MICROBIAL SYMBIOSIS OF MARINE SESSILE HOSTS-DIVERSITY AND FUNCTION

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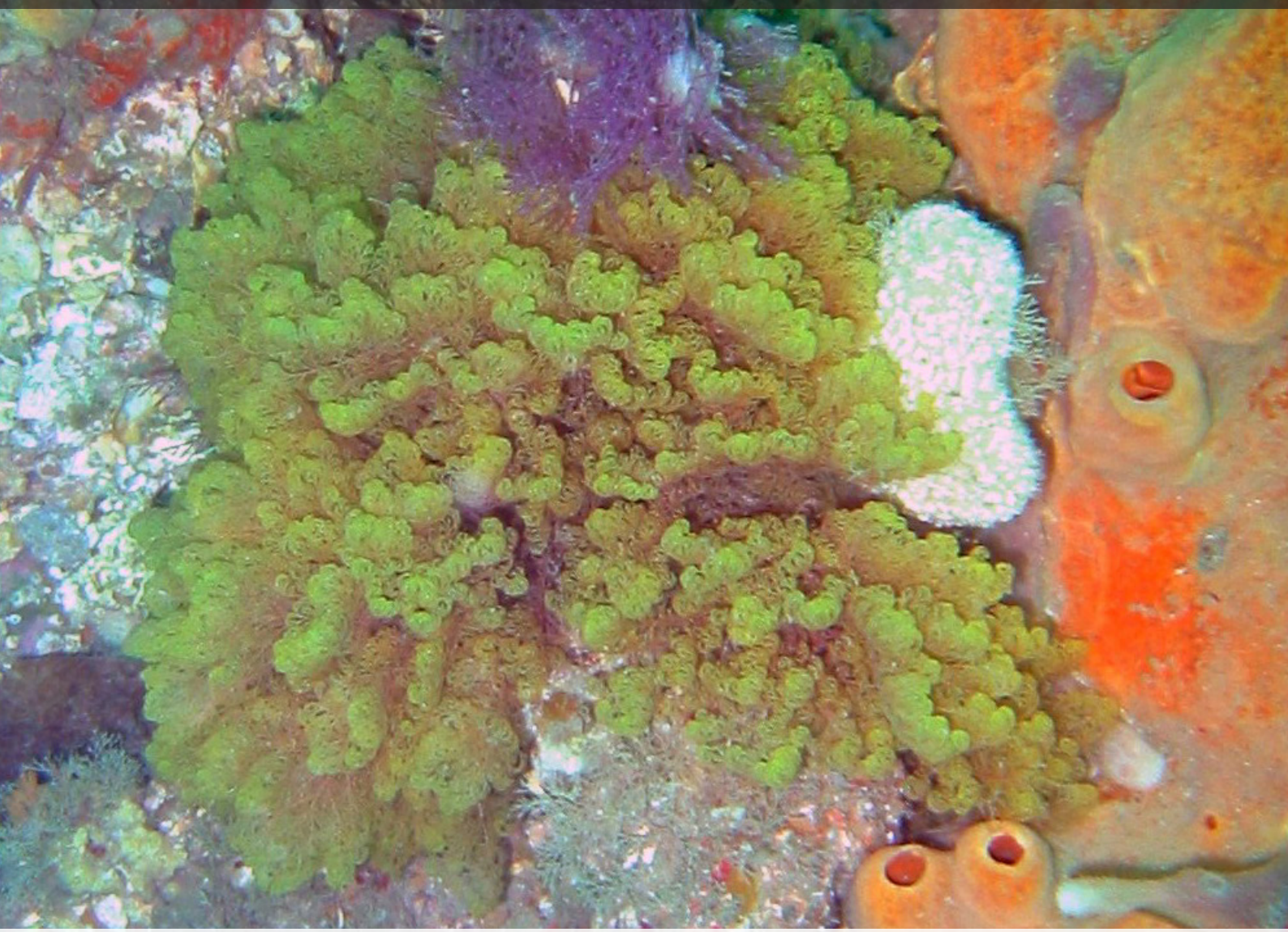

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MICROBIAL SYMBIOSIS OF MARINE SESSILE HOSTS- DIVERSITY AND FUNCTION

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Examples of the diversity and density of marine sessile host surfaces available to marine microorganisms. Photo taken 10 m depth NSW Australia.
Image by Suhelen Egan

Modern molecular –omics tools (metagenomics, metaproteomics etc.) have greatly contributed to the rapid advancement of our understanding of microbial diversity and function in the world's oceans. These tools are now increasingly applied to host-associated environments to describe the symbiotic microbiome and obtain a holistic view of marine host-microbial interactions.

Whilst all eukaryotic hosts are likely to benefit from their microbial associates, marine sessile eukaryotes, including macroalgae, seagrasses and various invertebrates (sponges, acidians, corals, hydroids etc), rely in particular on the function of their microbiome. For example, marine sessile eukaryotes are under constant grazing, colonization and fouling pressure from the millions of micro- and macroorganisms in the surrounding seawater. Host-associated microorganisms have been shown to produce secondary metabolites as defense molecules against unwanted colonization or pathogens, thus having an important function in host health and survival. Similarly microbial symbionts of sessile eukaryotes are often essential players in local nutrient cycling thus benefiting both the host and the surrounding ecosystem.

Various research fields have contributed to generating knowledge of host-associated systems, including microbiology, biotechnology, molecular biology, ecology, evolution and biotechnology. Through a focus on model marine sessile host systems we believe that new insight into the interactions between host and microbial symbionts will be obtained and important areas of future research will be identified. This research topic includes original research, review and opinion articles that bring together the knowledge from different aspects of biology and highlight advances in our understanding of the diversity and function of the microbiomes on marine sessile hosts.

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EDITORIAL
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Editorial for: Microbial symbiosis of marine sessile hosts- diversity and function

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Keywords: microbial interactions, microbial diversity, sponges, seaweeds, macroalgae, oysters, marine diseases, beneficial microorganisms

The marine surface environment is home to a large and often diverse community of microorganisms. Yet compared to terrestrial ecosystems we still know little about the diversity, degree of host-specificity, functional role or the molecular mechanisms of host-microbe interactions in marine systems. This research topic brings together 10 articles that highlight advances in our understanding of microbial communities associated with marine sessile eukaryotic hosts.

Many papers in this research topic have a particular focus on the stability and diversity of bacterial symbionts of marine sponges. Sponges are a diverse group of sessile organisms, which represent one of the earliest metazoan life forms and play an important role in benthic ecosystems due to their filter feeding activity (e.g., De Goeij et al., 2013). Many biogeochemical processes are carried out by sponge-associated microorganisms, which can comprise up to 35% of the sponge biomass (Hentschel et al., 2012). The evolutionary history and biological importance of the sponge-microbe interaction make them attractive models to study general concepts in marine microbial-host symbiosis. Three articles (Burgsdorf et al., 2014; Cuvelier et al., 2014; Easson and Thacker, 2014) use molecular approaches to elucidate the major factors that determine the composition of sponge symbiotic microbial communities. Burgsdorf et al. (2014) show that the local environment, rather than the host features, influences the community composition of distinct morphotypes of the sponge *Petrosia ficiformis*. In contrast, Cuvelier et al. (2014) conclude for the sponge *Cinachyrella* that the sponge host itself has the greatest influence on determining its microbial community composition. These seemingly opposing views are in part reconciled in the findings of Easson and Thacker (2014) that support the concept of a "core" microbial community in sponges, in line with previous studies (Schmitt et al., 2012), but also highlight that for individual sponge species the taxonomic identity of microbial symbionts can vary greatly.

Using seaweed as another marine model, Campbell et al. (2015) performed a local transplantation experiment to show that the symbiont community of the brown macroalgae *Phyllospora comosa* is primarily influenced by the local conditions, with some evidence for host-specificity. Thus, like sponges, the microbial community composition of seaweeds is also likely determined by a combination of environmental and host factors, a pattern that is emerging now of several sessile marine systems (Wahl et al., 2012; Egan et al., 2013).

Aside from diversity patterns, functional processes such as nitrogen fixation are also important for the dynamics of host-microbe symbiosis, as demonstrated in the research article by Zhang et al. (2014). This study found that expression of nitrogen fixation genes (*nifH)* occurred in two Caribbean sponges over the entire day-night cycle. Comparison between the two sponge species suggested that nitrogen fixation is dominated by a conserved group of cyanobacteria, with the heterotrophic bacterial community mainly contributing during the night.

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In contrast to these beneficial aspects of symbiosis, interactions can also be negative and thus result in disease (Webster, 2007; Burge et al., 2013; Egan et al., 2014). Two papers in this research topic examine negative interactions in different marine sessile hosts (Raftos et al., 2014; Zozaya-Valdes et al., 2015). Raftos et al. (2014) review the history and impact of microbial disease on shellfish and using QX disease in Sydney rock oysters, illustrate the complex interactions that exist between pathogens, the environment and hosts. Zozaya-Valdes et al. (2015) provides molecular evidence for the ecological importance of certain bacteria in the bleaching disease of the red macroalga *Delisea pulchra* and also highlight the possibility that multiple opportunistic bacterial pathogens exist.

During the last decade, next-generation sequencing technologies have rapidly advanced our understanding of microbial diversity in the marine environment (Gilbert and Dupont, 2011; Williamson and Yooseph, 2012). However, these culture-independent approaches should be complemented by the culturing of representative microorganisms, followed by detailed physiological, biochemical and genetic studies (Giovannoni and Stingl, 2007; Joint et al., 2010). Hardoim et al. (2014) tackle this challenge using a range of culturing, molecular and microscopy techniques to address cultivation bias when studying microbial communities in sponges. Using a "plate-washing method" they were able to culture an order of magnitude more bacterial species than previous cultivation studies (Hardoim et al., 2012). Approximately half of the bacterial species cultured were not detected in the sponge by culture-independent methods demonstrating the need for these complementary approaches to be used to fully characterize microbial diversity.

Having model bacteria is clearly important to explore mechanistic aspects of symbiosis. The article by Gardiner et al. (2014) illustrates this by showing that the seaweed-associated bacterium *P. tunicata* utilizes a surface lipoprotein (designated Ptl32) to attach to its host. Interestingly, Ptl32 shares homology to a conserved protein in *Leptospira* species, the causative agent of leptospirosis in animals (Murray, 2013), and this suggests that this attachment mechanism might be distributed between distantly related bacterial species via horizontal gene transfer (HGT). A clear link between organisms and their functions is important to define the importance of HGT in marine microbial symbiosis and this is further explored in the final article of this research topic. Here, Degnan (2014) raised a number of compelling points of how HGT between symbiotic microorganisms and marine invertebrates can shape the evolution of holobionts. HGT is clearly an underappreciated mechanism by which bacteria can influence their host (or vice versa).

The articles presented in this research topic highlight the diverse microbial communities associated with marine sessile macroorganisms play important roles in their health, function and evolution. Through the contributions of experts in classical microbiology (Gardiner et al., 2014; Hardoim et al., 2014), cell biology (Raftos et al., 2014), ecology (Campbell et al., 2015), evolution (Degnan, 2014) and molecular ecology (Burgsdorf et al., 2014; Cuvelier et al., 2014; Easson and Thacker, 2014; Zhang et al., 2014; Zozaya-Valdes et al., 2015), the papers in this research topic also demonstrate the benefits of using a multidisciplinary approach to understand the diversity, function and evolution of complex symbiotic systems.

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ORIGINAL RESEARCH ARTICLE
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Biogeography rather than association with cyanobacteria structures symbiotic microbial communities in the marine sponge *Petrosia ficiformis*

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The sponge *Petrosia ficiformis* is ubiquitous in the Mediterranean Sea and Eastern Atlantic Ocean, hosting a diverse assemblage of bacteria, including, in illuminated sites, cyanobacteria. Two closely related sponge color morphs have been described, one inside caves and at their entrance (white/pink), and one on the rocky cliffs (violet). The presence of the different morphs and their ubiquity in the Mediterranean (from North-West to South-East) provides an opportunity to examine which factors mostly affect the associated microbial communities in this species: (i) presence of phototrophic symbionts or (ii) biogeography. 16S rRNA gene tag pyrosequencing data of the microbial communities revealed that Chloroflexi, Gammaproteobacteria, and Acidobacteria dominated the bacterial communities of all sponges analyzed. Chlorophyll *a* content, TEM observations and DNA sequence data confirmed the presence of the cyanobacterium *Synechococcus feldmannii* in violet and pink morphs of *P. ficiformis* and their absence in white color morphs. Rather than cyanobacterial symbionts (i.e., color morphs) accounting for variability in microbial symbiont communities, a biogeographic trend was observed between *P. ficiformis* collected in Israel and Italy. Analyses of partial 18S rRNA and mitochondrial cytochrome c oxidase subunit I (COX1) gene sequences revealed consistent genetic divergence between the violet and pink-white morphotypes of *P. ficiformis*. Overall, data indicated that microbial symbiont communities were more similar in genetically distinct *P. ficiformis* from the same location, than genetically similar *P. ficiformis* from distant locations.

Keywords: *Petrosia ficiformis*, sponge-microbe symbiosis, biogeography, cyanobacteria, *Synechococcus feldmannii*, 454 amplicon pyrosequencing, microbial diversity, porifera

INTRODUCTION

The marine demosponge *Petrosia ficiformis* (Poiret, 1789) is a sponge species found across the Mediterranean and in the Eastern Atlantic (Guo et al., 1998). It has been the focus of diverse studies that investigated: (i) the chemistry of the sponge and its associated microorganisms (Seidel et al., 1986; Bringmann et al., 2004; Lopez-Gresa et al., 2009; Pagliara and Caroppo, 2011), (ii) the ability to produce primmorphs (e.g., Mussino et al., 2013; Pozzolini et al., 2014), (iii) the identity of cyanobacterial symbionts (Usher et al., 2004; Steindler et al., 2005), and (iv) the molecular mechanisms underlying the interaction between sponge host and cyanobacteria (Arillo et al., 1993; Steindler et al., 2007). *P. ficiformis* has usually been described with two different morphs (Sarà et al., 1998): (i) a massive, violet-pigmented form, living in illuminated habitats harboring a dense population of intracellular cyanobacteria in the sponge cortex (the superficial layer of the sponge); and (ii) a slender pinkish or white morph, commonly found in shaded habitats (pink) and particularly in dark caves (white), where the sponges are free of phototrophic symbionts (**Figure 1**). *P. ficiformis* has been considered particularly suitable for studies on the establishment and maintenance of sponge-microbe symbiosis due to this facultative symbiosis with cyanobacteria (Steindler et al., 2007). *P. ficiformis* shows striking changes in its morphology in the presence and absence of cyanobacterial symbionts. These changes have been considered adaptive and relate to the size (light-exposed specimens are much larger than their dark cave counterparts), shape, surface skeleton, density of pores, and the metabolism (Vacelet and Donadey, 1977; Sarà et al., 1998). In the large and tabular specimens living on light-exposed cliffs, inhalant pores are very rare. Light-sheltered specimens have a lower symbiont concentration and the sponge becomes cylindrical in shape (Sarà et al., 1998). These changes

affect the surface-to-volume ratio of the sponge whose values increase when chlorophyll *a* concentration decreases. In specimens with reduced cyanobacteria populations, a dense coat of vertical spicules is overlapped on the typical tangential spicular network. This arrangement could be tentatively interpreted as a light channeling system (Cattaneo-Vietti et al., 1996), likely improving light transfer in semi-dark habitats. In light-deprived specimens, the number of pores increases considerably, suggesting a more important role of the pumping system to support the feeding requirements. The aposymbiotic specimens (free of cyanobacteria) living in darkness can develop creeping branches or a spherical shape in case of low water movement, with an increased percentage of the surface covered by inhalant pores (Sarà et al., 1998). It has been shown that different morphs of *P. ficiformis* show differences in dehydrogenase activity and RNA content. It was proposed that in dark conditions *P. ficiformis* reacts to the absence of cyanobacteria by activating metabolic pathways able to maintain the cell reducing power.

P. ficiformis harbors a diverse and rich community of symbiotic bacteria (Schmitt et al., 2012a). The cyanobacterial symbiont, first described as *Aphanocapsa feldmannii* (Frémy in Feldmann, 1933) and later as *Synechococcus feldmannii* (Usher et al., 2004), is phylogenetically affiliated to free-living *Synechococcus*/*Prochlorococcus* species and not part of the widely distributed sponge-specific clade *Synechococcus spongiarum* (Steindler et al., 2005; Gao et al., 2014). The symbionts are absent from mature oocytes, spermatozoa, and embryos (Maldonado and Riesgo, 2009). Thus, microbes are likely acquired from ambient seawater by each new generation of juvenile sponges (Lepore et al., 1995; Maldonado and Riesgo, 2009). This differs from the vertical transmission of many sponge symbionts in other sponge species, including the transmission of the sponge-specific cyanobacterium clade *S. spongiarum* (e.g., Oren et al., 2005; Lee et al., 2009a; Webster et al., 2010; Gloeckner et al., 2013), and may also explain why most bacteria in the adult *P. ficiformis* sponge are found within bacteriocytes, as suggested in a more specific study on intergenerational transmission of microbial symbionts (Maldonado, 2007). Given the intracellular location (symbiosomes) of cyanobacterial symbionts in *P. ficiformis*, it is yet unknown how substrates are transferred from cortex to endosome (internal part of sponge), and whether certain heterotrophic bacteria within the sponge benefit from photosynthates and other substrates produced by cyanobacterial symbionts. The presence of cyanobacterial symbionts enhances the antioxidant defenses as compared to aposymbiotic specimens (Regoli et al., 2000) and may affect the heterotrophic bacterial community in such a way that different microbial communities could be expected in violet and white morphs of *P. ficiformis*.

The aim of this study was to determine which factors mostly affect the microbial community associated with *P. ficiformis*: presence of photosymbionts or biogeography. We compared the microbial communities of violet and white morphs of *P. ficiformis* from the northern Tyrrhenian Sea (Italy) and of violet morph in the north-western (Italy) and south-eastern (Israel) Mediterranean Sea. We took an additional violet sponge, and transferred half of it to a dark submarine cave, comparing the microbial communities of the violet and the "bleached" part (the half that lost its cyanobacterial symbionts after transfer to a cave). Finally, we amplified the 18S rRNA and cytochrome c oxidase subunit I (COX1) gene for triplicates of violet, pink (those growing at cave entrance), and white morphs of *P. ficiformis*.

FIGURE 1 | Specimens of *P. ficiformis* showing the three morphs: (A) the massive violet type living in lighted areas (B,C) the creeping type with the pink and white color living in dim-light and dark condition, respectively. Scale bars: **(A)** 5 cm, **(B,C)** 2 cm.

METHODS

SPONGE COLLECTION

Three violet colored (PV1, PV2, PV3), two pink (PP1, PP3), and two white (PW2, PW3) *P. ficiformis* sponge samples were

collected by SCUBA in August 2012 at depths of 10–13 m (violet), at the entrance (pink) and inside (white) of a submarine cave at 6 m in the Mediterranean Sea at Paraggi (GE), Italy (44°18′37.63″N; 9°12′48.01″E). An additional violet specimen (D), collected at 10 m depth, was cut into two halves. One half was transferred to the cave (Dt) and the other maintained at its original sampling location outside the cave. Dt was attached to the substrate inside the cave using an epossidic resin commonly used in aquariology. After almost 6 months (February, 2013), samples from these two sponge-halves (D, Dt) and additional white (PW1) and pink (PP2) *P. ficiformis* specimens were collected from the same cave. In May 2013, three violet colored *P. ficiformis* (termed here 106, 108, 111) were collected at a depth of approximately 20 m in Achziv on the Israeli Mediterranean coast (33°2′44.19″*N*; 35°5′7.58″E). After collection, all samples were immediately transferred to 100% ethanol and stored at −20°C until molecular analysis.

DNA EXTRACTION, DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) AND 454 AMPLICON PYROSEQUENCING

DNA was extracted from all sponge samples using the Power Soil DNA Isolation kit, MoBio Laboratories (Carlsbad, CA) according to the manufacturer's protocol. Cortex and endosome parts of samples 106, 108, and 111 were separated first and extracted separately. Initial comparisons of microbial communities in the cortex and endosome of these sponges were conducted using triplicate PCR reactions with a universal primer set for bacteria (341F-GC and 907R, Muyzer et al., 1993) followed by DGGE fingerprinting, as previously described (Green et al., 2004). For subsequent analyses (454 gene tag pyrosequencing), samples 106, 108, and 111 represent equal concentration mixtures of DNA derived from cortex and endosome. Cortex and endosome of sample 106 were also sequenced separately. Extracted DNA samples were delivered for pyrosequencing to Molecular Research LP (Shallowater, TX, USA). The V6–V8 hypervariable region of the 16S rRNA gene was amplified by PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) and primers 926f and 1392r (Engelbrektson et al., 2010) under the following conditions: 94°C for 3 min; followed by 28 cycles of: 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min; and a final elongation step at 72°C for 5 min. All amplicon products were purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments following manufacturer's guidelines.

QUALITY CONTROL AND OTU CLUSTERING OF 454 SEQUENCE DATA

Raw sequence data were processed using MOTHUR 1.33.0 (Schloss et al., 2009). The sequences were first trimmed of primer and tag sequences and de-noised by removing reads with low quality scores (using "average window," average quality >25, window size = 50 bp, maximum homopolymer number per sequence = 8 and length >200 bp). The remaining sequences were aligned using a reference database (Silva database). Pyrosequencing errors were diagnosed using a pseudo-single linkage algorithm (pre.cluster command). Chimera sequences were detected and removed from the dataset using UChime (http://www.drive5.com/uchime/). All sequences were classified according to RDP (PDS) version 9 and mitochondrial, eukaryotic and unknown sequences were removed. For the remaining sequences, a pairwise distance matrix between aligned sequences was calculated and the sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence identity (average neighbor algorithm). OTUs were classified according to both RDP version 9 and SILVA version 102. Ambiguous results were classified by manual BLASTn (Altschul et al., 1990) against SILVA 115 non-redundant database and a web based BLAST tool (http://blast.ncbi.nlm.nih.gov/). SILVA 115 non-redundant database was downloaded from the SILVA official web site (http://www.arb-silva.de).

STATISTICAL ANALYSES OF MICROBIAL DIVERSITY AND COMMUNITY STRUCTURE

DGGE images were analyzed with the Fingerprinting II® software (Bio-Rad Laboratories, Hercules, CA, USA), with cluster analyses calculated using unweighed pair group methods with arithmetic means (UPGMA) based on a Pearson r distance matrix. Significant differences in microbial community similarity between the cortex and endosome tissues were determined using permutational multivariate analyses of variance (PERMANOVA). All subsequent statistical methods refer to the 454 gene tag pyrosequencing dataset.

Good's estimator, the Chao1 estimator and rarefaction analyses were conducted, as implemented in MOTHUR, to assess how well the recovered microbial communities represented the total microbial community. This analysis was performed after removing singleton OTUs (OTUs containing only one read). Specifically, these metrics provide estimates of total coverage, total expected OTU diversity and saturation of OTU accumulation (i.e., collector's) curves, respectively.

Microbial community similarity among sponge hosts was compared using both OTU-dependent (Bray-Curtis, BC; partial least squares discriminant analysis, PLS-DA) and OTU-independent calculations (UniFrac, Lozupone and Knight, 2005). BC indices were calculated based on the relative abundances of OTUs and visualized in cluster plots using MOTHUR. Significant differences in microbial community similarity were determined using PERMANOVA for the factors biogeography (Israel vs. Italy) and color morph (violet vs. white) and visualized in cluster plots. Factors exhibiting significant differences were further investigated by similarity percentage analyses (SIMPER) to identify microbial OTUs contributing to community dissimilarity. All PERMANOVA and SIMPER calculations were performed using Primer v6 and PERMANOVA+ (Plymouth Marine Laboratory, UK). PLS-DA was performed using the METAGENassist web server tool (Arndt et al., 2012) and the 97% OTU dataset. UniFrac analysis (Lozupone and Knight, 2005) was performed on rarefied data sets using the MOTHUR pipeline and cluster plots were constructed from the weighted UniFrac pairwise distance matrix, based on a phylogenetic tree constructed using the Clearcut module (Evans et al., 2006). PLS-DA loadings plot was analyzed and OTUs that mostly effected a separation were described.

