in 5 Antarctic sponges was estimated at 15 to 86 different RFLP types, depending on host species (Webster et al. 2004). Relatively low levels of library coverage prevent accurate estimation of species richness for the Mediterranean sponge *Aplysina aerophoba* and the tropical Pacific sponge *Theonella swinhoei* (Hentschel et al. 2002), but examination of the sequence data in that study suggested diversity would be at least as high as that observed for *C. concentrica*. In terms of phylogenetic composition, the *C. concentrica* community was broadly similar to that of other studied sponges; *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Nitrospira* and *Proteobacteria* (*Alpha-*, *Delta-* and *Gamma-*) sequences are frequently recovered from marine sponges (reviewed by Hentschel et al. 2006, Taylor et al. 2007). The major differences included the recovery of several *Verrucomicrobia* sequences from *C. concentrica* (one of the first reports of this phylum from a marine sponge) and the presence of large numbers of diatoms (reported by Taylor et al. 2004, 2005). The latter is particularly interesting, as sponge-associated diatoms have seldom been reported from outside the polar regions.

A degree of caution is required when considering our alpha- and beta-diversity results within the context of other marine sponge studies. The sponge-specific, monophyletic sequence clusters first described by Hentschel et al. (2002) and found in multiple sponges from different geographic locations, at first glance imply low beta diversity (i.e. that these bacteria are not host-specific). However, such arguments are again heavily affected by the phylogenetic level being examined. Since the 16S rRNA gene sequences within some of these clusters vary by up to 23% (Hentschel et al. 2002), these are clearly not all from the same bacterial species, and at the species level, beta diversity may therefore be rather high. This notion of high beta diversity at the species level is consistent with an earlier study of *Cymbastela concentrica* (Taylor et al. 2005), in which DGGE banding patterns from southeastern Australia were very different from those obtained from Great Barrier Reef *C. concentrica* samples. Even if '*C. concentrica*' from the 2 locations are in fact 2 separate (sub)species (due to cryptic speciation; see Taylor et al. 2005), the data nonetheless suggest that at bacterial strain/species levels, the communities on 2 very closely related sponges are different. Remarkably, in another marine invertebrate (the nematode *Eubostrichus dianae*) bacterial diversity differed greatly even between individuals from the same location (Polz et al. 1999).

Similar comparisons among macroalgae are somewhat more constrained, due to the paucity of data on surface bacterial communities of marine plants and macroalgae. In one of the few existing studies, Meusnier et al. (2001) found various proteobacterial lineages (*Alpha-*, *Beta-*, *Delta-* and *Gamma-*), as well as representatives of the *Bacteroidetes*, *Planctomycetes* and *Cyanobacteria*, associated with the green macroalga *Caulerpa taxifolia*. A culture-based study of the green alga *Enteromorpha* sp. (Patel et al. 2003) identified a predominance of *Gammaproteobacteria* as well as representatives of the *Bacteroidetes*. 16S rRNA gene sequences retrieved from epiphytic bacteria on freshwater macroalgae belonged to several major lineages within the *Bacteria*: the *Alpha, Beta-* and *Gamma-* classes of the *Proteobacteria*, the *Bacteroidetes* and the *Actinobacteria* (Fisher et al. 1998). *Alphaproteobacteria* from the *Roseobacter* subgroup appear to be widely distributed among marine algae (Ashen & Goff 2000, R. Case et al. unpubl., this study) and have been implicated in gall (tumour) formation on macroalgae of the genus *Prionitis* (Ashen & Goff 2000). *Gammaproteobacteria* were isolated from the Australian red macroalgae *Amphiroa anceps* and *Corallina officinalis*, while additionally, *Alphaproteobacteria* and *Bacteroidetes* were isolated from *A. anceps* and *C. officinalis*, respectively (Huggett et al. 2006). Considering marine macrophytes more broadly, in the seagrass *Halophila stipulacea*, 103 16S rRNA gene clones comprised 58 different RFLP groups (Weidner et al. 1996), with bacterial lineages belonging predominantly to the *Gammaproteobacteria*, *Alphaproteobacteria*, *Verrucomicrobia* and *Planctomycetes* (Weidner et al. 2000). *Proteobacteria* and *Bacteroidetes* also occurred frequently in the *Delisea pulchra* and *Ulva australis* libraries presented here, suggesting that these typical marine bacteria are also part of the microbiota of marine plants and macroalgae. These observations beg the question, which has also been asked previously (e.g. Taylor et al. 2005), as to what level of taxonomic resolution is most meaningful. Minor differences in 16S rRNA sequence can correspond to major ecological differences (e.g. Ashen & Goff 2000, Johnson et al. 2006), so from a community function and stability perspective, our observations of high species-level differences among hosts may be much more important than the parallel observation of substantial phylum-level overlap.

Sequences recovered from the *Ulva australis* library in this study fell roughly into the same phylogenetic groups as the *Delisea pulchra* clone sequences, but the latter had greater representation within each group. Interestingly, and in contrast to the situation for marine sponges (Hentschel et al. 2002, 2006), the lack of overlap between bacterial communities on the 2 algae was striking, with little

evidence for algal-specific monophyletic sequence clusters. Whenever *D. pulchra*- and *U. australis*-derived sequences fell together, these were typically interspersed by other, non-algal source, sequences. Clearly, speculating on the existence of such clusters is premature given the current lack of information on macroalga-associated bacteria, and the situation will only become clearer as more sequence information becomes available.

The clone libraries analysed here represent a further attempt, following from the work of Taylor et al. (2004, 2005), to explore how associating with host eukaryotes affects marine microbial diversity. Several sources of potential variation in diversity have now been explored, including (1) different species of hosts at the same location, (2) the same species or taxa (e.g. phylum) at different locations, and (3) samples that differ in host and location. Although any comparisons are strongly constrained by the overall paucity of data and by differences in methods across different studies, the available data suggest a few patterns. First, bacterial communities associated with marine living surfaces seem broadly similar (at the phylum/class level) to those found in the surrounding seawater, but are often quite different at lower taxonomic (e.g. species) levels. A striking example of this is the community associated with the coral *Montastraea franksi*, which has a diverse bacterial assemblage with almost no overlap with that of the overlying water column (Rohwer et al. 2001). Second, even for one of the best-studied groups of host-associated bacteria, i.e. those from sponges, there is evidence both for striking similarities among communities, e.g. the monophyletic clusters of Hentschel et al. (2002), but also for differences in bacterial communities from the same or closely related species. Significant differences in bacterial diversity are also observed among different taxa of macrophytes (e.g. Weidner et al. 2000, Meusnier et al. 2001). Thus while it is clearly too early to say whether there are 'signature' communities for host-associated microbial communities in the sense that has been argued for planktonic communities, it does appear that microbial communities on different hosts often differ significantly both from each other and from those in the water column. This is consistent with a model of diversity in which at least some components of these communities are specifically adapted to particular hosts, making these hosts a potentially rich source of distinctive microbial diversity.

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