CHLOROPHYLL *a* MEASUREMENTS

Chlorophyll *a* (chl *a*) concentrations were determined following the method described in Erwin et al. (2012a). Briefly, 0.25 g of

ectosomal tissue (wet weight) from each sample was extracted overnight at 4°C with 5 ml of 90% acetone. Absorbance values of supernatant aliquots were determined at 750, 664, 647, and 630 nm and chl *a* concentrations were calculated using the equations of Parsons et al. (1984), standardized by sponge mass extracted. Chl *a* concentrations were compared between host sponge color morphs using One-Way analysis of variance (ANOVA) on ranked data (Kruskal-Wallis), since raw data deviated significantly from a normal distribution (Shapiro-Wilk, $P < 0.05$). Pairwise comparisons were conducted using the Student-Newman-Keuls (SNK) method. Statistical analyses were performed using the software SigmaPlot v11.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Sponge pieces of ca. 4 mm^3 from the violet (outside the cave), pink (cave entrance), and white (inside the cave) morphs of *P. ficiformis* were collected from a submarine cave located along the rocky cliffs of Paraggi (Portofino Promontory, Ligurian Sea, Italy). Each piece was fixed separately in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde buffered with filtered seawater. Samples were incubated overnight at 4°C, then rinsed several times with filtered seawater and stored at 4°C until processed. Ultrathin sections were obtained with an Ultracut Reichert-Jung ultramicrotome, mounted on gold grids, and observed on a JEOL JEM-1010 (Tokyo, Japan) coupled with a Bioscan 972 camera (Gatan, Germany) for the acquisition of digital images. Ultrathin sections and TEM observations were performed at the Microscopy Unit of the Scientific and Technical Services of the University of Barcelona, Spain.

MOLECULAR IDENTIFICATION OF *P. FICIFORMIS* SPONGES BELONGING TO DIFFERENT COLOR MORPHS

Sponges were identified by morphological analysis and sequencing of their 18S rRNA and cytochrome c oxidase subunit I (COX1) genes. A fragment of the 18S rRNA gene was amplified by PCR from Italian violet and pink *P. ficiformis* samples using primers: 18S_D1161b_PetrF (5′-TAGCGACTCCG TCGGCACCTCTC-3′) and 18S_PetrosiaV_1565_R (5′-AATCCT CCCTCGGCTAGAAAC-3′). The 18S rRNA gene from white *P. ficiformis* samples was amplified using the same forward primer, and the reverse primer 18S_PetrosiaW_1565_R (5′-AATCCT CCCTCGGCTTAGAAACC-3′). The PCR conditions were as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 3 min. A 222 bp alignment was used to build a maximum likelihood tree in MEGA 5.2. (Tamura et al., 2011), based on distance estimates calculated by the Kimura 2-parameter substitution model with gamma distributed rates among sites. This model was predicted as the best model for the current phylogenetic analysis by the Mega 5.2 best model prediction tool. Phylogenetic robustness was inferred from 1000 bootstrap replications.

The primers for amplifying the mitochondrial COX1 were LCO1490 (Folmer et al., 1994) and COX1-R1 (Rot et al., 2006). The conditions of PCR amplifications were: 95°C for 5 min; 35 cycles of 95°C for 40 s, 50°C for 50 s, 72°C for 1 min 30 s; and a final extension at 72°C for 10 min. The PCR products were gel purified on a 1% agarose gel and then extracted using the Promega Wizard® SV Gel and PCR Clean-UpSystem. A Maximum Likelihood tree based on distance estimates calculated by the Jones-Taylor-Thornton model with empirical frequencies was constructed in MEGA 5.2. This model was predicted as the best model for the current phylogenetic analysis by the Mega 5.2 best model prediction tool. Phylogenetic robustness was inferred from 1000 bootstrap replications.

Both PCRs were performed using 5× Red load Taq master (Larova) in a 50-µl PCR mixture with primer concentrations of 0.2 µM. For sequence alignments, additional 18S rRNA and COX1 genes sequences were downloaded from the NCBI nucleotide collection non-redundant database (http://www.ncbi.nlm.nih.gov/). Sequences were aligned using ClustalW (Thompson et al., 1994) as available in MEGA 5.2. 18S rRNA and COX1 amplicons were sequenced at Macrogen Europe (1105 AZ, Amsterdam, Netherlands).

RESULTS

MICROBIAL DIVERSITY AND CORE COMMUNITY

A total of 40,507 16S rRNA sequences were obtained by 454 amplicon sequencing. These sequences were assigned to 1055 OTUs at the species level (distance 0.03), after removing singletons (Table S1). Rarefaction curves began to reach asymptotes and coverage estimates were high (>96%) for all samples, with the exception of sample PW3. Only 197 reads forming 94 OTUs were counted for this sample, with an estimated 181 OTUs (Chao1) predicted and 66.3% coverage. Insufficient low depth of sequencing of this sample led us to remove it from further analyses.

Core microbial communities were defined as OTUs that appeared in all replicates of: (i) all *P. ficiformis* samples (70 OTUs), (ii) violet *P. ficiformis* from Italy (105 OTUs), and (iii) violet *P. ficiformis* from Israel (110 OTUs) (**Figure 2**). Chloroflexi and Gammaproteobacteria dominated all *P. ficiformis* samples, regardless of color morph and collection location (**Figure 3**), composed primarily of the Chloroflexi lineages SAR202 and TK10 and the Gammaproteobacteria lineages KI89A, Chromatiales and Xanthomonadales. Other phyla with OTUs present in all *P. ficiformis* samples included Acidobacteria (9.7%), Deferribacteres (7.1%), Actinobacteria (6.8%), Nitrospirae (4.5%), Deltaproteobacteria (3.6%), Gemmatimonadetes (3.3%), JTB23 (1.2%), Alphaproteobacteria (1%), Spirochaetes (0.3%) and Bacteroidetes (0.3%) (**Figure 2A**). The phylum Chlorobi (Cytophagales, OPB56) was present in higher abundances in violet sponges from Italy (PV1-3, **Figure 2C**) and comprised three OTUs (35, 677, 753).

COMMUNITY STRUCTURE ANALYSES

Community structure analyses were performed using different methods, all showing a similar result: violet and white morphs of *P. ficiformis* had similar microbial communities (PERMANOVA, $P = 0.18$), while biogeography played a significant role in determining the community structure ($P < 0.01$, Table S2). The microbial communities associated with *P. ficiformis* from Italy exhibited differences from those associated with *P. ficiformis* from Israel, with the exception of sample PV1 (**Figure 4**). **Table 1** (SIMPER) and Table S3 (PLS-DA) show the OTUs with the

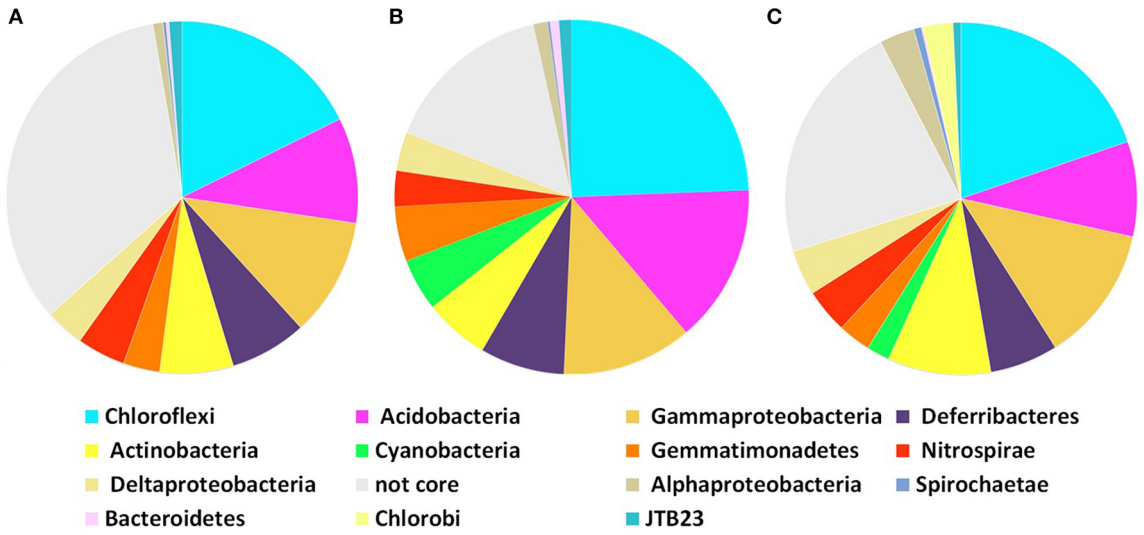

FIGURE 2 | Core community composition of microbial symbionts in *P. ficiformis* based on phylogenetic distribution of 97% OTUs at the phylum level (Proteobacteria are shown at the class level). Analysis does not include the experimental transplant specimens (D, Dt). Core symbiont communities in **(A)** all *P. ficiformis* samples, **(B)** violet *P. ficiformis* from Israel, and **(C)** violet *P. ficiformis* from Italy.

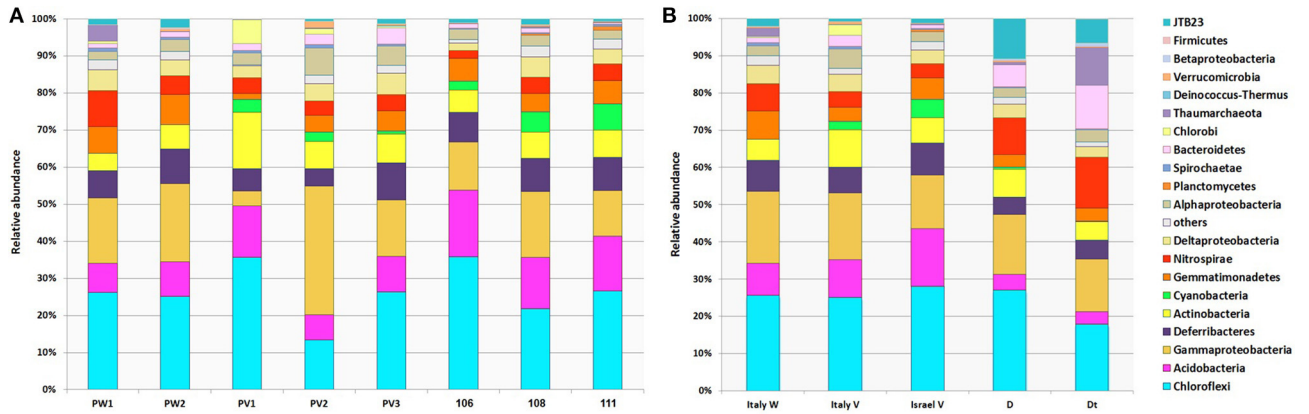

FIGURE 3 | Phylum-level composition of microbial symbionts in *P. ficiformis* among different color morphs and collection locations. Distribution and relative abundances of OTUs from different phyla are shown for **(A)** each *P. ficiformis* specimen, and **(B)** grouped by different *P. ficiformis* morphs and collection locations. White morphs from Italy (PW1, PW2; Italy W), violet morphs from both Italy (PV1, PV2, PV3; Italy V) and Israel (106, 108, 111, Israel V) and an additional violet specimen, half transplanted to a dark cave for 6 months (Dt) and the other half kept at its original location for control (D) are shown. Relative abundances shown in this figure were constructed using OTUs with distance of 0.03 at the phylum level (Proteobacteria are shown at the class level). Groups are shown in the order in which they are listed.

highest impact on the observed biogeographic grouping. OTUs 23, 33, 62, 69 belong to the SAR202 clade of Chloroflexi and were more abundant in Italian samples. OTUs 36 and 3 belonged to the Chloroflexi class Caldilineae and were more abundant in Israeli samples. OTU3 was also abundant in the Italian sample PV1. The Gammaproteobacteria OTUs 27 (KI89A clade) and 18 (E01-9C-26 marine group) were more abundant in Italian samples, while OTU43 (KI89A clade) showed higher abundance in Israeli samples. OTU45 (Marine group I, Thaumarchaeota) was unique to four Italian samples (D, Dt, PW1, and PW2), and its abundance was highly increased by translocation to the dark cave. OTU25 (Flavobacteria, Bacteroidetes) and OTU10 (JTB23, Proteobacteria) were prevalent in D and Dt. OTU6 (Nitrospira, Nitrospirae) was prevalent in Italian samples especially in D, Dt, and PW1. OTU13 (Acidobacteria, Acidobacteria) and OTU15 (PAUC34f, Deferribacteres) were prevalent in Israeli samples. OTU14 (Acidimicrobiales, Actinobacteria), OTU35 (Chlorobia, Chlorobi), and OTU19 (TK10, Chloroflexi) were prevalent in Italian sample PV1 and led to its separation on the PLS-DA ordination.

SPECIFICITY AND OVERLAP OF MICROBIAL OTUs

Maintenance vs. variation in microbial OTUs among sampled *P. ficiformis* sponges was further investigated and quantified with

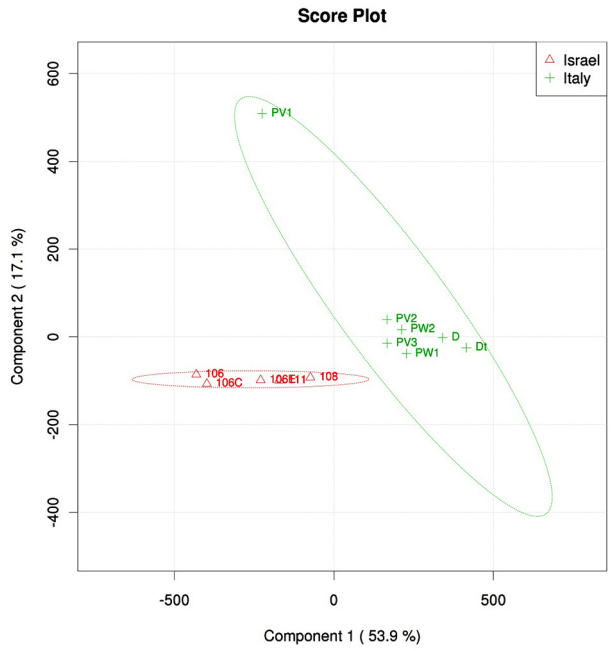

FIGURE 4 | PLS-DA score plot of microbial symbiont communities in *P. ficiformis* samples including aposymbiotic specimens (PW1, PW2, and Dt) and those hosting cyanobacteria (all the rest), and two geographic locations (red triangles for Israel and green cross for Italy). **Table 1** lists OTUs that had the highest impact on the PLS-DA grouping.

Venn diagrams (**Figure 5**). An OTU was considered shared if it was present in at least two replicates of both groups that were compared. An OTU was considered unique if it was present in at least two replicates of one group and absent in all replicates of the other group. Violet *P. ficiformis* from Israel and Italy ($n = 3$ per group) had 89 shared OTUs, while the numbers of unique OTUs of violet *P. ficiformis* from Israel and Italy were 62 and 57, respectively. Consistent with the observed biogeographical trend, violet *P. ficiformis* ($n = 3$) and white *P. ficiformis* ($n = 2$) from Italy shared more OTUs ($n = 109$) than violet morphs from Israel and Italy ($n = 89$). The numbers of unique OTUs of violet and white *P. ficiformis* were 35 and 28, respectively.

CHOROPHYLL *a* CONCENTRATIONS AND *SYNECHOCOCCUS FELDMANNII*

Chl *a* content differed significantly among color morphs (ANOVA, $P < 0.01$), with significant pairwise comparisons among violet ($79.9 \pm 31.5\,\mu g/g$), pink ($12.9 \pm 1.2\,\mu g/g$), and white ($2.4 \pm 1.4\,\mu g/g$) morphs (SNK, $P < 0.05$), confirming the presence of photosymbionts in violet and pink morphs of *P. ficiformis* and their absence in the white morph. Similarly, the 454 sequence dataset revealed the presence of the symbiotic cyanobacterium *S. feldmannii* in violet specimens, while it was absent from all white morphs of *P. ficiformis* (PW, **Figures 2B,C**). *S. feldmannii* (OTU9) was represented by 987 sequences and exhibited 99.5% similarity to previously described *S. feldmannii* from *P. ficiformis* (GenBank Acc. No. AY701297.1, Steindler et al., 2005). The transfer of half of the sponge D (violet *P. ficiformis*) to a cave caused the loss of coloration and disappearance of *S. feldmannii* (*S. feldmannii* represented 0.5% of the microbes in the violet specimen D and 0% in its bleached transferred half). When comparing cortex and endosome of specimen 106, *S. feldmannii* was present in the cortex (7.9%) and absent in the endosome (0%).

EFFECT OF PHOTOSYMBIONTS ON OVERALL MICROBIAL COMMUNITIES

Given the presence of cyanobacterial symbionts in the cortex of *P. ficiformis* specimens growing in illuminated sites, we tested whether the presence of cyanobacteria affected the microbial communities found in the tissue hosting them. No significant differences were observed between microbial communities found in the cortex and endosome of violet *P. ficiformis* (PERMANOVA, $P = 0.42$). In addition, the transplantation of a violet morph of *P. ficiformis* from an illuminated site to a dark cave had little effect on the microbial symbiont community, despite apparent bleaching and loss of cyanobacterial symbionts (**Figure 3**), as the control and transplanted portions of this sponge were more similar to each other than sympatric violet and white sponges (Bray-Curtis analysis, data not shown).

One major effect observed in the transplant experiment was a noticeable increase in Thaumarchaeota, Marine group I, *Candidatus* Nitrosopumilus in the bleached part (control D = 0.8%, transplant Dt = 10.2%). There were three different Thaumarchaeota OTUs, with more than 10 reads in specimen Dt. The OTU responsible for most of the shift (0.8–10.2%) was OTU45 (Thaumarcheota, Marine Group I, *Candidatus* Nitrosopumilus).

ULTRASTRUCTURAL OBSERVATIONS (TEM)

Micrographs of the three color morphs (violet, pink, and white) of *P. ficiformis* showed numerous bacteriocytes in the mesohyl (**Figures 6A,B**). Each bacteriocyte contained a high diversity of bacteria, some of which were actively dividing (**Figure 6C**) or interchanging cellular material (**Figure 6C**). Bacteria were also observed surrounding the bacteriocytes or being actively phagocytized by sponge cells (**Figure 6D**). As expected, cyanobacterial cells were only observed in the violet and pink morphs of *P. ficiformis* and were absent in the white form (**Figure 6A**). Besides the presence/absence of cyanobacteria, no other differences in sponge or bacterial cell abundance and morphology were detected for any of the sponge morphs.

HOST PHYLOGENETICS

18S rRNA and mitochondrial COX1 genes amplified from violet, pink and white specimens in triplicates exhibited consistent differences between *P. ficiformis* color morphs. Maximum likelihood phylogenies showed a more derived and genetically-distinct cluster of *P. ficiformis* comprised solely of pink/white morphs for both genes, with a bootstrap support >60% in both analyses (**Figure 7** and data not shown). The difference in COX1 gene sequences between the pink/white clade and the group formed by specimens from the violet morph was 3 nucleotides that resulted in variation of one amino acid (data not shown).

Table 1 | Similarity percentages (SIMPER) analysis showing the microbial OTUs that contributed most to the observed biogeographic trend in symbiont communities of *P. ficiformis*.

OTU	Phylum (lowest taxon)	Average rel. abund.(%)		Contribution to dissimilarity	
		Israel	Italy	Individual	Cumulative
3	Chloroflexi (Caldilineaceae)	13.19	2.83	9.42	9.42
8	Acidobacteria (Acidobacteria)	5.87	2.04	3.65	13.07
6	Nitrospirae (*Nitrospira*)	2.88	6.95	3.58	16.65
9	Cyanobacteria (*Synechococcus*)	4.44	0.97	3.43	20.07
13	Acidobacteria (Acidobacteria)	3.78	0.91	2.51	22.58
10	Proteobacteria (JTB23)	0.99	3.36	2.38	24.96

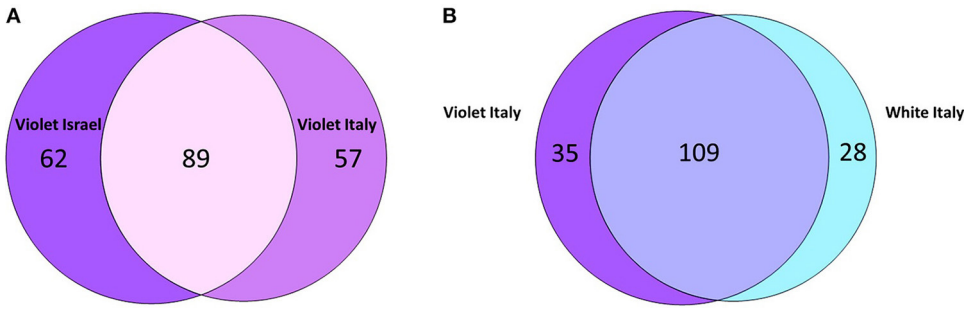

FIGURE 5 | Venn diagram comparing shared and unique 97% microbial OTUs in (A) violet *P. ficiformis* from Israel and Italy ($n = 3$ per group) and (B) violet ($n = 3$) and white ($n = 2$) *P. ficiformis* from Italy.

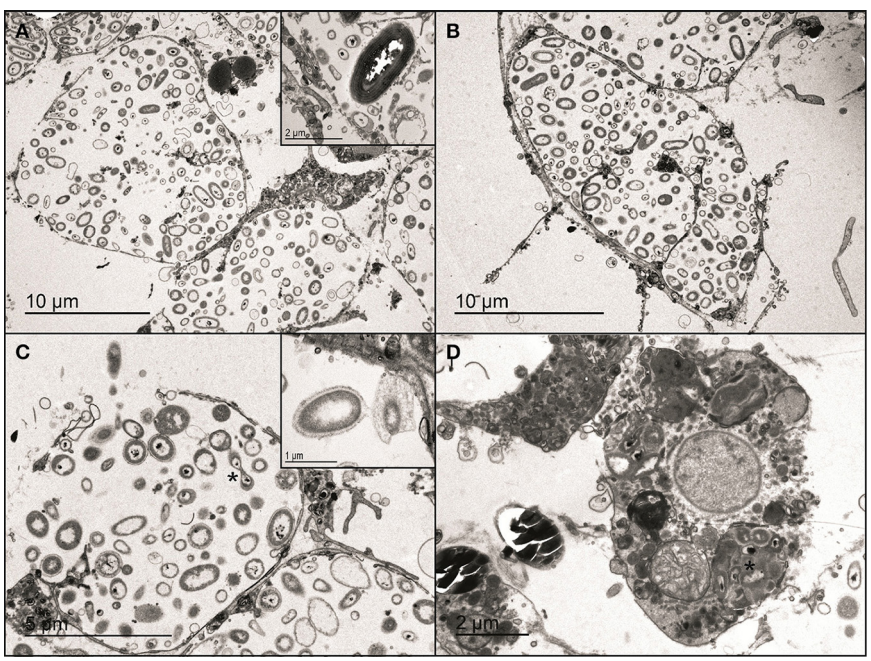

FIGURE 6 | Micrographs of violet, pink, and white morphs of the sponge *P. ficiformis*. (A) Bacteriocyte in the mesohyl of the violet morph and **(B)** the white morph. The inset shows a cyanobacterial cell from the violet morph. **(C)** Dividing bacteria (asterisk) in a bacteriocyte of the violet morph. The inset shows bacteria interchanging cellular material in the white morph. **(D)** Phagocytic sponge cell containing a partially-digested cluster of bacteria (asterisk) in the pink morph.

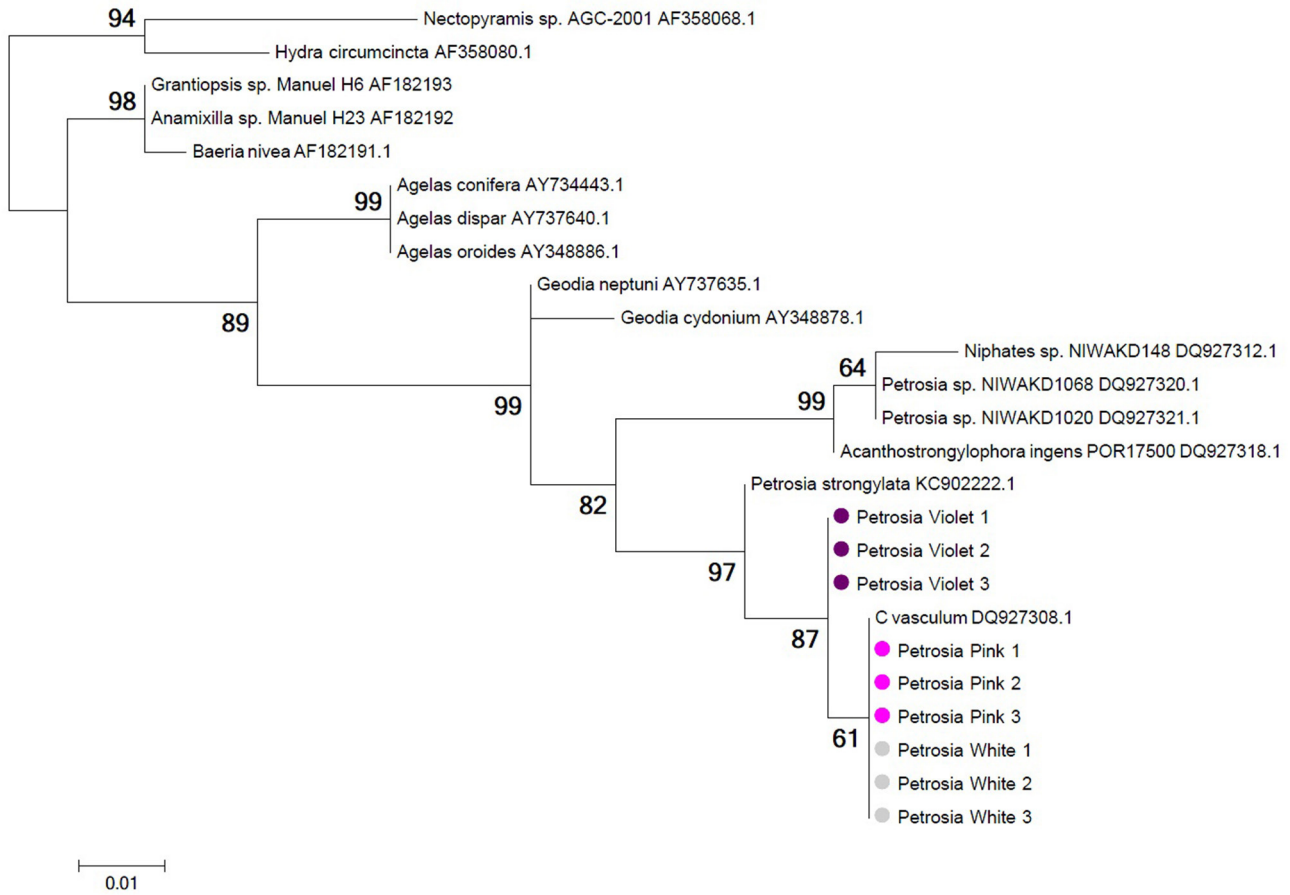

FIGURE 7 | Phylogeny of 18S rRNA gene sequences from white (gray circles), pink (pink circles), and violet (violet circles) morphs of *P. ficiformis*. Information on the geographic origin of the sponge specimens represented by the 18S rRNA sequences used in this tree is provided in Table S4 of Supplementary Material. Tree topology was constructed using maximum likelihood criteria and distance estimates calculated by the Kimura 2-parameter substitution model with gamma distributed rates among sites. Bootstrap values derive from 1000 replications and are shown at branch nodes. Tree construction was performed with MEGA5.2.

DISCUSSION

In this study, we investigated the structure of symbiotic microbial communities in *P. ficiformis* among color morphs and biogeographic locations, using a suite of molecular, chemical and microscopy analyses. Chlorophyll *a* content, TEM observations and DNA sequence data all confirmed the presence of cyanobacteria, specifically *S. feldmannii*, in violet and pink morphs of *P. ficiformis* and their absence in white morphs. Contrary to expectations, the presence/absence of these photosymbionts had little effect on overall microbial community composition, as evidenced through the comparison of white and violet morphs, the transplantation of a violet morph from an illuminated site to a dark cave habitat, and the comparison of cortex vs. endosome tissue from violet morphs. A previous study on various sponge species, including *P. ficiformis*, described high dissimilarities between microbial communities from cortex and endosome of high microbial abundance sponges. However, *P. ficiformis*, among others, was the species showing the least dissimilarity between tissue types (Gerce et al., 2011). The latter study analyzed a single specimen per analyzed sponge species, while in our investigation the comparison of cortex vs. endosome on triplicate samples did not show a grouping according to tissue type.

Rather than cyanobacterial symbionts (i.e., color morphs) accounting for variability in microbial symbiont communities, a biogeographic trend was observed between *P. ficiformis* collected in Israel and Italy. Surprisingly, DNA sequencing of ribosomal and mitochondrial markers revealed two genetically distinct clades of *P. ficiformis*: one composed of violet morphs and one of pink and white morphs. Taken together, these data show that microbial symbiont communities are more similar in genetically distinct *P. ficiformis* from the same location, than genetically similar *P. ficiformis* from distant locations. The difference in the sampling time of Italian and Israel samples (August and February for Italian and May for Israel specimens, respectively) was likely not the cause of the observed biogeographic effect, given that Italian samples from August and February grouped together (e.g., PW1 and PW2). In addition, temporal stability of sponge-associated microbial communities has recently been shown for other Mediterranean sponge species (e.g., Erwin et al., 2012b; Bjork et al., 2013; Hardoim and Costa, 2014).

Typical constituents of the sponge microbiome, previously described for many other sponge species (Schmitt et al., 2012b), were also found in *P. ficiformis*, with Chloroflexi (TK10, SAR202, Caldilinea, Anaerolineae; Schmitt et al., 2011; Bjork et al., 2013), Gammaproteobacteria (e.g., Xanthomonadales, Jackson et al., 2012) and Acidobacteria (e.g., PAUC26f, Hentschel et al., 2002) constituting approximately half of the microbiome. Additional typical sponge-specific clades included Actinobacteria (Sva0966 marine group) and Deferribacteres (e.g., PAUC34f, Moitinho-Silva et al., 2013). The main phyla found in the microbiome of *P. ficiformis* (Chloroflexi, Proteobacteria, and Acidobacteria) confirm results from a previous study (Schmitt et al., 2012a), however we found only few reads corresponding to Poribacteria, while in the sample of Schmitt et al. (2012a) Poribacteria represented 8% of the microbiome. A potential explanation for this discrepancy is that different primers (with different bias) were used in the two studies.

Most studies have shown high stability of sponge-associated microbial communities within the same sponge species collected across wide geographic distances (e.g., Pita et al., 2013a,b), though some exceptions have also been documented (Taylor et al., 2005; Lee et al., 2009b; Anderson et al., 2010). In the present study, we show that microbial communities in *P. ficiformis* are structured primarily by geographic location rather than host morph or presence/absence of cyanobacterial symbionts. Only one sample (PV1) did not conform to this biogeographic trend, instead it appeared to differ from all other samples, but to be closer to Israeli samples than Italian ones. This sample froze during storage (likely due to leakage of ethanol from the vial during transfer from Italy to Israel). Freeze-and-thaw of this sample may have biased the DNA extraction of specific bacterial groups resulting in the observed microbial diversity found for this sample. However, the alternative explanation, that a different general pattern may be observed if a larger sample size was to be inspected, cannot be excluded at this stage. The stability of microbial communities across distant locations found for other sponge species may be related to the location of symbiont within the host and the type of symbiont transmission utilized by the host. In most sponges, symbionts are found extracellularly within the mesohyl matrix and many bacteria are transferred to the next host generation through vertical transmission (i.e., transfer of symbionts via reproductive host cells: eggs, sperm, (e.g., Usher et al., 2001; Schmitt et al., 2008; Lee et al., 2009a; Gloeckner et al., 2013). In both these respects, *P. ficiformis* is quite an exceptional sponge: most bacteria are found intra-cellularly in bacteriocytes and embryos and larvae were found to be devoid of microbes (Maldonado, 2007). These characteristics may contribute to the biogeographic trends observed for microbial communities in *P. ficiformis* from Italy and Israel, with ambient bacterioplankton composition dictating which symbionts are available for colonization.

Before next generation sequencing (NGS) techniques were developed, it was suggested that sponge-specific bacteria may not be present in seawater and be only transferred vertically to the next sponge generation. NGS techniques revealed that sponge-specific bacteria are present in the seawater, though at very low concentration (Webster et al., 2010; Taylor et al., 2013). The fact that sponge-specific bacteria are found in *P. ficiformis* suggests that a yet undescribed recognition mechanism enables the acquisition of specific bacterial species from the ambient rare biosphere by sponges.

The greater influence of biogeography rather than association with cyanobacteria as structuring factor for symbiotic microbial communities in *P. ficiformis* contrasts our original hypothesis that photosymbionts (providers of an additional source of organic carbon) represent a keystone species in sponges. A possible explanation relates to the theory of functional equivalence between sponge microbiomes suggested by Fan et al. (2012). *P. ficiformis* also harbors ammonia-oxidizing archaea (*Nitrosopumilus* sp.) and ammonia-oxidizing bacteria (*Nitrosococcus*), thus the loss of carbon fixation processes by *S. feldmannii* may be compensated by a gain of chemoautotrophs. In fact, the transplant experiment revealed a major increase in the relative abundance of the *Nitrosopumilus* sp. (0.8–10.2%), an archaeon with ammonia-oxidizing capabilities. However, these remain hypotheses that require further replication of transplant experiments and physiological measurements to be appropriately tested.

The present study also provided molecular evidence for early speciation between the violet and the pink/white morphs of *P. ficiformis*. 18S rRNA and COX1 analyses showed that all samples from the violet morph of *P. ficiformis* (from Italy and Israel) grouped together and were basal to a clade formed by all the pink and white specimens. Notably, molecular evidence retrieved in this study supports previous morphological observations that described two distinct morphs of *P. ficiformis*: violet, tabular-shaped morphs on vertical relief habitats, and pink or white, cylindrical-shaped morphs growing inside or near the entrance of submarine caves (Vacelet and Donadey, 1977; Sarà et al., 1998). The molecular analyses presented herein also indicate that the pink-white, cave-associated morph is more derived, suggesting an earlier colonization of cave habitats by violet specimens, followed by subsequent diversification of the *P. ficiformis* species complex in cave habitats. This new condition seems to preclude the recolonization of the lighted habitats by the sponge larvae coming from caves as documented by necrosis of ectosome in case of transplant of adult *P. ficiformis* from inside the cave to the outside (Regoli et al., 2000). Environmental factors potentially involved in this speciation process include the different hydrodynamic conditions found externally to the cave vs. at its entrance and inside the cave. The slender and cylindrical morphology with higher pore numbers observed in cave specimens may have resulted from low hydrodynamic conditions where higher metabolic rates and filtration capacities are required to match the carbon requisites of the holobiont. While additional molecular, morphological and reproductive data are needed to confirm this interesting finding, the similarity in microbial communities among morphotypes of *P. ficifiormis* and their divergence across locations provide new insights into the factors that structure symbiont communities in marine sponges.

DATA DEPOSITION

All sequences have been deposited in GenBank, accession numbers KM452895-KM452903 (18S rRNA sequences) and KM452904-KM452912 (COX1 gene sequences). The raw 454

amplicon pyrosequencing data were deposited in the NCBI Sequence Read Archive under project number PRJNA259436. NCBI Biosamples and SRA experiments accession numbers are given in Table S5.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00529/abstract

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Two distinct microbial communities revealed in the sponge *Cinachyrella*

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Marine sponges are vital components of benthic and coral reef ecosystems, providing shelter and nutrition for many organisms. In addition, sponges act as an essential carbon and nutrient link between the pelagic and benthic environment by filtering large quantities of seawater. Many sponge species harbor a diverse microbial community (including Archaea, Bacteria and Eukaryotes), which can constitute up to 50% of the sponge biomass. Sponges of the genus *Cinachyrella* are common in Caribbean and Floridian reefs and their archaeal and bacterial microbiomes were explored here using 16S rRNA gene tag pyrosequencing. *Cinachyrella* specimens and seawater samples were collected from the same South Florida reef at two different times of year. In total, 639 OTUs (12 archaeal and 627 bacterial) belonging to 2 archaeal and 21 bacterial phyla were detected in the sponges. Based on their microbiomes, the six sponge samples formed two distinct groups, namely sponge group 1 (SG1) with lower diversity (Shannon-Wiener index: 3.73 ± 0.22) and SG2 with higher diversity (Shannon-Wiener index: 5.95 ± 0.25). Hosts' 28S rRNA gene sequences further confirmed that the sponge specimens were composed of two taxa closely related to *Cinachyrella kuekenthalli*. Both sponge groups were dominated by *Proteobacteria*, but *Alphaproteobacteria* were significantly more abundant in SG1. SG2 harbored many bacterial phyla (>1% of sequences) present in low abundance or below detection limits (<0.07%) in SG1 including: *Acidobacteria*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae*, PAUC34f, *Poribacteria*, and *Verrucomicrobia*. Furthermore, SG1 and SG2 only had 95 OTUs in common, representing 30.5 and 22.4% of SG1 and SG2's total OTUs, respectively. These results suggest that the sponge host may exert a pivotal influence on the nature and structure of the microbial community and may only be marginally affected by external environment parameters.

Keywords: marine sponge, symbionts, diversity, archaea, pyrosequencing, 16S rRNA, microbiome

INTRODUCTION

Sponges are one of the most primitive Metazoan life forms with fossils dating from at least 580 million years ago (Li et al., 1998; Ryan et al., 2013). Today, there are more than 8500 described extant sponge species, most of which are marine (van Soest et al., 2012). Marine sponges are ecologically important components of the benthic community due to their wide diversity and high biomass (Ilan et al., 2004; de Goeij et al., 2013). In addition, they play a key functional role linking benthic and pelagic ecosystems, as they efficiently remove particulate organic carbon from the seawater (Díaz and Rützler, 2001; Ilan et al., 2004; Webster et al., 2011). Indeed, these sessile invertebrates are able to filter considerable amounts of seawater; a 1 kg sponge can filter up to 24000 L of water per day (Vogel, 1977). Because they are efficient filter feeders, many sponges can live in nutrient-poor habitats such as tropical reefs. However, because of their feeding mode, they are also directly affected by water quality and are vulnerable to marginal environmental conditions (Webster and Blackall, 2009).

Many sponge species consistently harbor dense and diverse microbial communities including bacteria, archaea and eukaryotes (Taylor et al., 2007b). Symbionts can contribute up to 50% of the sponge biomass (Wilkinson, 1978a,b,c; Hentschel et al., 2006). Sponge-associated microorganisms include members of two archaeal lineages and >30 different bacterial and candidate phyla (Taylor et al., 2007b; Webster et al., 2008; Zhu et al., 2008; Sipkema et al., 2009; Schmitt et al., 2012). Many of these taxa form monophyletic sponge-specific clusters

Abbreviations: AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; LMA, low microbial abundance; HMA, high microbial abundance; SG1, sponge group 1; SG2, sponge group 2.

even though they are found in geographically and phylogenetically distinct sponge hosts (Taylor et al., 2007b; Simister et al., 2012).

Although sequencing technology has revealed much about the structural diversity of sponge associated microbiomes, relatively little is known about the specific ecological relationships and interactions among these sponge symbionts and their host (Taylor et al., 2007a; Webster and Taylor, 2012). While sponges are believed to provide a favorable environment to their symbionts, the contribution of the symbionts to the host is less well understood. However, phylogenetic inference suggests that associated bacteria and archaea are capable of a range of metabolic processes that can benefit their hosts such as ammonium-oxidation (Steger et al., 2008), nitrite-oxidation (Hentschel et al., 2002), nitrogen fixation (Wilkinson and Fay, 1979), sulfate reduction (Hoffmann et al., 2005), and photosynthesis (Wilkinson and Fay, 1979; Bayer et al., 2008; Hoffmann et al., 2009; Mohamed et al., 2010; Schläppy et al., 2010). However, it is possible that sponges and some, or all, of their microbes coexist in a more commensal or even parasitic style relationship with their hosts as opposed to a truly mutualistic one.

Further, how sponges distinguish between symbionts, food and pathogens is still unclear (Webster and Blackall, 2009). Recent studies have compared sponge microbial communities from phylogenetically distant hosts in the same location and from closely related sponges at different locations (Hentschel et al., 2002; Webster et al., 2010; Schmitt et al., 2012; Jeong et al., 2013; Montalvo et al., 2014; Kennedy et al., 2014). Thus, studies have established a "core microbial community" that would be present in many host taxa under various space and time conditions (Schmitt et al., 2012).

Here, we compare the microbial communities of different specimens of the sponge genus *Cinachyrella* collected from the same South Florida location at two different times of year. *Cinachyrella* (class *Demospongiae*), is common in coastal waters of South Florida as well as the Caribbean, with three species (*C. kuekenthali*, *C. alloclada*, and *C. apion*) present in these locations (Cárdenas et al., 2009). While *C. apion* is usually small and lives mainly near the mangrove area in shallow waters, *C. kuekenthali* and *C. alloclada* typically occur on reefs (Rützler and Smith, 1992; Cárdenas et al., 2009). However, these species are extremely difficult to visually differentiate and require careful examination of the spicules for identification at the species level (Cárdenas et al., 2009, personal observation).

Much debate currently exists concerning the identification of these species, with morphological diagnostic characters conflicting with molecular phylogenies created from marker genes. For example, using the 28S rRNA gene, *cox1* gene and a combination of the two former genes and 18S rRNA, Szitenberg et al. (2013) showed that, *Cinachyrella australiensis* contains several cryptic sympatric populations. Within the present study, we explore the microbiome of *Cinachyrella* specimens collected from the same natural environment. The purpose of the study was to describe the baseline microbial community of *Cinachyrella* in order to develop this sponge as a future experimental model. Interestingly, we discovered that based on different microbial communities, our samples formed two distinct groups of sponges, independent of the time of collection, indicating that *Cinachyrella* can harbor very distinct symbionts.

MATERIAL AND METHODS

SPONGE AND SEAWATER COLLECTION

Cinachyrella specimens were collected by SCUBA diving from the Inner Reef (as defined by Walker, 2012), Broward County, Florida, USA (N 26° 03′ 01″, W 80° 06′ 18″) at a depth of 6.1 m, on Aug 2, 2011, on Oct 24, 2011, and Feb 15, 2012, under a Florida Fish and Wildlife Conservation Commission Fishing License and a Special Activity License (-12-1372-372a). Sponges were identified as the genus *Cinachyrella* (family *Tetillidae*, Sollas, 1886; van Soest et al., 2014) given their characteristic orange to yellow color, subglobular shape and hispid surface. Water temperatures reached 30.3, 23.9, and 22.8°C in August, October and February, respectively. A total of 64 individuals were collected in total. Here, we present detailed results for six individuals consisting of three individuals on October and February (henceforth labeled as Sponge 1, 2, 3 (Sp1, Sp2, Sp3) Oct and Sponge 4, 5, 6 (Sp4, Sp5, Sp6) Feb. The other 58 individuals were subjected to various experimental conditions in aquaculture, and we provide a preliminary analysis of these samples (Supplementary Material). In-depth results of the different experiments for these samples are not shown. Individuals were cut at the base with a dive knife, placed in individual Nasco Whirl Pak bags filled with ambient seawater and brought to the surface. Samples were stored in the shade and maintained at ambient seawater temperature until transported back to the laboratory (within 2 h of collection). Surface seawater was also collected each time (one replicate in October and one replicate in February) from the dive site in 50 L carboys. These seawater samples were used to confirm that microbial communities associated with the sponge were specific to the sponges and not amplified from seawater DNA. Upon return to the laboratory, sponges were quartered with a sterile knife, frozen in liquid nitrogen, and placed at −80°C for long-term storage. Seawater (0.5 L) was filtered onto a 0.22 μm Supor filter (Pall Life Science, Ann Arbor, MI) by vacuum filtration (<10 mm Hg), the filters were frozen in liquid nitrogen, and stored at −80°C.

DNA EXTRACTION

Approximately ¼ of a sponge was used for DNA extraction. In a sterile petri dish, the sample was defrosted and the ectoderm (darker outer layer) was immediately removed using a sterile scalpel. The endoderm was transferred to a new petri dish and 5 ml of buffer (10 mM Tris pH = 7.6, 100 mM EDTA, 20 mM NaCl) was added. The sponge endoderm was minced, mixed in buffer, and the cell suspension collected into 1.7 mL tubes. These sponge suspensions were centrifuged for 15 min at 16,000 *g* at 4°C. Supernatant was decanted and the pellets transferred and extracted using the MO BIO PowerSoil DNA isolation kit according to the manufacturer's instructions (MO BIO, Carlsbad, CA).

Seawater filters also were extracted with the MO BIO PowerSoil kit to avoid yield discrepancy between DNA extraction protocols. The filters were placed into bead tubes (provided by the kit) and cut into fine pieces using sterile dissection scissors. DNA was extracted according to the manufacturer's instructions

using a 2 min bead-beating step (instead of 10 min vortexing step).

SPONGE 28S rRNA GENE PCR AND ANALYSIS

For molecular systematics, our methods followed those proscribed by the Porifera Tree of Life project (Thacker et al., 2013). Specifically, the 28S rRNA gene was amplified using the 28F63mod (5′- ACC CGC TGA AYT TAA GCA TAT HAN TMA G- 3′) and 28R2077sq (5′- GAG CCA ATC CTT WTC CCG ARG TT- 3′) (Thacker et al., 2013). PCR consisted of one reaction of 50 μL with: 1 μM each forward and reverse primer, 1 μL of template DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 unit of Taq (High Fidelity Taq, TaKARa Otsu, Shiga, Japan). Thermal cycling was initiated with denaturation at 94°C for 3 min, followed by 30 cycles of: 45 s at 94°C, 60 s at 55°C, and 72°C for 6 min and a final extension step for 10 min at 72°C. PCR products were visualized on a 1.5% agarose gel (containing Gel Red). PCR products were cloned and sequenced on an ABI 377 automated DNA sequencer at the University of Alabama, Birmingham using the primer: 28R1411 (5′-GTT GTT ACA CACTCC TTA GCG G-3′). Two samples (Sp5 Feb and Sp6 Feb) had low quality sequences and were removed from the study. The nearest relative for each sequence was determined using the NCBI BLASTn tool against the GenBank non redundant database.

16S rRNA GENE PCR AND ANALYSIS

Approximately 291 bp of the 16S rRNA gene was amplified by PCR using the universal bacterial and archaeal primers (targeting the V4 region of the gene): 515F (5′- GTGCCAGCMGCCG CGGTAA- 3′) and 806R (5′- GGACTACHVGGGTWTCTAAT- 3′) (Caporaso et al., 2011), which contained a unique barcode used to tag each PCR product. This primer set was chosen because it targets a broad range of bacterial and archaeal taxa with the exception of a few groups (Bates et al., 2011; Caporaso et al., 2011). PCR consisted of two reactions of 30 μL with (for each reaction): 1 μM each forward and reverse primer, 1 μL of template DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 unit of Taq (High Fidelity Taq, TaKARa Otsu, Shiga, Japan). Thermal cycling was initiated with denaturation at 94°C for 3 min, followed by 30 cycles of: 45 s at 94°C, 60 s at 50 and 72°C for 90 s and a final extension step for 10 min at 72°C. PCR products were visualized on a 1.5% agarose gel (containing Gel Red). Successful reactions (i.e., with a clear band, two reactions of 25 μL) were pooled and purified with the Agencourt AMPure kit (Beckman Coulter, Beverly, MA), using 1.8× vol. of AMPure bead slurry and eluted in 10 mM Tris pH 7.5. Each sample was quantified using PicoGreen dsDNA reagent (Invitrogen, Carlsbad, CA). Purified products were sequenced on a 454 Life Science Genome Sequencer FLX (Roche) at Advanced Genetic Technologies Center at the University of Kentucky.

Sequences were analyzed using QIIME version 1.6 (Caporaso et al., 2010b). Only sequences with a mean quality score >25 and of length >280 bp were included in the analysis. Sequences were then assigned to each barcode and denoised using the denoise_wrapper option (Reeder and Knight, 2010) in QIIME. Operational Taxonomic Units (OTU) were picked using the UCLUST method (Edgar, 2010) and sequences with ≥97% identities were considered as one OTU. A representative sequence was chosen for each OTU and the taxonomic identity of each representative was assigned (in QIIME) using the RDP Classifier (Wang et al., 2007) against the Greengene 12_10 database (McDonald et al., 2012). Chimera sequences were removed using the ChimeraSlayer option (Haas et al., 2011). Sequences were aligned (using PyNAST with default paramaters set in QIIME, Caporaso et al., 2010a) and screened with Lane mask to remove gaps and hypervariable regions (Lane, 1991). A representative phylogenetic tree was built using FastTree (Price et al., 2010) and used for further analysis in QIIME (alpha, beta diversity from weighted UniFrac, Lozupone and Knight, 2005 and principal coordinate analysis generated from the UniFrac distances). *T*-tests (Microsoft Excel) were used to compare the relative abundance of each microbial phylum present in the samples of SG1 and SG2. A P value less than 0.05 was considered statistically significant. A principal coordinate analysis generated from the weighted UniFrac distances and an analysis of similarity (ANOSIM, 999 permutations) were generated in QIIME for all the 64 sponge individuals.

RESULTS

MOLECULAR PHYLOGENETICS CONFIRM SPONGES ARE *CINACHYRELLA*

All the partial 28S rRNA gene sequences obtained were most similar to the single *C. kuekanthali* 28S rRNA sequence present in Genbank (KC869490.1). Two 28S rRNA gene sequences (Sp5 and Sp6) could not be included in this study because of poor quality. Sp1 Oct and Sp4 Feb displayed 97% identity to *C. kuekanthali* and Sp2 Oct and Sp3 Oct had 99% identity to the same sequence (*C. kuekanthali)*. Results showed that Sp1 Oct and Sp4 Feb were most closely related to each other (99.3% identity compared to ∼97% identity to the other two samples). Similarly, Sp2 Oct and Sp3 Oct were 100% identical to each other respectively, but only ∼97% identical to the other two samples (**Table 1**). Based on the 28S rRNA gene sequences, the samples therefore form two groups, one group including: Sp1 Oct and Sp4 Feb and another group including: Sp2 Oct and Sp3 Oct. These are similar to the two groups observed after analysis of the microbiomes (see below).

CINACHYRELLA SPECIMENS HARBOR A DIVERSITY OF UNIQUE BACTERIA AND ARCHAEA

After quality control and chloroplast sequence removal, a total of 16,811 sequences were analyzed including 13,947 from

Table 1 | Percent identity between the 28S rRNA gene partial sequences of *Cinachyrella* samples (Sp1- 4: sponge 1- 4) collected in October 2011 (Oct) and February 2012 (Feb) from South Florida and *C. kuekenthali* (*C. kuek.*; GenBank: KC869490.1; Panama).

	Sp1 Oct	Sp2 Oct	Sp3 Oct	Sp4 Feb	*C. kuek.*
Sp1 Oct	100				
Sp2 Oct	97.3	100			
Sp3 Oct	97.3	100	100		
Sp4 Feb	99.3	96.9	96.9	100	
C. kuek	96.9	99.8	99.8	96.5	100

sponges (ranging from 1185 to 3616 sequences/animal) and 2864 from seawater (ranging from 1340 to 1524 sequences/sample) (**Table 2**). Results indicated that *Cinachyrella* specimens harbor a diverse community of symbionts, including members of all three Domains of life (Bacteria, Archaea and Eukaryotes). Here, the analysis of the eukaryotic community is not presented. In total, 951 OTUs (measured at 97% identity) were identified among all samples (including seawater), of which 19 were archaeal and 932 were bacterial. A total of 639 OTUs (12 archaeal and 627 bacterial OTUs) were present in the sponge symbiont community, and OTU richness in the sponges was lower than the seawater except for one sample (Sp3 Oct, 341 OTUs). The seawater microbial community contained a total of 450 OTUs (10 archaeal and 440 bacterial OTUs), and OTU richness was similar in both samples (246 vs. 285 OTUs) across sampling times (**Table 2**).

CINACHYRELLA CONTAIN DISTINCT AND CANALIZED MICROBIOMES COMPARED TO SEAWATER

Rarefaction analysis demonstrated that for some samples (Seawater Oct, Seawater Feb, Sp2 Oct and Sp3 Oct), the diversity was high enough such that sequencing depth was likely not sufficient to evaluate the rarer members of the community and that further sequencing would be necessary to reveal the true diversity (**Supplementary Figure 1**). Yet the rarefaction analysis here confirmed that most sponge samples' microbiome was less diverse than seawater (**Supplementary Figure 1**). Chao1 richness estimates for sponges varied from 124 to 529 phylotypes and 440 and 510 OTUs for the seawater ($t = -1.9$, $0.05 < P < 0.1$). Similarly, the Shannon-Wiener indices for the *Cinachyrella* samples were lower on average (3.1–6.2), but not statistically different than for the seawater (6.2 and 6.3; **Table 2** $t = -1.9$, $0.05 < P < 0.1$).

Comparatively, 21 bacterial and 2 archaeal phyla and candidate phyla were detected in the sponges vs. 27 bacterial and 2 archaeal phyla and candidate phyla in the seawater. Here, we use the term "candidate phylum" to define a phylum that can be identified from genetic sequences, but lacks cultured representatives (Hugenholtz et al., 1998). Most bacterial sequences were classified, but a small portion (2.7 ± 0.9% in sponges and 2.8 ± 0.003% in seawater samples) could not be assigned to any known phylum.

Table 2 | Overview of the number of sequences, OTUs (97% identities) and diversity indices for six sponges (Sp1- 6: sponge 1- 6) and seawater (SW) samples collected in October 2011 (Oct) and February 2012 (Feb).

Sample ID	Total #reads	Total OTUs[#]	Chao1*	Observed OTUs*	Shannon*
SEAWATER					
SW Oct	1340	246 (0)	440	221	6.2
SW Feb	1524	285 (1)	510	239	6.3
GROUP "SG1"					
Sp1 Oct	1185	90 (2)	176	86	3.7
Sp4 Feb	2386	179 (0)	267	119	4.2
Sp5 Feb	1755	105 (1)	191	79	3.3
Sp6 Feb	3616	115 (1)	124	61	3.1
GROUP "SG2"					
Sp2 Oct	2254	220 (1)	289	156	5.7
Sp3 Oct	2751	341 (0)	529	203	6.2

[#] *Number in parentheses denotes the number of unclassified OTUs included in the total.*

* *1100 reads were subsampled to calculate diversity indices.*

MICROBIAL COMMUNITY COMPOSITION DEFINES TWO *CINACHYRELLA* TAXA

Both sequence taxonomy (**Table 1**) and PCoA analyses (**Figure 1**) suggest that the *Cinachyrella* specimens in this study form two distinct groups and may represent different taxa of sponge. We defined here these groups as Sponge Group 1 (SG1) and Sponge Group 2 (SG2; **Figure 1**). SG1 incorporates samples that spanned both seasons (Sp1 Oct, Sp4 Feb, Sp5 Feb, and Sp6 Feb) while SG2 is composed of just two samples from one season (Sp2 Oct and Sp3 Oct). In addition, the PCoA analysis for all 64 sponges samples (See Material and Methods) confirmed that Sp1-6 were split among two groups of sponges defined by their microbial communities (**Supplementary Figure 2**), even though 58 of these samples were placed in aquaculture under various conditions (results of experiments not shown). ANOSIM analysis (using all 64 sponge samples) confirmed that these were statistically different ($R = 0.9926$, $P = 0.001$).

The marked differences in these two groups are demonstrated by comparisons of the diversity of microbial taxa in each. SG2

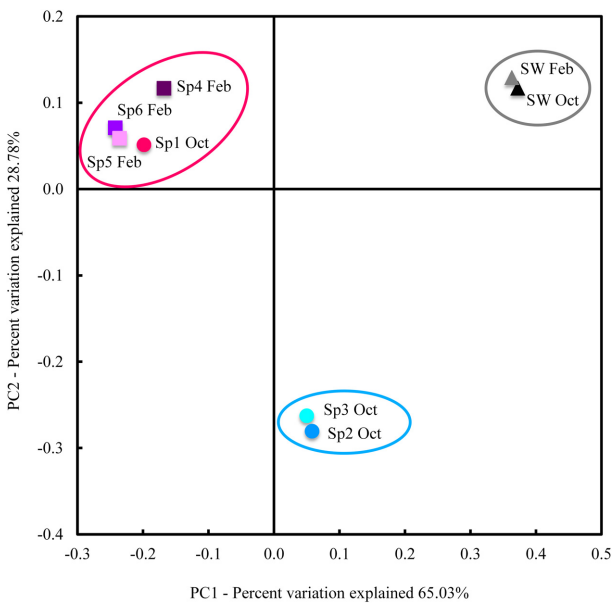

FIGURE 1 | PCoA analysis of weighted UniFrac distance. UniFrac measures phylogenetic distances between OTUs sets within a phylogenetic tree. Here, we used weighted UniFrac, which takes into account relative abundances of OTUs (as opposed to presence/absence only). Samples formed three groups: a seawater group (samples circled in gray), SG1 (samples circled in red) and SG2 (samples circled in blue). Ovals circling samples are for visual guidance and do not represent any statistical grouping.

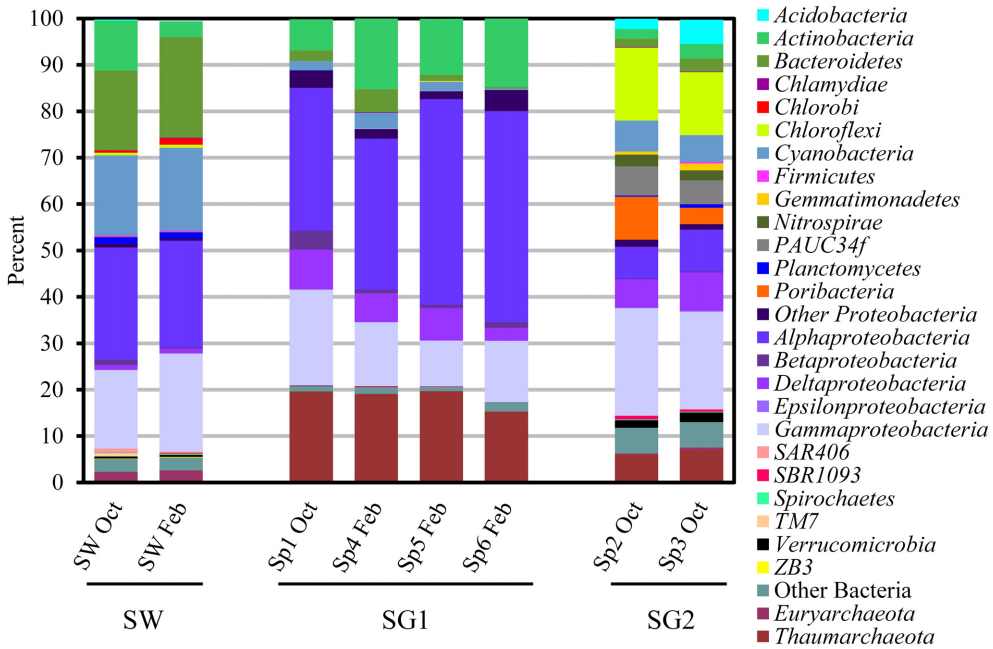

FIGURE 2 | Relative abundance of pyrosequencing reads at the phylum (or classes in the case of *Proteobacteria*) level present in six sponges (Sp1- 6: sponge 1- 6) and seawater (SW) samples collected in October 2011 (Oct) and February 2012 (Feb). Phyla comprised of <0.1% of sequences per sample are not shown. Based on the microbial community structure, samples were placed into two groups: sponge group 1 (SG1: Sp1 Oct, Sp4 Feb, Sp5 Feb and Sp6 Feb) and sponge group 2 (SG2: Sp2 Oct and Sp3 Oct).

samples harbored a more diverse community of microbes as measured by a mean Shannon-Wiener diversity index of 5.95 ± 0.25 (s.e.m.) compared to 3.73 ± 0.22 in the SG1 community ($t = -6.8$, $P < 0.01$; **Table 2**). Further, SG2 contained taxa from 21 different bacterial phyla and candidate phyla and 2 archaeal phyla; SG1 contained about half that with 12 bacterial and candidate phyla, and 2 archaeal phyla.

Overall, both sponge groups were dominated by *Proteobacteria* (SG1: 63.5 ± 2.9%; SG2: 38.9 ± 1.0%), but *Alphaproteobacteria* were more abundant ($t = 5.23$, $P < 0.01$) in SG1 (38.3 ± 3.8%) than in SG2 (7.9 ± 0.2%). *Proteobacteria* in SG2 were dominated by the *Gammaproteobacteria* (22.1 ± 1.1%, **Figure 2**). *Actinobacteria* were also present in both sponge groups, but were in significantly greater numbers ($t = 3.23$, $P < 0.05$) in SG1 (12.2 ± 2.0%, **Figure 2**) than SG2 (2.6 ± 0.6%, **Figure 2**). SG2 harbored the candidate phylum *Poribacteria* (6.4 ± 2.9%) that was first discovered from sponge tissues and can be widespread in these invertebrates (Fieseler et al., 2004; Lafi et al., 2009). In contrast *Poribacteria* was below the detection limit in SG1 ($t = -3.67$, $P < 0.05$; **Figure 2**).

Only a few bacterial phyla or classes were not significantly different in abundance between SG1 and SG2: *Bacteroidetes* ($t = -0.049$, $P > 0.05$), *Chlamydiae* ($t = -2.08$ $P > 0.1$), *Firmicutes* ($t = -1.63$, $P > 0.1$), *Beta-* ($t = 1.22$, $P > 0.1$), *Delta-* ($t = 0.08$, $P > 0.1$), *Gammaproteobacteria* ($t = -2.23$, $P > 0.05$), and SAR406 ($t = -0.42$, $P > 0.1$, **Figure 2**). On the contrary, many phyla were present in SG2 at >1% (mean), but in very low abundance (<0.07% mean) or below detection limits in SG1 and included: *Acidobacteria* ($t = -4.03$, $P < 0.02$), *Chloroflexi* ($t = -22.09$, $P < 0.001$), *Gemmatimonadetes* ($t = -4.154$, $P < 0.02$), *Nitrospirae* ($t = -18.01$, $P < 0.001$), PAUC34f ($t = -17.63$, $P < 0.001$) and *Verrucomicrobia* ($t = -11.99$, $P < 0.001$, **Figure 2**).

In SG1, a few OTUs noticeably dominated the community and composed >10.0% of all the sequences. These included one unclassified *Alphaproteobacteria* OTU (30.0 ± 4.4%), one OTU in the *Cenarchaeaceae* family (18.3 ± 1.1%; **Supplementary Figure 3**), and one unclassified *Actinobacteria* OTU (11.9 ± 2.0%). In SG2, none of the OTUs represented more than 10% of all the community.

Another striking difference in the communities was the relative abundance of archaeal sequences. Archaeal sequences represented a large portion (18.5 ± 1.1%) of all the sequences recovered from SG1 samples, but only 6.9 ± 0.7% for SG2 samples ($t = 9.23$, $P < 0.01$; **Figure 2**). In SG1, one archaeal OTU in *Cenarchaeaceae* family (mentioned above) was dominant (99.3 ± 0.3%). In SG2, 68.2 ± 15.0% of archaeal reads also fell into one *Cenarchaeaceae* family OTU, but this OTU was different from the main one in SG1. A small proportion (5.8 ± 2.3%) of the SG2 archaeal sequences were assigned to the phylum *Thaumarchaeota*, which was almost absent (except for three sequences) from SG1 ($t = 7.48$, $P < 0.01$). These data indicate that the sponges collected in our study, while physically reminiscent, in the same genus, and from the same environment harbor distinct enough microbial communities to warrant a re-evaluation of their phylogenetic relationship.

SEAWATER ARCHAEAL AND BACTERIAL COMMUNITIES ARE DISTINCT FROM SPONGES'

In the overlying seawater, *Proteobacteria* (45.0 ± 0.9%)—and particularly *Alpha-* (23.6 ± 0.6%) and *Gamma-* (19.0 ± 2.1%)—were the most abundant taxa of bacteria. In addition, *Bacteroidetes* (19.4 ± 2.2%), *Cyanobacteria* (17.6 ± 0.3%), and *Actinobacteria* (7.0 ± 3.7%) were the only other bacterial phyla that comprised >2% of all the reads.

The seawater-derived archaeal sequences represented 2.5 ± 0.2% of sequences and mostly belonged to the *Thaumarchaeota,* in particular the Marine Group II or Marine Group III. Marine Group II represented 89.3 ± 4.4% of all seawater archaeal sequences with a single OTU with pronounced dominance (58.5 ± 6.0%; **Supplementary Figure 2**).

CINACHYRELLA'S CORE AND VARIABLE MICROBIAL COMMUNITIES

To further examine the distinct microbial communities, core and variable members of each group were compared. The numbers of common OTUs between SG1 and SG2 was relatively low, with only 95 shared OTUs representing 22.4% of the OTUs in SG2 and 30.5% in SG1. This was approximately equivalent to the numbers of OTUs the seawater shared with SG1 (94 OTUs) and SG2 (103 OTUs, **Figure 3A**).

Within each sponge group, 136 common OTUs were found in SG2 samples, as compared to 21 shared in the two SG1 samples (**Figures 3B,C**). In SG2, these common OTUs belonged to 2 archaeal and 14 bacterial phyla, with the most abundant being (≥8 shared OTUs): *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, and *Proteobacteria*. Interestingly, the samples in SG2 also shared 12 unclassified bacterial OTUs. In SG1, the shared OTUs belonged to the *Crenarchaeota*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Proteobacteria*. The diversity among sponge samples of the same group was similar at the class level, but not shared at the family or genus level. In most cases, many of the OTUs were present in only one of the sponge samples. Out of all the sponge samples, 83 OTUs were present in at least 50% of the samples and 23 OTUs in at least 70% of the sponge samples. Within SG1 and SG2, 107 and 424 OTUs respectively were present in at least 50% of the samples and 55 and 135 OTUs respectively were present in at least 70% of the samples. The only 11 OTUs common to all the sponge samples (i.e., the core community) were assigned to the *Proteobacteria* (*Alpha-* and *Gamma-*) as well as *Cyanobacteria*, the *Bacteroidetes* and the *Actinobacteria*.

MICROBIAL COMMUNITY FUNCTIONAL INSIGHTS

QIIME analysis of the 16S rRNA gene sequences revealed that microbes with potential contribution to the nitrogen cycle were present. SG2 samples contained OTUs belonging to the genera *Cenarchaeum* (18.4 ± 12.1% of the archaeal reads) and *Nitrosopumilus* (6.9 ± 5.9% of archaeal reads). These genera are part of the ammonia-oxidizing archaea (AOA) that oxidize ammonia to nitrite (Preston et al., 1996; Walker et al., 2010). In SG1, AOA sequences belonging to the family *Cenarchaeaceae* were also present. Bacteria involved in the second step of nitrification, the oxidation of nitrite to nitrate were present in SG2 samples. These belonged to two OTUS in the family *Nitrospiracea* (phylum

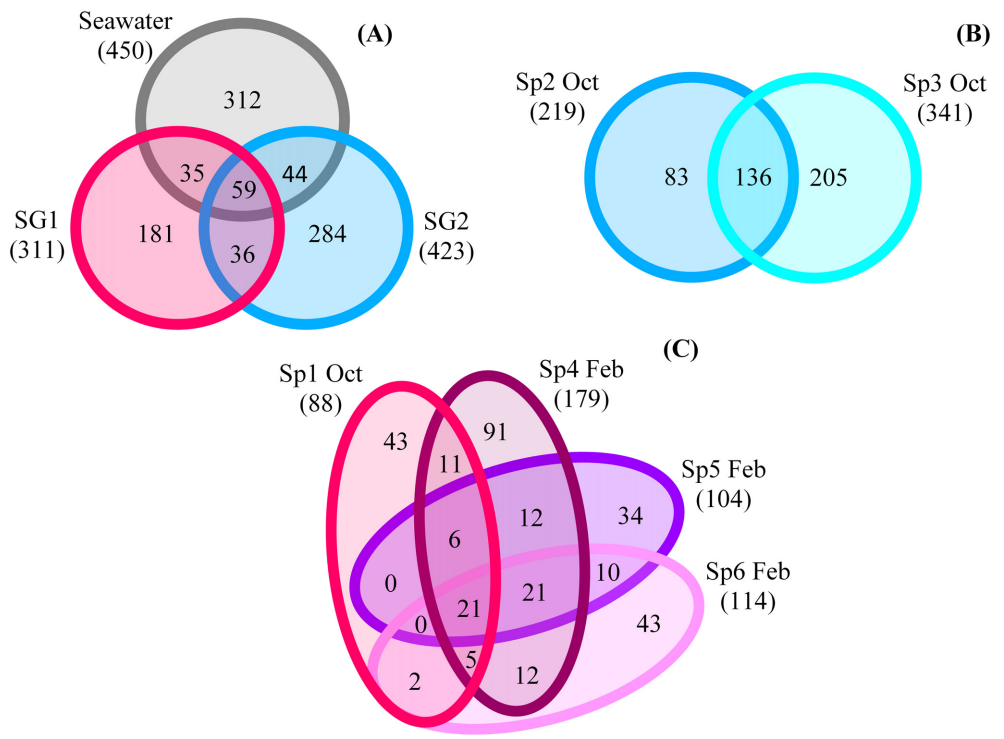

FIGURE 3 | Venn diagrams of specific and shared classified OTUs between. (A) Sponge group 1 (SG1)-SG2-seawater (SW); **(B)** SG1 samples; **(C)** SG2 samples.

Nitrospirae, 2.3 ± 0.2%; **Supplementary Figure 3**) with 98.3 ± 0.02% of these reads affiliated to one OTU. This OTU had 99% identity to sponge-derived sequences

In the *Chloroflexi* (which was almost absent from SG1), two classes were abundant in SG2: *Anaerolinae* (7.4 ± 1.6%) and SAR202 (6.4 ± 0.6%), with most OTUs in the latter class belonging to sponge-specific clusters.

The most abundant *Cyanobacteria* OTU in SG1 (1.6% ± 0.6%) and SG2 (4.7% ± 0.1%) was 100% identical to *Synechoccocus* strain WH8109. This also was the second most abundant OTU in seawater. One noteworthy finding related to *Cyanobacteria* involved the numerically dominant OTU in seawater, which was 100% identical to a UCYN-A clone, *Candidatus Atelocyanobacterium thalassa* (Thompson et al., 2012), a widespread cyanobacterium and likely a significant contributor to N_2-fixation in marine waters (Zehr et al., 2001; Moisander et al., 2010).

In the phylum *Proteobacteria,* many OTUs were obtained that could not be further classified, but had little overlap between SG1 and SG2. In SG1, the unclassified sequences in each of the *Proteobacteria* class had one clear dominant OTU. In the *Alpha-*, *Beta-*, *Delta-* and *Gammaproteobacteria* sequences, this OTU encompassed 77.4 ± 4.2%, 92.1 ± 3.2%, >95.5 ± 1.4%, 55.4 ± 5.9% of the reads in each class, respectively. In contrast, in SG2, none of the unclassified OTU at the class level included more than 37.1 ± 0.2% of the sequences and a few abundant OTUs were usually present. In all sponge samples, many unclassified OTUs (at the class level) were closely related to uncultured bacteria derived from sponge tissues. In particular, the most abundant unclassified *Alpha-* and *Gammaproteobacteria* OTUs were 99 and 100% identical, respectively, to a sequence from *Cinachyra* sp. from India. Within the classified *Alphaproteobacteria*, the families *Rhodobacteraceae* and *Rhodospirillaceae* were common and diverse in both sponge groups and seawater. As expected *Pelagibacteraceae* were the most abundant *Alphaproteobacteria* in the seawater.

DISCUSSION

Since our field collections were confined to a relatively small portion of the reef, we did not intend or expect to collect two apparently divergent *Cinachyrella* taxa. The sponges in this study were collected as part of a broader study involving greater number of specimens used for aquaculture. Upon analysis of the all the samples, it became clear that sponges formed two groups based on their microbial communities. The sponges in aquaculture (data not shown) were subject to different conditions. We therefore decided to present here only the data from sponges collected from the reef and never kept in aquaculture. In the present study, although we have confirmed that these specimens belong to the genus *Cinachyrella*, their exact taxonomic and phylogenetic identification goes beyond the scope of this paper, as the taxonomy of this genus and family (*Tetillidae*) is still under much debate (see introduction and Szitenberg et al., 2013). However, our findings are consistent, but not totally sufficient (due to low sample number and the low 28S rRNA sequence quality of two of our six samples) to prove the idea presented by Cárdenas et al. (2012) that microbiome signatures may be useful traits to delineate some sponge taxa. Thus, additional samples and a more comprehensive histology and electron microscopy analyses of the spicules would be needed to confirm the species identity of these sponge individuals. However, given the clear differences in the microbiomes of these sponge taxa, a simple PCR diagnostic of one or more variable members of the sponges' microbiota could also be used.

Overall, our results are similar to those of Chambers et al. (2013). There, the authors showed that two sponge morphs initially assigned to the genus *Paratetilla* (Demospongiae, Tetillidae) had different microbial communities, sharing less than 43% similarity. Within each morph group, microbial community similarity varied between 65 and 94% between individuals. Using *COI* gene, the authors confirmed that one of the sponge morphs actually belonged to the genus *Cinachyrella*, "challenging the value of the morphological characters used in the classification of these genera" (Chambers et al., 2013). Similar to our results, the bacterial communities were different for the two groups, even for specimens collected from the same location.

DIVERSE MICROBES ARE PRESENT IN *CINACHYRELLA*

Multiple studies have shown that marine sponges can harbor a large diversity of microbes and the microbial taxa richness present in our *Cinachyrella* tissue samples (90–341 OTUs) was within the range of other sponge species. An extensive study targeting 32 species from eight different locations worldwide revealed each sponge carried between 225 and 364 OTUs (at 97% identity) with sequence coverage similar to our study (Schmitt et al., 2012). As expected, when sequencing depth was much greater, OTU richness was higher, reaching numbers between 1099 and 2996 OTUs (95% identity) in three Pacific sponge species (Webster et al., 2010). Total taxon richness (at a higher sequencing depth) was also greater in *C. australiensis* sampled from the coast of Indonesia, in which 800 phylotypes were present (Cleary et al., 2013). In subtropical waters of Key Largo, FL, USA (close to our study site), the barrel sponge *Xestospongia muta* had Shannon diversity indices comparable to the lower range of our *Cinachyrella* samples (Montalvo and Hill, 2011). However, *Cinachyrella* contained fewer OTUs than *Axinella corrugata* (at least 1000 OTUs per specimen) collected less than a few miles away from our study site (White et al., 2012). Compared to the coral *Orbicella faveolata* (formerly *Montastraea faveolata*; Kimes et al., 2013), our sponge samples showed similar diversity, for which 943 bacterial clones contained 178 OTUs (97% similarity threshold), with Chao1 estimates of 307 ribotypes (Sunagawa et al., 2009). Similarly, the coral *O. annularis* sampled from various sites at Curaçao Island harbored 163–323 bacterial OTUs (Barott et al., 2011).

CINACHYRELLA HARBOR FUNCTIONALLY DIVERSE MICROBES

A small percentage of the bacterial 16S rRNA gene fragments could not be further classified indicating that some of the bacterial diversity remains unexplored. This number was much lower than those reported for *A. corrugata* collected nearby, in which 36% of the reads obtained by amplification of the 16S rRNA gene V1-V3 regions were not assigned to any bacterial phylum (White et al., 2012). In their pyrosequencing study of *C. australiensis* and

Suberites diversicolor microbiomes, Cleary et al. (2013) also found 34% of bacterial OTUs unclassified at the phylum level. There, the primers used targeted the V3-V4 regions while the V4 region was used for this *Cinachyrella* study.

In sponges, the dominant microbial phyla can vary with taxonomy and across geographical location or habitat. High microbial abundance (HMA) sponges usually harbor many bacterial taxa while low microbial abundance (LMA) sponges typically have one or few numerically dominant taxa and a few less abundant ones (Hentschel et al., 2003; Giles et al., 2013). In this study, SG1 samples contained few taxa with pronounced dominance, resembling LMA sponges in terms of microbial equitability, but also encompassed many other phylotypes, atypical of LMA sponges. SG2 samples clearly harbored a more diverse microbial community, similar to HMA sponges. It is important to note that the similarity of these sponge groups to HMA and LMA was inferred solely based on the structure of the microbiomes and an in-depth histological study was not performed on these samples to confirm microbial abundance.

SG2 samples contained the candidate phylum *Poribacteria*, but this taxon was below detection limits in both SG1 and seawater. This is notable because *Poribacteria* are typical members of sponge microbiomes, but have mostly been detected in HMA sponges (Hochmuth et al., 2010). This taxon can be diverse, as shown by Schmitt et al. (2012) who detected a total of 437 *Poribacteria* OTUs in the 32 sponges species studied, with up to 79 different *Poribacteria* OTUs (97% identity) per species. In our *Cinachyrella*, *Poribacteria* were only classified as two OTUs. This lower diversity related to *Poribacteria* might be distinctive of *Cinachyrella* because only four OTUs were present in *C. autraliensis* specimens from open ocean habitats in Indonesia and similar to our SG1, *Poribacteria* were undetected in specimens collected from nearby marine lakes (Cleary et al., 2013).

Chloroflexi also was below detection limits in SG1. This again might be typical of LMA sponges as the *Chloroflexi* were absent in LMA sponges from the Red Sea, the Caribbean Sea and the South Pacific Ocean and present in low numbers in other LMA sponges (Schmitt et al., 2011; Giles et al., 2013). In SG2, *Chloroflexi* sequences were grouped into 12 OTUs, close to the range (14–21 OTUs) Schmitt et al. (2011) reported for HMA sponges, but lower than the 502 OTUs (97% identity) retrieved from another 32 sponge species (Schmitt et al., 2012).

Giles et al. (2013) studied the microbiomes in six species of LMA sponges using clone libraries and found that the phyla *Acidobacteria, Chloroflexi* and *Gemmatimonadetes* were not detected. Here, SG1 samples also were missing these phyla (with the exception of three sequences of *SAR202-Chloroflexi* and two sequences in the *Gemmatimonadetes*). These three bacterial phyla were also missing in eight of the 13 species analyzed by Jeong et al. (2013). The other five species contained a high microbial diversity with a large proportion of *Chloroflexi* (this group was called the CF group because of the *Chloroflexi*).

We also found a large portion of unclassified *Proteobacteria* in the sponge, but not in the seawater suggesting that it was not a consequence of the analysis. In the sponges *Raspailia ramosa* and *Stelligera stuposa,* 32 and 17% of the *Proteobacteria* sequences, respectively, were unclassified as opposed to only 1% in the seawater (Jackson et al., 2012). Further exploration suggests that many of our unclassified *Proteobacteria* OTUs are sponge-specific and the presence of large clusters of sponge-specific and sponge- and coral-specific bacteria in the invertebrates have been described (Simister et al., 2012). Interestingly, our results related to *Proteobacteria* were similar to Cleary et al. (2013). In their study, *Alphaproteobacteria* were more abundant in *C. australiensis* from marine lakes than open ocean habitats. In our *Cinachyrella* samples, *Alphaproteobacteria* were significantly more abundant in the SG1 than SG2. These might again be typical of some LMA sponges as Kamke et al. (2010) also recovered a large portion of *Alphaproteobacteria* clones from LMA sponges.

Cinachyrella symbionts also belonged to the Archaea (6.9–18.5%), in proportions within the wide range recorded for four deep water (4–65%) and three shallow water sponges from the Red Sea (4–28%) (Lee et al., 2011; Kennedy et al., 2014). All of the archaeal sequences in *Cinachyrella* fell within two phyla: *Thaumarchaeota* and *Euryarchaeota*, with most of the archaea belonging to the *Thaumarchaeota*, which is widespread in sponges (Webster et al., 2001; Margot et al., 2002; Lee et al., 2011; Kennedy et al., 2014; Polónia et al., 2014). Archaeal reads grouped into a low number of OTUs, with a few numerically dominant ones, similar to the four species sampled by Kennedy et al. (2014), which had 70% of the *Thaumarchaeota* sequences separated in three OTUs. The phylum *Thaumarchaeota* includes AOA performing the first step of nitrification using ammonium excreted by sponges as a metabolic waste product (Jiménez and Ribes, 2007; Bayer et al., 2008; Hoffmann et al., 2009). Ammonia oxidation by archaea is believed to be widespread in marine environments (Francis et al., 2005; Könneke et al., 2005; Schleper et al., 2005) and was detected both the LMA and HMA sponges (Schläppy et al., 2010). In addition to the AOA, nitrite-oxidizing bacteria catalyzing the second step of nitrification were found in SG2. Hentschel et al. (2002) detected early on clones affiliated with nitrite-oxidizing phylum *Nitrospirae* in sponges. The proportion of this phylum varies greatly between host species, ranging from 0.6% in *X. testudinaria* from the Red Sea (Lee et al., 2011) to 24% in *Stelligera stuposa* from Irish waters (Jackson et al., 2012). Overall, in the present study, it appears that only one group of *Cinachyrella* (SG2) harbors the microbes required for both steps of nitrification.

THE TWO SPONGE GROUPS ONLY SHARE A SMALL CORE MICROBIOME

Symbionts in SG1 and SG2 were very different at the OTU level with both groups only sharing a small core microbial community as seen in many sponges. For example, *C. australiensis* from open ocean habitat and marine lakes only shared 9.4% of their OTUs (Cleary et al., 2013), lower than the percentage shared between SG1 and SG2. In contrast, the sponge genus *Xestospongia* often showed exceptionally high overlap in OTUs. For example, *X. muta* (collected from Florida) and *X. testudinaria* (from Indonesia) shared 85% of the reads (=245 OTUs) between the two species (Montalvo et al., 2014). However, after surveying 32 sponge species, Schmitt et al. (2012) concluded that phylogeny of the host (i.e., how closely related sponges were) did not correlate with the bacterial composition. Similarly, host sponge

phylogeny—except for the genus *Xestospongia*—did not affect the similarity of the symbionts communities in sponges from Orpheus Island (Webster et al., 2013). Nevertheless, when triplicate individuals of the same species (including *Cinachyra* sp.) were analyzed, conserved (>65% similarity) microbial communities were observed (Webster et al., 2013). This is consistent with the pyrosequencing characterization of *A. corrugata* symbiont communities in S. Florida (White et al., 2012), which showed relatively high similarities among multiple individuals and across hundreds of km. In *Cinachyrella*, the numbers of shared OTUs between SG1 samples (12–24%) and SG2 samples (39–62%) was low. Giles et al. (2013) and Schmitt et al. (2012) suggest environmental factors such as temperature, salinity or nutrient levels might impact symbionts population structures. In their study, species from tropical waters had more similar bacterial communities. This did not hold true at a smaller scale as we observed distinct communities in the two sponge groups from the same environment, independent of spatial or temporal scales.

Considering many sponges (including *Cinachyrella*) have a reduced core and large variable microbial community, it would be reasonable to assume that different OTUs perform distinct functions within the sponge. However, using a metagenomic approach, a recent study showed that taxonomically divergent sponges can harbor phylogenetically diverse symbionts with functional equivalence (Fan et al., 2012). The authors were able to show that six sponge species possess similar functional profiles distinct from the ones obtained for the seawater microbial communities (Fan et al., 2012). These findings suggest that key functions in marine sponges might be performed by different microbial taxa and a phylogenetically similar "core microbial community" may therefore not be essential to meet the sponge requirements. Moreover, perhaps the concept of a "core" microbiome, for *Porifera* at least, may have to be redefined altogether to emphasize *function* over symbiont identity. This view may not be so far fetched when considering that bacteria can often drastically change their metabolic activities through horizontal gene transfers (Costa et al., 2009).

Together with recent and ongoing molecular microbiome analyses of adjacent coastal waters and reef invertebrate hosts (unpublished), this study contributes to a growing spatio-temporal profile of microbiome dynamics in subtropical South Florida (Negandhi et al., 2010; White et al., 2012). These results also help provide a baseline characterization for *Cinachyrella*, which may be developed for further experimental studies, due to its hardiness in aquaculture, relative ease of collection and maintenance.

AUTHOR CONTRIBUTIONS

Marie L. Cuvelier, Emily Blake, Rebecca L. Vega Thurber, Peter J. McCarthy, and Jose V. Lopez designed research; Marie L. Cuvelier, Emily Blake, and Jose V. Lopez performed sampling; Marie L. Cuvelier performed DNA extractions and 16S rRNA amplicon preparation; Emily Blake performed sponge taxonomy analysis; Rebecca Mulheron performed 28S rRNA PCR; Marie L. Cuvelier, Rebecca L. Vega Thurber, and Jose V. Lopez analyzed data; Marie L. Cuvelier, Emily Blake, Peter J. McCarthy, Patricia Blackwelder, Rebecca L. Vega Thurber, and Jose V. Lopez wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00581/abstract

Supplementary Figure 1 | Rarefaction curves (note: SW Feb is under the Sp6 Feb line).

Supplementary Figure 2 | PCoA analysis of weighted UniFrac distance. UniFrac measure phylogenetic distances between OTUs sets within a phylogenetic tree. Here, we used weighted UniFrac, which takes into account relative abundances of OTUs (as opposed to presence/absence only). 64 sponge individuals were used in total and Sp1 through Sp6 are labeled.

Supplementary Figure 3 | Number of OTUs, abundance and classification of all the sequences present in the seawater (Oct and Feb), Sponge Group 1 (Sp1 Oct, Sp4 Feb, Sp5 Feb, Sp6 Feb) and Sponge Group 2 (Sp2 Oct and Sp3 Oct). Each OTU is classified at the lowest ranking.

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Phylogenetic signal in the community structure of host-specific microbiomes of tropical marine sponges

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Sponges (Porifera) can host diverse and abundant communities of microbial symbionts that make crucial contributions to host metabolism. Although these communities are often host-specific and hypothesized to co-evolve with their hosts, correlations between host phylogeny and microbiome community structure are rarely tested. As part of the Earth Microbiome Project (EMP), we surveyed the microbiomes associated with 20 species of tropical marine sponges collected over a narrow geographic range. We tested whether (1) univariate metrics of microbiome diversity displayed significant phylogenetic signal across the host phylogeny; (2) host identity and host phylogeny were significant factors in multivariate analyses of taxonomic and phylogenetic dissimilarity; and (3) different minimum read thresholds impacted these results. We observed significant differences in univariate metrics of diversity among host species for all read thresholds, with strong phylogenetic signal in the inverse Simpson's index of diversity (*D*). We observed a surprisingly wide range of variability in community dissimilarity within host species (4–73%); this variability was not related to microbial abundance within a host species. Taxonomic and phylogenetic dissimilarity were significantly impacted by host identity and host phylogeny when these factors were considered individually; when tested together, the effect of host phylogeny was reduced, but remained significant. In our dataset, this outcome is largely due to closely related host sponges harboring distinct microbial taxa. Host identity maintained a strong statistical signal at all minimum read thresholds. Although the identity of specific microbial taxa varied substantially among host sponges, closely related hosts tended to harbor microbial communities with similar patterns of relative abundance. We hypothesize that microbiomes with low *D* might be structured by regulation of the microbial community by the host or by the presence of competitively dominant symbionts that are themselves under selection for host specificity.

Keywords: coevolution, community ecology, diversity, microbial symbioses, phylogeny, Porifera

INTRODUCTION

Marine sponges are globally distributed and perform critical ecological functions in benthic ecosystems (Rützler, 2012; Van Soest et al., 2012). Sponges are active participants in the carbon, nitrogen, and sulfur cycles, performing aerobic and anaerobic processes that benefit the broader community (Taylor et al., 2007; Schläppy et al., 2010; Maldonado et al., 2012; Schöttner et al., 2013). In addition, sponges play a critical role in pelagic–benthic coupling, transferring pelagic carbon and nitrogen to benthic food webs (Lesser, 2006; De Goeij et al., 2013). The diverse communities of microbial symbionts hosted by marine sponges are hypothesized to be the primary drivers of these essential nutrient cycles (Maldonado et al., 2012; Thacker and Freeman, 2012). For example, approximately one-third of Caribbean reef sponges host photosynthetic symbionts (Erwin and Thacker, 2007) that convert dissolved inorganic carbon to organic molecules that are available to heterotrophs, including the sponge host (Freeman and Thacker, 2011). The diversity of sponge-associated microbiomes is unmatched by other invertebrate hosts, such that their complexity is frequently compared to that of mammalian gut microbiomes (Webster et al., 2010; Reveillaud et al., 2014).

Sponges can be broadly classified into two groups based on the abundance of their associated microbial communities. High microbial abundance (HMA) sponges contain diverse and abundant microbial communities that are distinct from the microbial communities found in the surrounding seawater (Hentschel et al., 2003). HMA sponges are also characterized by lower pumping rates and a higher frequency of hosting photosynthetic symbionts (Weisz et al., 2007). Conversely, low microbial abundance (LMA) sponges contain significantly lower abundances of associated microbes that tend to be more similar to the microbial communities found in the surrounding water column (Erwin et al., 2011; Giles et al., 2013). LMA sponges are also characterized by higher pumping rates, with a higher rate of heterotrophic feeding on particulate organic matter (Weisz et al., 2008; Schläppy et al., 2010; Freeman and Thacker, 2011).

Recent work has blurred this distinction between HMA and LMA sponges, emphasizing instead the presence of "core" microbial taxa within symbiotic communities, containing microbiota that are widely shared across diverse sponge hosts, "variable" microbial taxa shared by at least two sponge species, and "host-specific" microbial taxa that are reported from a single sponge species (Schmitt et al., 2011). Studies using a variety of microbial community fingerprinting techniques [such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphisms (TRFLPs), and automated ribosomal intergenic spacer analysis (ARISA)] as well as clone library sequencing have reported a high degree of host-specificity in both HMA and LMA sponges (Anderson et al., 2010; Erwin et al., 2011, 2012a; Pita et al., 2013; Schöttner et al., 2013; Olson et al., 2014). Quantitative analyses of ARISA data revealed a significant association between microbiome similarity and host sponge species and families (Schöttner et al., 2013), indicating that these communities have likely co-evolved with their hosts. Next generation sequencing (NGS) approaches have increased the precision and quantity of information sampled from sponge-associated microbial communities (Schmitt et al., 2011; Webster and Taylor, 2012; Reveillaud et al., 2014). Multiple studies employing NGS approaches have also demonstrated that sponge microbiomes are largely host-specific, though some seasonal, environmental, and geographic variation has been noted within host species (Hardoim et al., 2012; White et al., 2012; Cleary et al., 2013).

Sponge-specific bacteria, defined as bacterial lineages found only in sponges and not in ambient seawater or sediments, were initially identified through clone library sequencing, but have also been documented using NGS approaches (Taylor et al., 2007, 2012). Together with the direct observation of vertical transmission of some microbial symbionts, these sponge-specific lineages provide additional evidence for co-evolution, and potentially co-speciation, between host sponges and their microbial symbionts (Thacker and Freeman, 2012). However, NGS approaches have also reported "sponge-specific" bacterial lineages from seawater (Taylor et al., 2012). Likewise, more thorough analyses of GenBank sequences have indicated that several bacterial taxa thought to be specific to sponges also occur in other habitats, such as sediment and in other host organisms (Simister et al., 2012; Taylor et al., 2012). While the absolute "sponge-specific" nature of these taxa is debatable, with a recent study suggesting the use of the term "sponge-enriched" instead (Moitinho-Silva et al., 2014), most studies have found the sponge host to be the single strongest influence on the composition of the associated bacterial community (Lee et al., 2010; Webster et al., 2010; Schmitt et al., 2011).

NGS datasets are often extremely large and difficult to manipulate using standard computing power. Limiting the size of the dataset can help remove error and noise, but can also remove meaningful information about rare members of the microbiome (Sogin et al., 2006; Huse et al., 2010). In addition, investigators quantifying the "rare biosphere" have reported evidence of host-specificity even in the extremely rare members of the sponge microbiome (Reveillaud et al., 2014). This pattern holds true even for LMA species, in which a single microbial lineage can dominate host-species-specific microbiomes (Giles et al., 2013).

In the current study, we characterized the diversity and dissimilarity of microbiomes associated with 20 species of tropical marine sponges to test whether host phylogeny significantly impacts symbiotic microbial community structure. We assessed host phylogenetic relatedness using DNA sequences obtained by the Porifera Tree of Life project (Redmond et al., 2013); this approach contrasts with previous comparative studies of sponge microbiomes that relied on taxonomic names to describe host relatedness (Schmitt et al., 2011; Schöttner et al., 2013). We focused our investigation over a relatively narrow geographic range to limit potential biogeographic effects on microbiome community structure. First, we tested whether univariate measures of the diversity of symbiotic microbial communities displayed significant phylogenetic signal across the host phylogeny. Second, we examined both host identity and host phylogenetic relatedness as factors in multivariate analyses of both taxonomic and phylogenetic dissimilarity among microbiomes to determine whether host relatedness influences microbiome community structure in addition to host identity. Finally, we investigated how measures of diversity and dissimilarity change when using different read count thresholds.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DNA EXTRACTION

We collected tissues from 100 sponge specimens representing 20 host species (5 specimens per species) by snorkeling and using SCUBA at several shallow dive sites near Bocas del Toro, Panama, between 2006 and 2012 (Supplementary Table 1). Species identities were confirmed by microscopic examination of morphological characters (Hooper and van Soest, 2002). Samples were collected into sterile bags, then preserved in 95% ethanol at the Smithsonian Tropical Research Institute (STRI) and stored at 4°C until extraction. DNA was extracted from combined ectosomal and choanosomal tissue using the PowerSoil DNA isolation kit (MoBio Laboratories, Inc.), following the standard EMP protocol (http://www.earthmicrobiome.org/emp-standard-protocols/dna-extraction-protocol/).

NEXT-GENERATION SEQUENCING

Sequencing of the samples in our study was completed in collaboration with other researchers as part of the EMP (http://www.earthmicrobiome.org/). Our collaborators at EMP amplified and sequenced the V4 region of the 16s rRNA gene using the bacterial/archaeal primer pair 515F/860R and following previously published methods (Caporaso et al., 2012). Amplicons were fused to Illumina barcodes and sequencing was completed on an Illumina platform.

QUALITY CONTROL, FILTERING, AND TAXONOMIC ASSIGNMENTS

Raw sequences were quality-filtered (average quality score = 30, window size = 5 bases, maximum number of homopolymers = 8) and trimmed to a minimum length of 100 base pairs. We removed 10 samples from our dataset that did not meet these quality standards. We aligned the sequences to a trimmed SILVA database (v102, trimmed to the V4 region 11894–25319; Schloss et al., 2009). The aligned sequences were then checked for chimeras, removing all that were found. Sample sequences

were then classified based on the SILVA reference database, with a minimum cutoff of 60% identity. The classified sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity cutoff, yielding a data table containing each sample and its respective OTUs.

We extracted the 90 samples specific to our study from the full EMP dataset using four custom Perl scripts (Supplementary File 1). We used the first script (matchRows.pl) to extract specific rows (those containing the pertinent samples) from the full EMP dataset based on user-provided criteria. We used the second script (RemoveColumnByThreshold.pl) to remove all columns with a column sum of zero from the extracted rows (i.e., deleting OTUs that were not found in samples specific to the current study). Since the second script allowed users to set any value for column sums, we also used this script to reduce the dataset to specific sequence read thresholds. We used the third script (SavedOTUdatabase.pl) to match the new OTU occurrence matrix with the OTU database file obtained from mothur, generating a reduced OTU database file, which contained the OTU identifier, the OTU sequence, and the taxonomic classification of each OTU. Finally, we used a fourth script (deleteSpecificColumns.pl) to remove metadata columns not needed for analyses in *R*.

We performed statistical analyses on three versions of the same dataset, using minimum OTU read thresholds (i.e., the minimum number of reads required for a particular OTU to be included in the dataset) of 1, 5 (Supplementary File 2), and 500 reads. To reduce the size of the dataset, and remove noise and potential error, we focus the remainder of the text on the analysis of the dataset with a minimum read threshold of 500 reads. The use of minimum read threshold values is considered a more conservative approach to standardizing an NGS dataset compared to approaches such as regularization and convex minimization (Dunn et al., 2013).

MICROBIAL COMMUNITY DIVERSITY

Using the R package vegan (Oksanen et al., 2014), we converted OTU abundance to relative abundance to minimize the possibility of false positives in our analyses (McMurdie and Holmes, 2014). We also used vegan to calculate three univariate measures of the diversity of the microbial community associated with each host specimen: OTU richness (*S*), the Shannon–Weaver index (H'), and the inverse Simpson's index (*D*). We compared these metrics among host species using analyses of variance (ANOVA).

PHYLOGENETIC RECONSTRUCTIONS

We constructed a phylogeny of sponge hosts by obtaining sequences of the gene encoding the small subunit (18S) of nuclear ribosomal RNA for each host species from GenBank (Supplementary Table 2). We aligned the sequences using the default options of MAFFT 7.017 (Katoh et al., 2002), as implemented in Geneious 6.1.6 (Biomatters Limited). We constructed the host phylogeny by implementing a relaxed-clock model in MrBayes version 3.2.1 (Ronquist et al., 2012), employing the computational resources of iPLANT (Goff et al., 2011). The options set in MrBayes included constraining the clade containing the genera *Aiolochroia, Aplysina,* and *Chondrilla* (all members of subclass Myxospongiae) as an outgroup and implementing the independent gamma rate relaxed clock model with a birth–death process. This analysis included three parallel runs of 10 million generations, each using four Markov chains and sampling every 100 generations. We assessed convergence of the chains by examining the average standard deviation of split frequencies, which reached a value of 0.003. Following a burn-in of 25%, we summarized the output of the three runs as a consensus phylogeny.

To enable analyses of microbiome phylogenetic dissimilarity, we constructed a maximum likelihood phylogeny of bacterial OTUs. We aligned OTU sequences using the default options of MAFFT and constructed the phylogeny using Fasttree2 (Price et al., 2010), as implemented by iPLANT, using the default settings.

PHYLOGENETIC SIGNAL

Phylogenetic signal describes the degree to which more closely related organisms share more similar traits (Blomberg et al., 2003). We used the phylosignal function of the R package picante (Kembel et al., 2010) to test whether *D* displayed significant phylogenetic signal given the host sponge phylogeny (i.e., whether more similar values were associated with more closely related hosts more often than expected by chance).

TAXONOMIC AND PHYLOGENETIC DISSIMILARITY

We calculated microbial community taxonomic dissimilarity among specimens using the Bray–Curtis index of dissimilarity (BCD). We calculated mean BCD among specimens within host species to assess the variability of microbiomes within host species. We compared these values between LMA and HMA sponges using a *t*-test, designating LMA/HMA status based on previous studies (Weisz et al., 2007).

We used the R package picante (Kembel et al., 2010) to calculate phylogenetic dissimilarity among microbiomes, which reflects the genetic variation among the microbial OTUs present in each community. This analysis was only conducted on the two reduced datasets, as the original dataset yielded a phylogenetic distance matrix that exceeded the integer limit of R. We used the adonis function of the R package vegan (Oksanen et al., 2014) to quantify the impact of host species identity on BCD and phylogenetic dissimilarity. Since adonis could not simultaneously treat host identity and host phylogeny as factors, we used Mantel tests to assess the correlation between each of these individual factors and BCD, as well as a partial Mantel test to assess the effect of host phylogeny on BCD given host identity. We conducted similar Mantel tests to examine the correlations between host identity, host phylogeny, and microbial phylogenetic dissimilarity.

We calculated the percentage contribution to BCD of specific OTUs using SIMPER (Oksanen et al., 2014) for only the 390 OTUs present given a minimum threshold of 500 reads. Since SIMPER can only perform pairwise comparisons, the microbial community of each host species was compared to the microbial community of the remaining hosts pooled together, thereby contrasting an individual host species to all other hosts species and placing emphasis on the OTUs unique to each host. The output of this analysis revealed the percentage contribution of each OTU to this contrast. When employing lower minimum read thresholds,

individual microbial OTUs excluded by the 500 read threshold contributed nearly zero percent to host species contrasts.

EFFECT OF READ THRESHOLDS

After filtering the dataset by using minimum read threshold values of 1, 5, 10, 50, 100, 500, 1000, and 5000, we used ANOVA to calculate the F-ratio associated with variation in S among host species. We used a polynomial regression to test whether these F-ratios were significantly related to threshold values.

REPRODUCIBILITY OF ANALYSES

All statistical analyses were performed in R v. 3.1.1. Supplementary File 3 contains a set of R commands that allow the user to reproduce all of the analyses described in this manuscript.

RESULTS

The raw data for this EMP study are available at http://www.earthmicrobiome.org/. From the starting set of 100 sponge specimens, 90 specimens met all quality control standards, yielding 88,395 unique OTUs (defined as 97% sequence similarity) representing 20 bacterial phyla (based on SILVA classification), with a maximum of 8357 unique OTUs in a single host specimen. Minimum thresholds of 5 and 500 reads per OTU yielded 21,395 and 390 unique OTUs, respectively. Proteobacteria was the most abundant microbial phylum, accounting for approximately 47 % of all unique OTUs, consistent with previous studies investigating sponge microbial communities (**Figure 1**). Other notably abundant phyla included Actinobacteria, Chloroflexi, and Cyanobacteria. A few host species displayed surprisingly low phylum-level diversity, including *Iotrochota birotulata*, *Tedania ignis*, and *Lissodendoryx colombiensis*, while others hosted high phylum-level diversity, including the verongid species *Aiolochroia crassa*, *Aplysina cauliformis*, and *Aplysina fulva*. Classification of these microbial communities according to the criteria of Schmitt et al. (2011) revealed that only 1.5% of the community consisted of "core" taxa and only 11% could be considered "host-specific" taxa. The majority of the microbial community in our sample set occurred in several host species, but not ubiquitously. Interestingly, ten of the twenty host species contained no species-specific microbial OTUs, including four of the seven HMA species. In addition, within some host species, a relatively large percentage of OTUs were not classified when referencing the SILVA database. For example, at a minimum threshold of 500 reads, 91 of 390 OTUs (23%) were not classified. After referencing the Greengenes database (DeSantis et al., 2006), 10 of these 91 reads could be classified as Archaea, 67 as Bacteria, and 14 remained unclassified.

At a minimum threshold of 500 reads, the mean OTU richness (S) of sponge microbiomes ranged from 811 in *T. ignis* to 5263 in *Erylus formosus* (**Table 1**; summaries for minimum thresholds of 1 and 5 reads are presented in Supplementary Tables 3, 4, respectively). Comparisons among host species revealed significant differences in S, (ANOVA: $df = 19$, $F = 17.82$, $P < 0.001$), H' (ANOVA: $df = 19$, $F = 24.46$, $P < 0.001$), and D (ANOVA: $df = 19$, $F = 14.31$, $P < 0.0001$). We observed

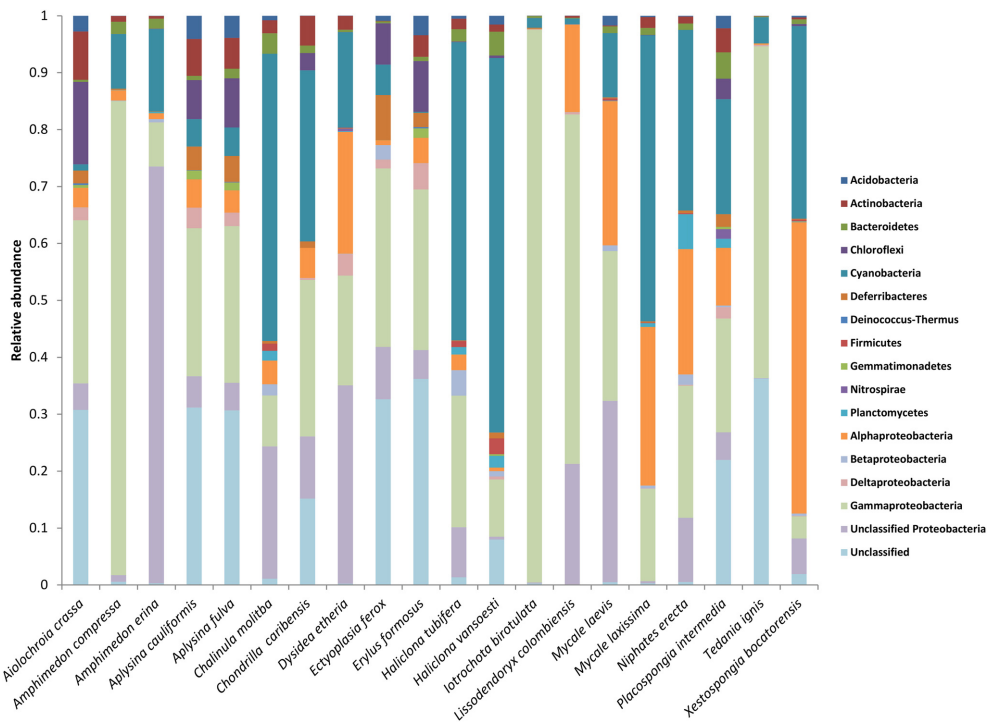

FIGURE 1 | Mean relative abundance of microbial taxa within each host species. Microbial phyla are displayed to the right of the chart, with the phylum Proteobacteria split into classes.

Table 1 | Mean ± standard error of univariate measures of microbiome diversity for each host species, analyzed with a minimum threshold of 500 reads, and defining OTUs by 97% sequence similarity.

Species	*S*	*H′*	*D*	Within-host BCD	SIMPER OTUs	*n*
Aiolochroia crassa	132.2 ± 3.88	4.09 ± 0.07	39.91 ± 5.21	33.7 ± 7.9	25	5
Amphimedon compressa	70.4 ± 6.02	1.17 ± 0.28	1.76 ± 0.28	16.2 ± 5.1	1	5
Amphimedon erina	84.2 ± 18.74	0.91 ± 0.51	2.34 ± 1.2	33.7 ± 19.2	2	5
Aplysina cauliformis	162.4 ± 3.50	4.39 ± 0.05	54.5 ± 5.38	26 ± 6	27	5
Aplysina fulva	150.6 ± 4.11	4.24 ± 0.04	47.95 ± 3.69	27.7 ± 6.6	25	5
Chalinula molitba	61.67 ± 1.45	2.77 ± 0.12	9.14 ± 1.06	24.1 ± 10.8	9	3
Chondrilla caribensis	68.4 ± 9.10	2.71 ± 0.15	9.24 ± 2.35	29.2 ± 9.1	12	5
Dysidea etheria	90 ± 14.32	2.49 ± 0.30	8.16 ± 2.69	66.4 ± 15.8	15	5
Ectyoplasia ferox	95.2 ± 3.31	3.44 ± 0.07	21.98 ± 2.15	24.6 ± 6.2	17	5
Erylus formosus	172.2 ± 6.16	4.38 ± 0.12	53.48 ± 10.69	29.7 ± 7	30	5
Haliclona tubifera	76.25 ± 10.09	2.38 ± 0.46	7.38 ± 2.19	52.5 ± 18.3	9	4
Haliclona vansoesti	54 ± 1.00	2.95 ± 0.26	11.35 ± 5.52	23.9 ± 19.5	11	2
Iotrochota birotulata	66 ± 5.02	0.4 ± 0.11	1.13 ± 0.04	4 ± 1.5	1	4
Lissodendoryx colombiensis	64 ± 10.28	1.2 ± 0.08	2.42 ± 0.25	42.4 ± 18.5	4	5
Mycale laevis	76.8 ± 9.65	2.06 ± 0.26	5.72 ± 2.03	48.8 ± 15.6	6	5
Mycale laxissima	82.8 ± 1.85	2.11 ± 0.18	4.26 ± 0.61	51.8 ± 13.2	8	5
Niphates erecta	79.2 ± 4.65	2.61 ± 0.12	7.72 ± 1.03	41.4 ± 10.4	10	5
Placospongia intermedia	69.25 ± 14.05	2.75 ± 0.47	15.15 ± 7.62	73 ± 21.8	17	4
Tedania ignis	49 ± 4.38	1.08 ± 0.11	2.33 ± 0.34	35.5 ± 9.9	3	5
Xestospongia bocatorensis	62 ± 3.61	2.02 ± 0.50	5.95 ± 2.90	35.9 ± 17.8	6	3

S, OTU richness; H′, Shannon index; D, inverse Simpson index; within-host BCD, intraspecific percentage Bray–Curtis dissimilarity; SIMPER OTUs, number of OTUs explaining 40% of Bray–Curtis dissimilarity; n, sample size.

significant differences in these univariate metrics for all read thresholds (Supplementary File 2). A plot of mean OTU richness vs. the inverse Simpson index (**Figure 2**) provided a visualization of the substantial variation in these metrics among host species. Four HMA host species with high values of these metrics were clearly separated from a cluster of LMA host species with low values; however, two HMA host species (*Chondrilla caribensis* and *Xestospongia bocatorensis*) were similar to the LMA host species. Notably, both of these host species contain abundant populations of photosynthetic bacteria (Erwin and Thacker, 2007).

The reconstructed phylogeny of host species (**Figure 3**) was a well-supported subset of the phylogeny presented by Redmond et al. (2013). We found significant phylogenetic signal in *D* ($K = 0.591$, $P = 0.003$, **Figure 3**), with three representatives of order Verongida (*A. cauliformis*, *A. crassa*, and *A. fulva*), along with *E. formosus* (order Astrophorida), all displaying relatively high values of *D*, while five representatives of order Poecilosclerida (*I. birotulata*, *L. colombiensis*, *Mycale* spp., and *T. ignis*) all displayed very low values of *D*.

We observed a wide range (4–73%) of within-host-species variability in BCD (**Table 1**); surprisingly, this variability was not related to the HMA or LMA classification of the host species (mean ± SE, HMA: 29.5 ± 1.5, LMA: 39.5 ± 5.4; $t = 1.768$, $df = 14$, $P = 0.099$). LMA or HMA classification also had no effect on the number of unique OTUs found in a particular host species (mean ± SE, HMA: 2.4 ± 1.3, LMA: 1.9 ± 0.6; $t = 0.35$, $df = 9$, $P = 0.737$). We visualized variation in community structure among host species using both a heat map of the relative abundance of the 100 most abundant OTUs (**Figure 4**) and a hierarchical clustering dendrogram displaying average linkages among host species (**Figure 5**). Analysis using adonis provided strong support for the effect of host identity on BCD (adonis: $df = 19$, $F = 10.241$, $R^2 = 0.735$, $P < 0.001$). We also used adonis to perform a *post-hoc* comparison of three verongid hosts (*A. cauliformis*, *A. crassa*, and *A. fulva*) that contained visually similar communities (**Figure 4**). Despite the high phylogenetic relatedness of these hosts, and their similar values of *D* (**Figure 3**), the microbiomes of these three host species displayed highly significant differences in BCD (adonis: $df = 2$, $F = 4.62$, $R^2 = 0.435$, $P < 0.001$). Analysis using Mantel tests found that, when tested individually, host identity (Mantel: $r = 0.422$, $R^2 = 0.178$, $P < 0.001$) and host phylogeny (Mantel: $r = 0.602$, $R^2 = 0.362$, $P < 0.001$) each explained a significant amount of variability in BCD. Testing the effect of host phylogeny given host identity greatly reduced the explanatory power of phylogenetic relatedness, but remained significant (Partial Mantel: $r = 0.182$, $R^2 = 0.033$, $P < 0.001$).

The phylogeny of microbial OTUs (Supplementary File 4) constructed for analyses of phylogenetic dissimilarity is not the true phylogeny of these microbial taxa, but instead represents the genetic variation present in the microbial communities and is appropriate for analyses of beta-diversity (Hamady and Knight, 2009). Microbial phylogenetic dissimilarity was significantly impacted by host identity (adonis: $df = 19$, $F = 57.541$, $R^2 = 0.940$, $P < 0.001$, **Table 2**). Analysis using Mantel tests revealed that when tested individually, host identity (Mantel: $r = 0.331$, $R^2 = 0.109$, $P < 0.001$) and host phylogeny (Mantel: $r = 0.382$, $R^2 = 0.146$, $P < 0.001$) each explained a significant

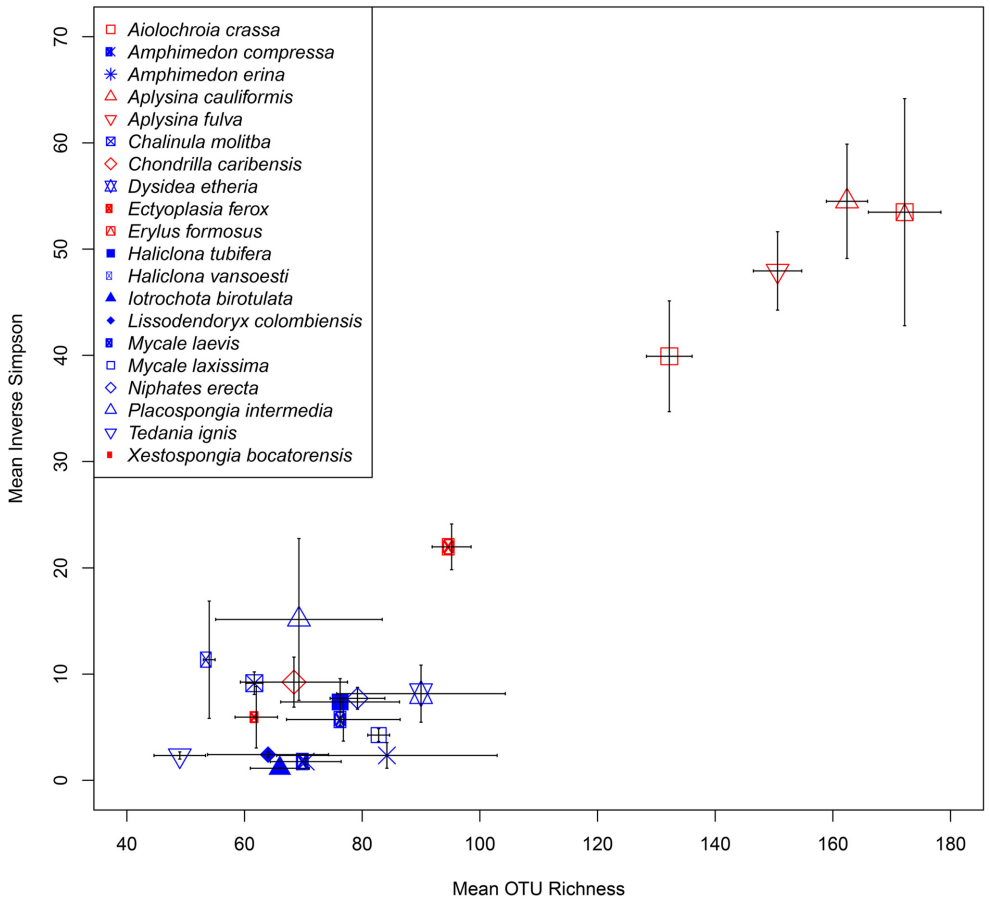

FIGURE 2 | Scatterplot of mean (± SE) OTU richness (*S*) and inverse Simpson's index (*D*) for each sponge host. High microbial abundance (HMA) and low microbial abundance (LMA) classifications are displayed as red and blue symbol colors, respectively.

amount of phylogenetic dissimilarity. Testing the effect of host phylogeny given host identity reduced the explanatory power of phylogenetic relatedness, but remained significant (Partial Mantel: $r = 0.268$, $R^2 = 0.072$, $P < 0.001$). BCD and phylogenetic dissimilarity are not necessarily independent of one another, and these two metrics were significantly correlated (Mantel test, $r = 0.660, R^2 = 0.436, P < 0.001$). SIMPER analyses revealed the OTUs specific to each host species that were the primary drivers of the observed differences in BCD and phylogenetic dissimilarity (**Supplementary Figure 1**). The number of bacterial taxa comprising 40% of the observed BCD variation ranged from 1 to 30 OTUs among host species and reflected observed trends in *D* (**Table 1**).

We observed significant differences in *S* among host species across an array of minimum read thresholds, and the *F*-ratio of this test was significantly impacted by minimum read threshold (polynomial regression: $df = 2$, $F = 24.03$, $P = 0.006$; **Supplementary Figure 2**). In all cases, *S* displayed a significant amount of variability among hosts, indicating that the effect of host identity on *S* is robust. The significance of the *F*-ratio varied among minimum read thresholds across all three diversity indices, but was most substantial when comparing *S*. The effect of host species identity was highest for all diversity metrics at a minimum threshold equal to or greater than 500 reads (Supplementary File 2).

DISCUSSION

Previous researchers have used a wide variety of techniques to document that sponge-associated microbial communities are largely host specific (e.g., Erwin et al., 2012a; Reveillaud et al., 2014), but host phylogenetic relatedness has only rarely been included as a specific factor influencing microbiome community structure (Schöttner et al., 2013). Our analysis of the microbiomes of 20 host taxa over a narrow geographic range adds further evidence to the high degree of host specificity observed in these microbial communities. Host identity and host phylogeny were each significant individual influences on Bray–Curtis dissimilarity (BCD) and phylogenetic dissimilarity; however, when examined together, host identity explained much more variance than host phylogeny. In our dataset, this outcome is largely due to closely related host taxa harboring extremely different microbiomes. Despite these striking differences in microbial community composition, one aspect of community structure, the inverse Simpson index of diversity (*D*), displayed significant

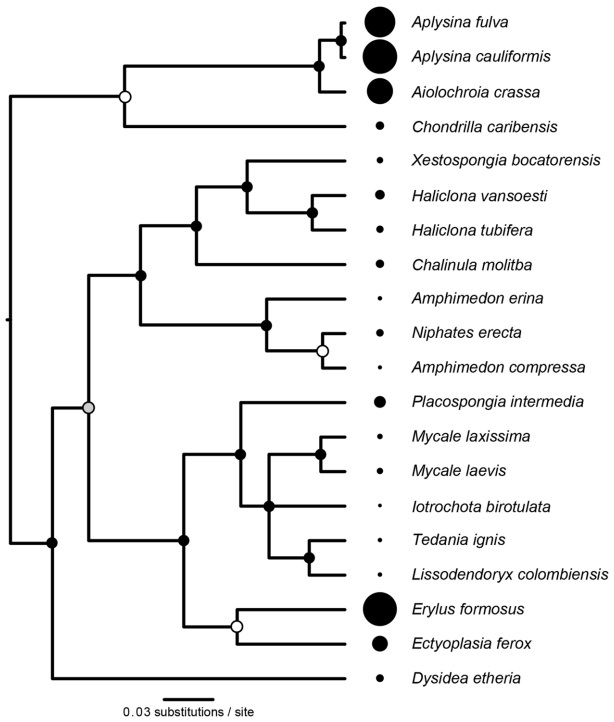

FIGURE 3 | Phylogenetic signal in the inverse Simpson's index (*D*). The phylogeny of host sponge species is based on 18S rRNA gene sequences. The scale bar indicates the number of nucleotide substitutions per site, while circles at the nodes of the phylogeny indicate percentage Bayesian posterior probabilities (PP): black, 100% PP; gray, 95–99% PP; white, <95% PP. Circles at the tips of the phylogeny are sized in proportion to the average value of the inverse Simpson's index (*D*) for each host (**Table 1**), which displayed significant phylogenetic signal ($K = 0.591$, $P = 0.003$).

phylogenetic signal across the host phylogeny. *D* is frequently described as an index of dominance because it is most strongly influenced by the relative abundance of the most common taxa in a community (Magurran and Magurran, 1988; Haegeman et al., 2014). Thus, although the identity of specific microbial OTUs varied substantially among host sponges, more closely related sponge species tended to harbor microbial communities with more similar patterns of relative abundance and dominance.

Early studies of sponge-microbe associations investigated fewer host species and used methods such as clone library construction that identified fewer OTUs per host; however, several of these early studies proposed the hypothesis of a uniform microbial community associated with sponges (Hentschel et al., 2002, 2006; Hill, 2004; Montalvo and Hill, 2011). Later studies proposed the occurrence of sponge-specific "sequence clusters" in phylogenies of microbial taxa (Taylor et al., 2007; Thiel et al., 2007; Webster et al., 2010; Simister et al., 2012), indicating that, in many cases, sponge-associated bacteria were found in monophyletic groups. Continued work on this topic has provided strong support for the hypothesis that microbial communities are largely host-species-specific (Taylor et al., 2004; Erwin et al., 2012b; Pita et al., 2013), while placing less emphasis on the occurrence of sponge-specific lineages and instead describing these taxa as "sponge-enriched" (Taylor et al., 2012; Moitinho-Silva et al., 2014). The host-specific nature of sponge-associated microbial communities is now well-established, and next-generation sequencing techniques continue to document this specificity in an increasing number of host taxa (Lee et al., 2010; Schmitt et al., 2011; Reveillaud et al., 2014).

In a strict sense, the terms HMA and LMA refer to the abundance of microbes resident within a sponge host, but these terms are often used to infer characteristics of diversity and microbial specificity (Weisz et al., 2007), with HMA sponges being associated with highly diverse communities (Schmitt et al., 2008; Erwin et al., 2012a) and highly specific communities (Hentschel et al., 2003; Schläppy et al., 2010; Gerçe et al., 2011). Furthermore, several LMA sponges have previously been hypothesized to be more reflective of the surrounding environment than HMA sponges (Weisz et al., 2007; Erwin et al., 2011). In our study, we were surprised to observe strong host specificity even in sponges characterized as LMA species. Giles et al. (2013) also found a large amount of specificity in LMA hosts. Our investigation demonstrated that several LMA sponge species harbor communities with moderately high OTU richness, while some HMA species host microbiomes with considerably lower OTU richness. The two HMA species hosting the lowest OTU richness, *C. caribensis* and *X. bocatorensis*, both host dense populations of photosynthetic cyanobacteria (*Synechococcus spongiarum* and *Oscillatoria spongeliae*, respectively; Thacker and Freeman, 2012). However, it is unclear whether these photosymbionts can structure the remainder of the microbiome, since two other HMA species hosting *S. spongiarum* (*A. cauliformis* and *A. fulva*) displayed among the highest values of OTU richness. In addition, some LMA hosts displayed extremely low values of *D*, indicating that these sponges were not hosting a random microbial assemblage; instead, there seems to be strong evolutionary selection for some sponge lineages to host an extremely specific microbial community that is dominated by a relatively low number of OTUs. These results are similar to those of Poppell et al. (2013), who used DGGE banding patterns to assess diversity in a set of 8 HMA and 7 LMA species and observed significantly lower diversity (and values of *D*) in the LMA species.

We employed multivariate approaches to further explore the nature of these microbial associations. High levels of community dissimilarity are often noted between host sponges (Lee et al., 2010; Reveillaud et al., 2014), and although not often directly tested, dissimilarity often decreases within taxonomic and phylogenetic groupings. This observation is also suggestive of a phylogenetic signal in the structuring of microbiomes. Schöttner et al. (2013) tested this idea directly and noted a significant effect of host species and family on the types of microbial taxa found in specimens of species within the family Geodiidae. When testing the influence of phylogenetic or taxonomic relatedness, it is most appropriate to either test taxonomic groups as nested factors or to use a phylogenetic or taxonomic distance matrix as a factor (Kembel et al., 2010). We used adonis to assess the impact of host identity on microbiome community and phylogenetic dissimilarity, finding that this factor accounted for the majority of variation in these measures. However, adonis could not simultaneously estimate the impact of host identity and host

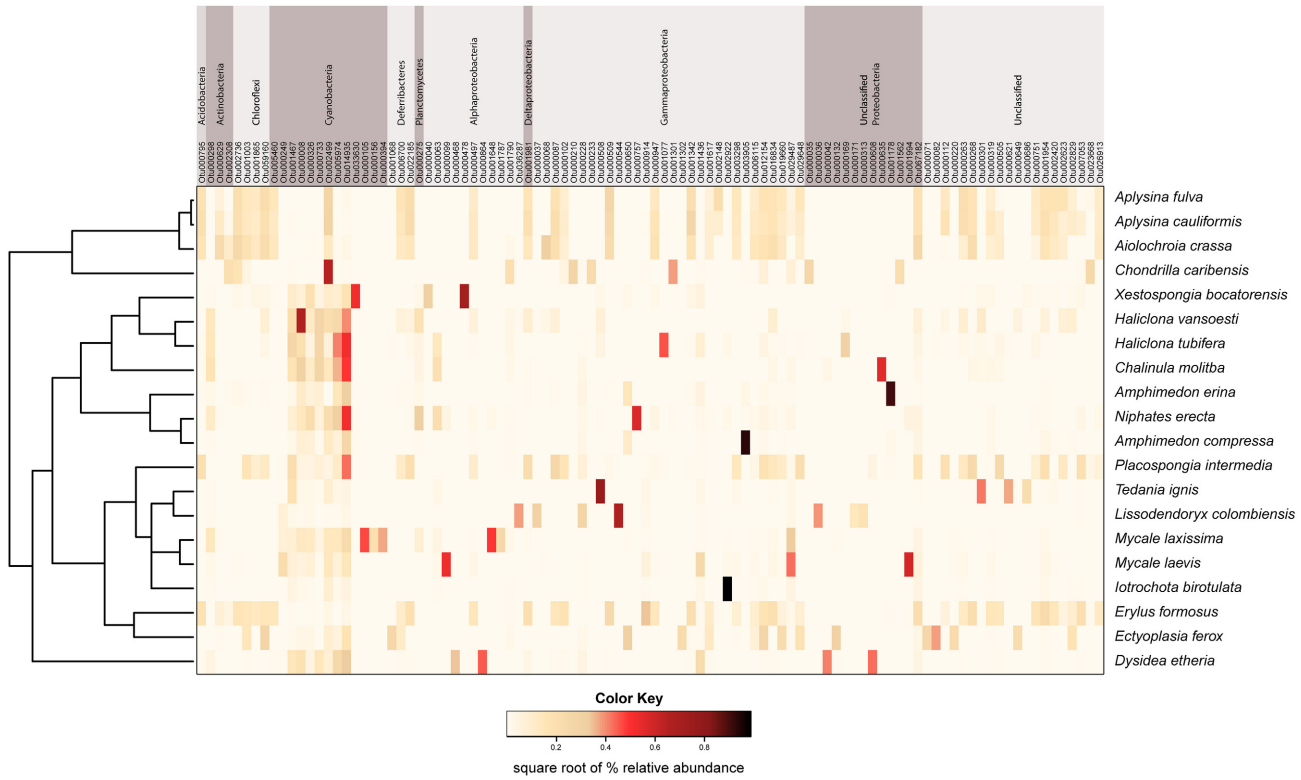

FIGURE 4 | Mean relative abundance heat map of the 100 most abundant microbial OTUs in each host sponge species. These data are square-root transformed for ease of visualization. OTUs are grouped by phylum across the top of the figure, with the exception of Proteobacteria, which is split into classes. The host sponge phylogeny is displayed to the left of the heat map for ease of reference.

phylogeny (or host relatedness). To assess the relative impact of these factors, we used a partial Mantel test, finding that host phylogeny explained very little variation in community dissimilarity after accounting for host identity. These data suggest that the strong selective forces for divergent microbiome community composition remain strong even among closely related hosts, suggesting that symbiotic microbes might play critical roles in niche differentiation among host species.

We observed an extremely wide range of intraspecific variability in community structure, with some LMA species displaying less than 5% BCD and others displaying more than 50% BCD among individuals. Surprisingly, this range was not correlated with the HMA or LMA classification of the host species. Thus, although some LMA species with extremely high intraspecific variability might be more reflective of the surrounding environment, other LMA species appear to be under strong selective pressures to limit membership in their microbiomes. Furthermore, our sampling strategy focused on representing both ectosomal and choanosomal tissue from each specimen. Species with high intraspecific variability, such as *Dysidea etheria* and *Placospongia intermedia* (**Table 1**), might reflect zonation of microbial symbionts among microhabitats within the host. Future studies could explicitly test this hypothesis of microbiome zonation by carefully excising distinct tissue layers and cell types.

SIMPER analysis of BCD highlighted the wide variation in host-microbial associations. Host species with high values of *D* harbored more even communities, where no one OTU accounted for a large proportion of the BCD (**Supplementary Figure 1**). Conversely, some host species were dominated by one or a few microbial taxa, and these specific OTUs contributed to a large proportion of the contrast of BCD among species (**Supplementary Figure 1**). Indeed, the 5 highest proportional contributions of single OTUs were observed in 5 LMA species (*A. compressa, A. erina, I. birotulata, L. colombiensis,* and *T. ignis*). Importantly, these proportional contributions are not necessarily related to unique membership in a particular community. Though all of the sponge species in our study hosted significantly dissimilar communities, half of these species possessed no "species-specific" microbial taxa. Given the strong statistical signal for host identity, our results suggest that the observed significant dissimilarity among host species was largely driven by differences in relative abundance, with each host species harboring specific microbial assemblages rather than strictly unique OTUs. This pattern was also reported by an earlier study by Erwin et al. (2012a), which described this type of community structure as a "specific mix of generalists."

We found that host identity maintained a strong statistical signal at all minimum read thresholds tested in our study. The significance of host identity decreased with lower minimum read thresholds, revealing that the microbial OTUs that distinguish hosts from one another, although not necessarily unique to a particular host, are often among the most dominant members

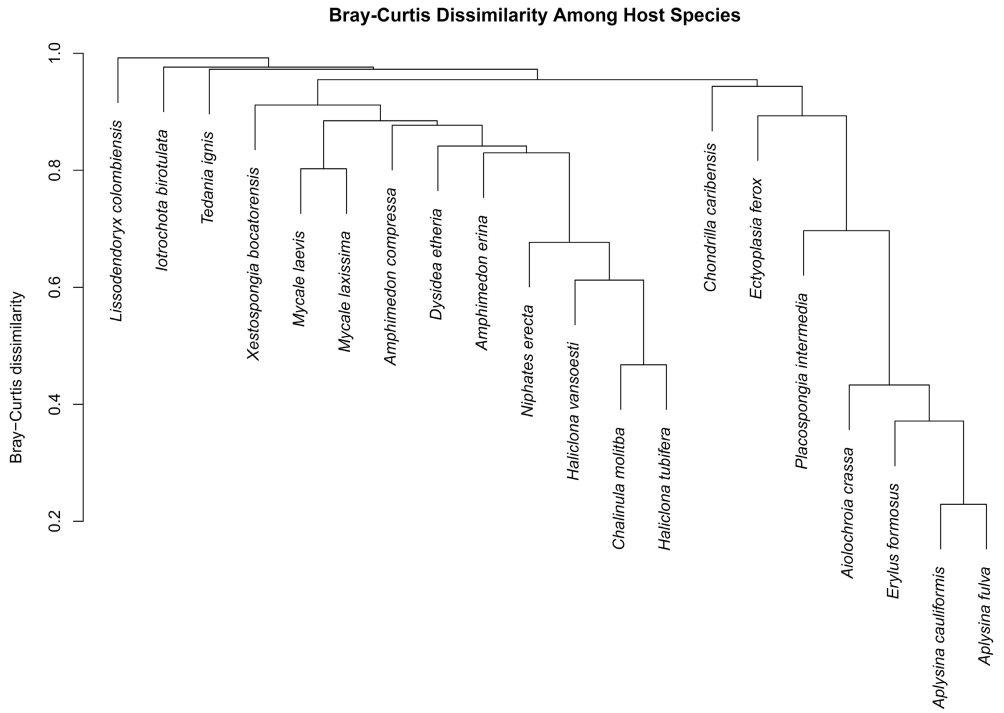

FIGURE 5 | Dendrogram displaying Bray–Curtis dissimilarity among host sponge species. Host species exhibited varying degrees of dissimilarity from one another, but all hosts were significantly different from each other (adonis: $df = 19$, $F = 10.241$, $R^2 = 0.735$, $P < 0.001$).

Table 2 | Analyses of Bray–Curtis dissimilarity and phylogenetic dissimilarity among host species using the *R* function adonis.

	df	Sum of squares	Mean squares	*F*-ratio	R^2	*P*-value
BRAY–CURTIS DISSIMILARITY						
Host species identity	19	28.47	1.498	10.241	0.735	<0.001
Residuals	70	10.242	0.146		0.265	
PHYLOGENETIC DISSIMILARITY						
Host species identity	19	1.72162	0.091	57.541	0.940	<0.001
Residuals	70	0.166	0.0023		0.088	

of their community. This finding suggests that removing OTUs with lower abundance reduced noise in our dataset, likely due to the presence of microbes found more broadly in the community. Additionally, increasing the minimum read threshold added confidence to our analysis by ensuring that the observed taxa are of biological origin, and not a product of error (Reveillaud et al., 2014). The relevance of rare microbial OTUs in large NGS datasets is still an area of much debate and the use of minimum read thresholds is considered a conservative way to reduce false positives while maintaining the majority of the biological diversity (Dunn et al., 2013).

In addition to the statistical advantages of using minimum read thresholds, some practical issues must be considered when analyzing NGS datasets, since these data are often extremely large and are potentially unmanageable without significant computing power. In our dataset, limiting the minimum read threshold to 5 reads reduced the number of OTUs by 76%. This reduction not only reduced the amount of computing power needed to process these data, but it also permitted us to conduct community phylogenetic analyses in *R*. Although the full dataset generated phylogenetic distance matrices that far exceeded *R*'s current integer limit (R Development Core Team, 2008), analyses of the reduced dataset still exceeds most standard computing power. Our study made use of the cyber-infrastructure provided by iPlant to perform analyses on a super-computing platform. As Internet-based tools such as iPlant become more widely available, these limitations will become less important, but the practical processing of these large datasets remains a challenge today.

Our results lead us to consider the designations LMA and HMA to reflect two ends of a continuum in sponge microbiome community structure. Although the four highest values of *S* and *D* were found in four of the seven HMA species in our study, two HMA species displayed very low values of *D*. Both of these sponges host photosymbionts, so these low values of *D* potentially reflect strong selection for the nutritional benefits received from these partners (Thacker and Freeman, 2012). Similar host selection for symbiont-derived benefits might also occur for other microbial OTUs in LMA sponges that display lower values of *D*. We observed strong phylogenetic signal for *D*, but BCD and phylogenetic dissimilarity were influenced more by host species identity than host phylogenetic relatedness. In contrast to previous studies, we found a low number of species-specific microbial OTUs, as well as an unexpectedly large range of intraspecific variation in BCD. In future research on these microbiomes, these metrics of community structure can be used in combination with

microbial abundance to assess trends in the evolution of microbiomes. Based on our current dataset, broad-scale microbial diversity within a host sponge appears to be strongly influenced by host phylogeny, but the specific members of each host's microbial community appear to be structured by unique interactions within each host species.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00532/abstract

Supplementary Figure 1 | Mean percentage contribution of the 103 OTUs contributing at least 40% of the SIMPER contrast of Bray–Curtis dissimilarity among host species. These data are square-root transformed for ease of visualization. OTUs are grouped by phylum across the top of the figure, with the exception of Proteobacteria, which is split into classes. The host sponge phylogeny is displayed to the left of the heat map for ease of reference.

Supplementary Figure 2 | *F*-ratio of OTU richness across host species plotted against an array of minimum read thresholds. The *F*-ratio was significantly influenced by minimum read threshold (polynomial regression: $df = 2$, $F = 24.03$, $P = 0.006$).

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Spatial variability of microbial assemblages associated with a dominant habitat-forming seaweed

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Macroalgal surfaces support abundant and diverse microorganisms within biofilms, which are often involved in fundamental functions relating to the health and defense of their seaweed hosts, including algal development, facilitation of spore release, and chemical antifouling. Given these intimate and important interactions, environmental changes have the potential to negatively impact macroalgae by disrupting seaweed–microbe interactions. We used the disappearance of the dominant canopy-forming fucoid *Phyllospora comosa* from the metropolitan coast of Sydney, NSW, Australia as a model system to study these interactions. We transplanted *Phyllospora* individuals from nearby, extant populations back onto reefs in Sydney to test whether bacterial assemblages associated with seaweed surfaces would be influenced by (i) the host itself, independently of where it occurs, (ii) the type of habitat where the host occurs, or (iii) site-specific differences. Analyses of bacterial DNA fingerprints (terminal fragment length polymorphisms) indicated that assemblages of bacteria on *Phyllospora* were not habitat-specific. Rather, they were primarily influenced by local, site-specific conditions with some evidence for host-specificity in some cases. This could suggest a lottery model of host-surface colonization, by which hosts are colonized by 'suitable' bacteria available in the local species pool, resulting in high variability in assemblage structure across sites, but where some species in the community are specific to the host and possibly influenced by differences in host traits.

Keywords: seaweed-microbe interaction, biofilm, colonization, succession

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Introduction

Marine macroorganisms live in persistent contact with diverse microorganisms that are abundant and ubiquitous in the surrounding seawater (Reinheimer, 1992) and within biofilms on their surfaces (Wahl, 1989). Although our understanding of the functional importance of biofilm-associated microorganisms in the lives of the higher organisms they live upon is still evolving (Egan et al., 2008), emerging evidence points to their fundamental involvement in the development, functioning, and defense of diverse macroorganisms (e.g., Armstrong et al., 2001; Lindquist et al., 2005; Wahl et al., 2012).

The phylogenetic structure of biofilm assemblages often has a high degree of organismal- (e.g., Longford et al., 2007), species- (e.g., Taylor et al., 2004), and tissue-specificity (e.g., Thiel et al., 2007;

Campbell et al., 2011; Fernandes et al., 2012). More recent work has also provided evidence for functional redundancy within microbial consortia in biofilms (Burke et al., 2011a), where various combinations of phylotypes are capable of providing a core set of functions as required by the host (Burke et al., 2011b).

Functional redundancy within microbial biofilms could be beneficial to host organisms that rely on them for development, function, or defense, if a disturbance disrupts the composition of a biofilm, because lost functions could be restored by functionally (but not necessarily taxonomically) similar microbes available in the local species pool. Marine ecosystems are undergoing rapid change on a global scale, with rates of ocean warming higher than on land (Burrows et al., 2011), and worldwide increases in coastal development (Small and Nicholls, 2003; Bullieri and Chapman, 2010) leading to widespread degradation in coastal marine ecosystems (Jackson, 2001; Lotze et al., 2006; Airoldi and Beck, 2007). Environmentally mediated impacts have already affected diverse marine macroorganisms at multiple spatial scales, including species distributional range shifts (Parmesan, 2006), habitat fragmentation (Goodsell et al., 2007), higher incidences of disease (Harvell et al., 1999), and species extinctions (Sala and Knowlton, 2006). Evidence suggests that environmental change is also having profound and alarming effects on the abundance (Sarmento et al., 2010), distribution (Cook et al., 2011), and function (Wohlers et al., 2009) of planktonic marine microorganisms. However, we know almost nothing about how environmental change can affect the composition or function of epibiotic, biofilm-associated microorganisms (Wahl et al., 2012).

Much of the available information on environmental impacts on host–biofilm interactions comes from studies into coral holobionts (Mouchka et al., 2010). The composition (Ben-Haim et al., 2003; Bourne et al., 2007) and functional gene profile (Thurber et al., 2009) of coral-associated microbial assemblages can change with changes in the environment. Higher temperatures are often correlated with the detection of pathogenic strains within coral-associated microbial communities (Ben-Haim et al., 2003; Bourne et al., 2007), the production of lytic compounds (Ben-Haim et al., 2003), or the up-regulation of genes involved in pathogenicity (Thurber et al., 2009). The involvement of bacteria (and other microorganisms) in coral bleaching and subsequent coral reef decline is now a major focus of environmental microbiology, given the ecological and socio-economic importance of these ecosystems.

On temperate coasts, macroalgae are the dominant habitat-forming primary producers, playing analogous ecological roles to corals on tropical reefs (Steneck et al., 2002). Similar to corals, large, canopy-forming macroalgae are declining from many temperate rocky reefs (e.g., Steneck et al., 2002; Airoldi and Beck, 2007; Connell et al., 2008; Wernberg et al., 2011), but these systems receive far less attention than their tropical counterparts. Consequently, our understanding of the importance of bacteria to macroalgae and the effects of environmental change on macroalgal–bacterial interactions is less well developed (see Egan et al., 2012; Minich and Dinsdale, 2014). However, recent work highlights the importance of bacteria to the development (e.g., Marshall et al., 2006), function (e.g., de Oliveira et al., 2012), and defense (Egan et al., 2008) of some seaweeds. Like corals, there is evidence that seaweed-associated bacterial communities can be species-specific (Lachnit et al., 2009), fluctuate seasonally (Lachnit et al., 2011), and as a function of host condition (Campbell et al., 2011; Fernandes et al., 2012).

When investigating microbes associated with a green alga (*Ulva australis*) Burke et al. (2011a) found a high degree of within-species variability with respect to biofilm composition, but conservation of the functional gene profiles of microbial assemblages among samples. Their findings suggest macroalgae like *U. australis* rely on functions provided by their surface biofilms. Thus, any environmentally mediated disruption to the biofilm could have negative impacts for the macroalga, if lost functions cannot be restored rapidly. Although seasonally correlated changes in macroalgal-associated biofilms have been recorded (Lachnit et al., 2011) and some laboratory experiments have demonstrated that the composition of biofilms associated with macroalgae are affected by environmental factors [e.g., temperature, Stratil et al. (2013); and salinity, Stratil et al. (2014)], these ideas have not been tested experimentally in the field.

We experimentally investigated how changing an alga's environment affected the composition of its surface biofilms, using a large, brown fucoid alga, *Phyllospora comosa* (hereafter '*Phyllospora*'). Like many other large canopy-forming macroalgae, *Phyllospora* is showing signs of decline and has disappeared from *ca.* Seventy kilometers of coastline adjacent to the metropolitan area of Sydney, NSW, Australia's largest city, but remains dominant on shallow subtidal reefs north and south of the city (Coleman et al., 2008). The local disappearance of this species was linked to poor water quality in the region due to sewage pollution. Since the 1980s, sewage treatment has improved and deep ocean outfalls have been constructed, which has vastly enhanced water quality in the region (Scanes and Philip, 1995). Despite this improvement, *Phyllospora* has failed to recover and remains absent in this area. Genetic analyses of populations of *Phyllospora* north and south of this range fragmentation suggest a high degree of connectivity and genetic exchange across its gap in distribution. We hypothesized that environmental conditions adjacent to Sydney might affect *Phyllospora*-associated biofilms and that this may be one factor that has contributed to its failure to re-establish naturally in the region. To investigate this, we transplanted *Phyllospora* individuals from extant populations back onto reefs within the Sydney region and compared the bacterial communities that developed.

Materials and Methods

Field Experiments

To determine whether bacterial communities on *P. comosa* individuals were (i) host-specific (i.e., that all *Phyllospora* individuals will have similar communities, regardless of where they occur or are moved to); (ii) influenced by a particular environment (i.e., will change when they are moved from a '*Phyllospora*' to a 'non-*Phyllospora*' habitat); or (iii) site-specific (i.e., they will change when they are moved to a particular place, regardless of whether it is a '*Phyllospora*' habitat or not), we transplanted adults from two extant populations on the periphery of Sydney

(donor habitats) into two physically similar reef habitats within metropolitan Sydney where *Phyllospora* has been absent for several decades (recipient habitats; Coleman et al., 2008; Campbell et al., 2014).

Full details of the experimental design and procedure can be found in Campbell et al. (2014) but briefly, the donor populations on the periphery of Sydney were in Cronulla (Cr; 34°03′23″ S 151°09′23″ E) and Palm Beach (PB; 33°35′58″ S 151°19′43″ E). Shallow rocky reefs at these places are characterized by a mosaic of patches of *Phyllospora* forests (size-range: 7–40 m^2), barrens, turfing corallines, and 'fringe' habitats, with few individuals of the kelp *Ecklonia radiata*. The recipient habitats in Sydney were in Long Bay (LB; 33°57′58″ S 151°15′27″ E) and Cape Banks (CB; 33°59′57″ S 151°14′52″ E). Reefs at these recipient sites are very similar to those in donor places, except that patches of *Ecklonia* forests are more abundant and *Phyllospora* forests are absent. Collections and transplantations were carried out under a Scientific Collection Permit (# P00/0054-6.0) issued to the authors by the New South Wales Department of Primary Industries (Fishing and Aquaculture).

Experiments were done twice. In the first experiment (February 28 to May 9, 2011), 40 adults were collected haphazardly (collected individuals were typically 1–3 m apart) at the same depth (1–2 m) from each donor habitat by carefully detaching the holdfast from the substratum. Individuals were kept in 50 L containers with seawater for ∼2–3 hs during transportation until reattachment.

Phyllospora individuals from the two donor habitats were randomly allocated to one of three treatments (as per Campbell et al., 2014): (i) Transplanted individuals ('TP'; $n = 20$), which were moved to one of the recipient habitats in Sydney (individuals from Cronulla were moved to Long Bay, while those from Palm Beach were moved to Cape Banks); (ii) Disturbed individuals ('D'; $n = 10$), which were disturbed in the same manner as required for transplantation, but were returned to their original donor habitat; or (iii) Translocated individuals ('TL'; $n = 10$), which were similarly disturbed, but were taken to the other donor site (i.e., an environment in which extant, natural populations of *Phyllospora* persist – from Cr to PB and *vice versa*). Undisturbed individuals ('U'; $n = 20$) were haphazardly selected and marked *in situ* at each donor site but otherwise not handled. Disturbance and translocation treatments allowed for us to distinguish between the effects of transplantation to a different environment from the possible effects of the transplantation procedure or the effects of simply moving the algae from one place to another, regardless of environment (Marzinelli et al., 2009).

Algae that were removed from the substratum (TP, D, and TL individuals) were re-attached using cable-ties to 0.25 m^2 plastic meshes, which were 0.5–2 m apart and had been previously attached to bare rock in barren patches approximately at 1–2 m depth. Five individuals were attached to each mesh to approximate natural densities (mean density 6.7 ± SE 1.1 per 0.25 m^2), creating a patch of ∼4–5 m^2 area at each place. After 2 months, one blade from each of 2–5 *P. comosa* individuals from each treatment at each location was sampled. Sampling size varied among treatments because several individuals were lost during the experiment. Blades were selected at random from the algae and cut approximately 30 cm from the tip of the blade. Each blade was sealed underwater in an individual plastic, press-seal bag. Blades were taken to the surface, rinsed with filter-sterilized seawater to remove any unattached epibionts (Millipore 0.2 μm filter) and using a sterile cotton swab, microbial assemblages from the algal surfaces were sampled (approximately 10 cm^2 of thallus surface was gently swabbed for 30 s). The cotton tip of each swab was aseptically transferred into individual sterile 2.0 ml cryogenic storage tubes. Tubes were closed the flash frozen onsite in liquid nitrogen then stored at −80°C until processing.

The experiment was repeated in late winter/spring (started August 9, 2011). In this second experiment, algae from both donor populations were transplanted to each recipient site to test for differences between algae from different sources at the same destination. Sixty algae were collected from each donor place (Palm Beach and Cronulla) and randomly assigned to three treatments: (i) individuals TP to Long Bay ($n = 20$ from each donor site), (ii) individuals TP to Cape Banks ($n = 20$ from each donor site), (iii) TL individuals to the other donor place ($n = 20$). U individuals ($n = 20$) were haphazardly selected and marked *in situ* (four sub-patches of five individuals each to resemble replication in the other treatments). Algae were attached to meshes as described above. Total patch-sizes ranged between 4 and 8 m^2 at each place. Bacteria on blades from each of five individuals were sampled after 5 months (January 17, 2012) as described above.

DNA Fingerprinting of *Phyllospora*-Associated Bacterial Assemblages

To compare the composition of bacterial assemblages from *Phyllospora* in different treatments, we used a polymerase chain reaction (PCR) based DNA fingerprinting technique (terminal restriction fragment length polyorphisms [TRFLP; Liu et al., 1997]). TRFLP is popularly used by molecular ecologists to characterize and compare the composition and diversity of microbial communities (Walker et al., 2004), theoretically with a species resolution, but typically to the widely accepted 'operational taxonomic unit' (OTU; Nocker et al., 2007). To carry-out a TRFLP analysis, phylogenetic marker genes within the sample DNA were amplified using PCR with a fluorescent dye attached to the 5′ end of the forward primer. PCR products were then digested using restriction enzymes, which resulted in DNA fragments of variable length. These fragments were then physically separated in sequencing capillaries and the labeled terminal fragments were detected using a laser, producing an electropherogram. A size-standard labeled with a different fluorophore was also analyzed, allowing the fragment lengths to be estimated with a resolution of one lase pair (Liu et al., 1997; Nocker et al., 2007). Each OTU is represented by a different fragment length and thus the composition and diversity of the community can be estimated based on the polymorphism of the terminal restriction fragment lengths from a sample of community DNA. Swab samples were thawed on ice. DNA from each sample was extracted and isolated using the Powersoil DNA Isolation Kit (Mo Bio Laboratories #12888-100) then stored in a −20°C freezer.

PCR and Fragment Analysis

A fragment (∼500 bp) of the bacterial 16S rRNA gene was amplified in each sample using the community DNA as a template. Primers 27F and 519R (sequences 5′-AGAGTTT GATCMTGGCTCAG-3′ and 5′-GWATTACCGCGGCKGCTG-3′, respectively), which encompass the 16S Variable regions V1–V3, were used. Primers were fluorescently labeled on the 5′ end with phosphoramidite dyes (27F labeled with 6-FAM and 519R labeled with VIC; Applied Biosystems).

The PCR reaction mixtures (25 μl) contained 5 pmol of the labeled 27F and 519R primers, 12.5 μl Econotaq 2x MasterMix (Lucigen), 3 μl of community template DNA, and molecular grade H_2O. DNA amplification was performed with a PCR Express thermal cycler (Thermo Hybaid) using the following program: a 3 min start at 94°C, 30 cycles consisting of 94°C denaturation for 30 s, 56°C annealing for 30 s, and 72°C extension for 5 min. The program continued with a final extension at 72°C for 5 min. Successful amplification was verified by gel-electrophoresis of 2 μl PCR products on 1% agarose gels with gel red, visualized under UV-light. PCR products were purified and concentrated using the DNA Clean and Concentration -5 Kit (Zymo Research, D4014). Purified product was eluted in 12 μl molecular grade H_2O and quantified using a nano-drop ND1000 (Thermo Scientific).

Terminal fragment length polymorphisms was conducted using the restriction enzyme HAEIII (NE Biolabs) and standard methodology. Fragments were visualized by fluorophore color of 6-FAM (blue) and VIC (green). TRFLP data was first analyzed using Peak Scanner (Applied Biosystems). Fragment size was determined by comparison to internal size standard Liz-600, and fragments of <30 or >600 bp were excluded. Using the program T-REX (BMC Bioinformatics), background noise was removed to distinguish true fragments from background fluorescence.

Statistical Analyses

Bacterial TRFs data were compared among *Phyllospora* from different treatments using permutational multivariate analyses of variance (Anderson, 2001) with the PERMANOVA add-on in PRIMER v6 (Anderson et al., 2007). Similarity matrices based on Bray–Curtis distances of square-root transformed relative abundances or on Jaccard distances (presence/absence) were generated for the analyses, which used 9,999 permutations of residuals under a reduced model. Multivariate dispersion, which is an estimate of variance used to test for homogeneity among groups, was also compared among treatments (for both relative abundances and presence/absence of TRFs) using the PERMDISP analysis within the PERMANOVA add-on in PRIMER v6 (Anderson et al., 2007). *P*-values were calculated using 9,999 permutations. To visualize multivariate patterns in bacterial TRFs assemblages, non-metric multi-dimensional scaling (nMDS) was used as an ordination method using PRIMER v6 (Clarke and Gorley, 2006).

To test hypotheses about influences of the host, the environment, or the sites on surface-associated bacterial communities, we analyzed the data from the point of view of the origin of the algae and also their destination. Analyses from the point of view of the origin of the algae had two factors: Treatment, which was fixed with four levels (first experiment: U, D, TL, TP; second experiment: U, TL, TP to LB, TP to CB), and Place of origin, random, with two levels (Cr, PB). In addition, comparisons of surface-associated bacteria between algae that ended up in the same place of destination were also conducted. Analyses from the point of view of the destination of the algae in the first experiment had two factors: Treatment, fixed with three levels (U, D, TL), and Place of destination, random with two levels (Cr, Pb). Two analyses from the point of view of the destination were done for the second experiment. The first analysis was similar to that of the first experiment, with two factors: Treatment, fixed with two levels (U, TL), and Place of destination, random with two levels (Cr, Pb). The second analysis compared bacteria on algae originally from different donor populations that ended up in the same Sydney metro destination: treatment was a fixed factor with two levels (TP from PB, TP from Cr), and Place of destination was random with two levels (CB, LB). All analyses are detailed in each Table.

The model that surface-associated bacterial communities are influenced by the host leads to the prediction that the structure and composition of bacterial communities on translocated algae to sites within the *Phyllospora* habitat and on those transplanted to the Sydney metro habitat will not differ from undisturbed algae at the site of origin. Under the model of differences in the environment, we predict that bacterial communities on transplanted algae to the Sydney metro habitat will differ from those translocated to sites within the *Phyllospora* habitat, which will remain similar to undisturbed algae at the site of origin. Finally, the model that communities are site-specific leads to the prediction that the communities on translocated algae to sites within the *Phyllospora* habitat and on those transplanted to the Sydney metro habitat will differ from each other and from undisturbed algae at the site of origin, and will become similar to algae in the same place of destination.

Results

We detected a total of 795 microbial TRFs in this study. TRF sizes were searched against the RDP and SILVA databases using MICA (Shyu et al., 2007). No fragment was found to correspond with the predicted size of chloroplast or plastid 16S rRNA genes contained within the two databases. This indicates that the TRFLP profiles are not contaminated by host DNA or epiphytic algae and thus contain only bacterial or archaeal 16s rRNA gene information. In the first experiment, the structure and composition of bacterial fingerprints differed among treatments, but these differed according to the transplants place of origin (i.e., there was Treatment × Place interaction, **Table 1A**). Bacteria on algae transplanted from Palm Beach to Sydney metro differed from those on individuals that remained in Palm Beach undisturbed, on disturbed individuals returned to Palm Beach and on those individuals translocated to Cronulla. Although bacteria on translocated algae differed from those on undisturbed algae, there were no differences between the translocated and disturbed treatments (**Table 1A**). Algae originally from Cronulla had different bacterial TRFLP across all treatments (**Table 1A**; **Figure 1**).

TABLE 1 | PERMANOVAs based on Bray–Curtis (BC) similarity measure for square-root transformed relative abundances or Jaccard similarity measure (composition) of bacterial TRF profiles associated with *Phyllospora* in the first experiment from the point of view of (A) the origin of the algae or (B) the destination.*

		Square-root transformed BC			Jaccard		
Source	*df*	MS	pseudo-*F*	*p*(perm.)	MS	pseudo-*F*	*p*(perm.)
(A) Origin							
Treatment (Tr)	3	2456	1.06	0.426	3149	0.95	0.569
Place (Pl)	1	1608	2.13	**0.002**	2462	1.68	**0.004**
Tr × Pl	3	2326	3.08	**<0.001**	3324	2.27	**<0.001**
Residual	26	756			1463		
Pairwise tests		PB: TP ≠ TL = D ≠ U Cr: TP ≠ TL ≠ D ≠ U			PB: TP ≠ TL = D ≠ U Cr: TP ≠ TL ≠ D ≠ U		
(B) Destination							
Tr	2	2041	1.35	0.291	2728	1.23	0.330
Pl	1	2272	3.13	**<0.001**	3942	2.74	**<0.001**
Tr × Pl	2	1517	2.09	**0.001**	2226	1.55	**0.002**
Residual	18	725			1440		
Pairwise tests		PB: TL = D ≠ U Cr: TL ≠ D ≠ U			PB: TL = D ≠ U Cr: TL = U ≠ D		

(A)* *Treatment was fixed with four levels (U, Undisturbed; D, Disturbed; TL, Translocated; TP, Transplanted), Place of origin was random with two levels (CR, Cronulla; PB, Palm Beach).* *(B)*** *Treatment was fixed with three levels (U, D, TL), Place of destination was random with two levels (Cr, PB). The replicates were the Phyllospora individuals (n = 2–5). P-values were calculated using 9,999 permutations under a reduced model. Bold indicates statistical significance (at alpha = 0.005).*

When we compared bacterial fingerprints from the point of view of the site of destination of transplants, algae that ended up in PB differed from those that ended up in Cr (**Table 1B**). At each destination place, bacteria on translocated algae were, however, different from those on undisturbed algae, except at PB where translocated algae originally from Cr did not differ from disturbed algae (which originated in Palm Beach; **Table 1B**). There were no differences in dispersion of the structure or composition of bacterial TRFs among treatments (PERMDISP: $F_{3,30} = 1.92$, $p = 0.24$; $F_{3,30} = 1.18$, $p = 0.49$, respectively; **Figure 1**).

In the second experiment, bacterial TRFs on algae originally from Palm Beach differed across all treatments. The same trend was observed for bacteria on algae originally from Cronulla, although pairwise comparisons could not resolve where these differences occurred and hence there was a significant Treatment × Place interaction (**Table 2**; **Figure 2**).

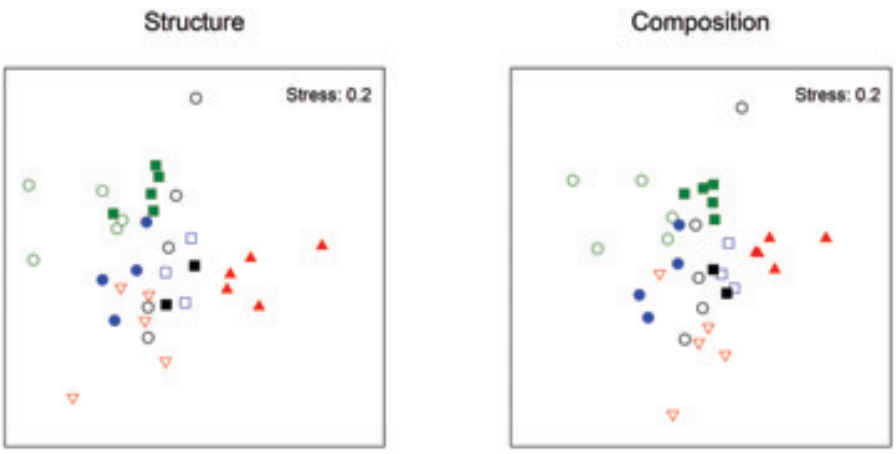

FIGURE 1 | nMDS based on Bray-Curtis measure of square-root transformed relative abundances (structure) or Jaccard measure (composition) of bacterial TRFLP on *Phyllospora comosa* originally from Cronulla (empty symbols) or Palm Beach (filled symbols) in the first experiment. Treatments: Undisturbed (green symbols), Disturbed (black symbols), Translocated (blue symbols), Transplanted (red symbols). Destination places: Cronulla (circles), Palm Beach (squares), Long Bay (downward triangle), Cape Banks (upward triangle).

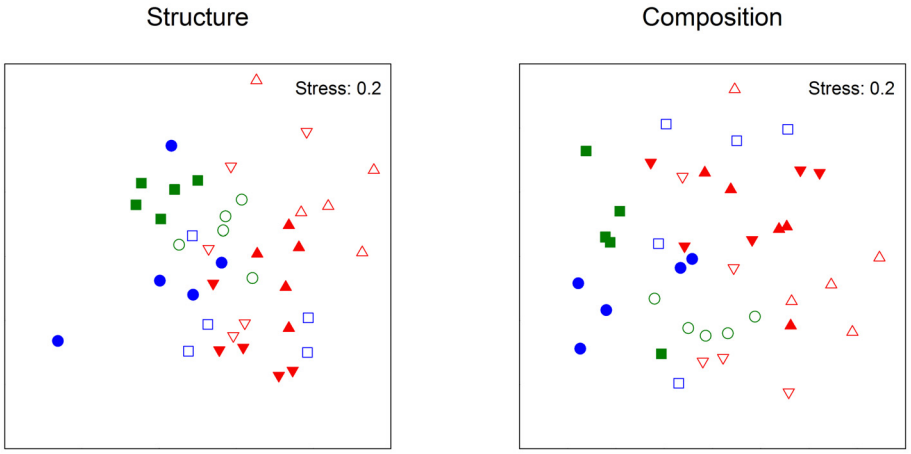

FIGURE 2 | nMDS based on Bray-Curtis measure of square-root transformed relative abundances (structure) or Jaccard measure (composition) of bacterial TRFLP on *Phyllospora comosa* originally from Cronulla (empty symbols) or Palm Beach (filled symbols) in the second experiment. Treatments: Undisturbed (green symbols), Translocated (blue symbols), Transplanted (red symbols). Destination places: Cronulla (circles), Palm Beach (squares), Long Bay (downward triangle), Cape Banks (upward triangle).

Bacterial TRFs on algae that ended up in PB differed from those that ended up in Cr (**Table 3A**). At each destination place, however, bacteria on translocated algae remained different from those on undisturbed algae (**Table 3A**). Bacteria on transplanted algae also differed between destination places in Sydney metro; however, bacteria on algae transplanted from Palm Beach differed from those on co-occurring algae transplanted from Cronulla at both destination places (**Table 3B**; **Figure 2**). There were no differences in dispersion of the structure or composition of bacterial TRFs among treatments (PERMDISP: $F_{3,36} = 1.63$, $p = 0.28$; $F_{3,36} = 1.29$, $p = 0.34$, respectively).

Discussion

To our knowledge, this is the first field manipulation of hosts which assesses impacts of environmental change on microbial communities associated with a large, habitat-forming macroalga. We found that in most cases, *Phyllospora*-associated microbial communities were more strongly and consistently affected by local conditions (i.e., were site-specific) than the type of environment or habitat they occurred in (i.e., '*Phyllospora*' vs. 'non-*Phyllospora*' habitat). In the first experiment, algae that were moved from Palm Beach seemed to respond to a change of habitat (into the Sydney region) rather than to a specific site, but this pattern was not observed in individuals moved from Cronulla in the same experiment, or those from either site in the second experiment, despite some handling or 'disturbance' effects. This high degree of site-specificity (and lack of consistent 'environment/habitat' effect) suggests that simply moving algae to a different place independent of the habitat, will result in changes of their bacterial communities. We also found some evidence for host-specificity in *Phyllospora*-associated biofilms: algae translocated from one site to another did not adopt communities similar

TABLE 2 | PERMANOVAs based on BC similarity measure for square-root transformed relative abundances or Jaccard similarity measure (composition) of bacterial TRFs on *Phyllospora* in the second experiment from the point of view of the origin.*

		Square-root transformed BC			Jaccard		
Source	***df***	**MS**	**pseudo-*F***	***p*(perm.)**	**MS**	**pseudo-*F***	***p*(perm.)**
Tr	3	4048	1.85	0.076	4539	1.55	0.093
Pl	1	2302	2.41	**0.001**	3512	2.01	**<0.001**
Tr × Pl	3	2192	2.29	**<0.001**	2933	1.68	**<0.001**
Residual	32	956			1746		
Pairwise tests		PB: TP–CB ≠ TP–LB ≠ TL ≠ U Cr: TP–CB ≠ TL ≠ U TP–LB ≠ TP–CB ≠ TL U = TP–LB			PB: TP–CB ≠ TP–LB ≠ TL ≠ U Cr: TP–CB ≠ TL ≠ U TP–LB ≠ TP–CB ≠ TL U = TP–LB		

**Treatment was fixed with four levels (U, Undisturbed; TL, Translocated; TP–CB, Transplanted to Cape Banks; TP–LB, Transplanted to Long Bay), Place of origin was random with two levels (Cr, Cronulla; PB, Palm Beach). The replicates were the Phyllospora individuals (n = 5). P-values were calculated using 9,999 permutations under a reduced model. Bold indicates statistical significance (at alpha = 0.005).*

TABLE 3 | PERMANOVAs based on BC similarity measure for square-root transformed relative abundances or Jaccard similarity measure (composition) of bacterial TRF profiles associated with *Phyllospora* at their destination place in (A) donor populations or (B) Sydney metro in the second experiment.*

		Square-root transformed BC			Jaccard		
Source	***df***	**MS**	**pseudo-*F***	***p*(perm.)**	**MS**	**pseudo-*F***	***p*(perm.)**
(A)							
Tr	1	2744	1.40	0.254	3578	1.32	0.259
Pl	1	2929	3.20	**<0.001**	3732	2.19	**<0.001**
Tr × Pl	1	1966	2.15	**0.004**	2702	1.58	**0.004**
Residual	16	914			1706		
Pairwise tests		PB: TL ≠ U Cr: TL ≠ U				PB: TL ≠ U Cr: TL ≠ U	
(B)							
Tr	1	2327	2.35	**0.004**	3126	1.74	**0.006**
Pl	1	4288	4.33	**<0.001**	4987	2.79	**<0.001**
Tr × Pl	1	879	Pooled		1875	Pooled	
Residual	19	997			1786		

(A) *Treatment was fixed with two levels (U, TL), Place of destination was random with two levels (Cr, PB).* **(B)** *Treatment was fixed with two levels (TP from Palm Beach or Cronulla), Place of destination was random with two levels (CB, LB). The replicates were the Phyllospora individuals (n = 5). P-values were calculated using 9,999 permutations under a reduced model. Non-significant interaction terms with p > 0.25 were pooled. Bold indicates statistical significance (at alpha = 0.005).*

to undisturbed individuals at the destination site. Furthermore, communities on transplanted individuals with different origins that ended-up at the sample place, still supported different communities even after 5 months.

The lack of consistency in microbial communities associated with *Phyllospora* across all treatments and sites in our study does not agree with some previous studies, which compared different algal hosts and found consistent, species-specific microbial communities among places, and seasons (Lachnit et al., 2009). However, Lachnit et al. (2009, 2011) used a method with lower resolution than TRFLP fingerprinting (denaturing gradient gel electrophoresis; DGGE). Although in many studies, TRFLP and DGGE fingerprinting techniques yield similar results (Smalla et al., 2007; Campbell et al., 2011; Fernandes et al., 2012), TRFLP is a more sensitive technique (Moeseneder et al., 1999; Enwall and Hallin, 2009), and so smaller differences will be better detected in fingerprints generated by this method than DGGE, particularly in ecological studies with multiple factors.

Furthermore, Lachnit et al. (2009) compared microbial communities from several algal species. Differences among species may outweigh site- or treatment-specific differences within a single species. We did not include an 'out-group' (i.e., comparison with a different species or water-borne microorganisms), in part because such comparisons in our experience – particularly when using surrounding seawater as the comparator – reveal such large differences as to be uninformative with respect to the macroalgal communities (Burke et al., 2011b and below). However, comparisons with other algae could have provided a larger conceptual scale against which to compare *Phyllospora* individuals from different sites and/or treatments, and such comparisons are currently underway.

In contrast, Burke et al. (2011b) compared microbial communities from replicate samples of the green alga *U. australis* occurring in separate rock pools at a single site and also compared algal-associated communities to those within the surrounding water column, by creating and sequencing sophisticated 16S rRNA gene clone libraries. As well as finding almost no similarity between algal-associated and water-borne microbial communities, they detected a very high degree of variability among communities associated with algal samples from different rock pools (despite the 'out-group' comparison with water samples). They proposed that microorganisms could colonize algal surfaces using a 'competitive lottery model,' in which multiple species could colonize algal surfaces, so long as a core set of functions (rather than a core phylogeny) was represented within the microbial consortia. Indeed, there was a high degree of conservation of functional gene profiles expressed by the microbial communities from replicate algal samples (Burke et al., 2011a). Interestingly, previous work on the same system using the lower-resolution technique DGGE, suggested that a temporally and spatially consistent, species-specific community was present on *U. australis* (Tujula et al., 2010). In the latter study, a core set of OTUs was always present, despite some spatial and temporal variation. Our results suggest that *Phyllospora* may require a core set of functions from its biofilm, rather than a core set of 'species' (or OTUs) and that local, site-specific conditions rather than habitat-type will influence the phylogenetic composition of *Phyllospora*'s biofilm.

However, because translocated individuals did not come to resemble undisturbed individuals and furthermore, because transplanted individuals from different origins did not conform to a 'destination site-specific' community, this suggests some degree of host-specificity – that is, microbial communities are influenced by more than simply the site in which they occur (or to which they are moved). This may simply be an issue of timing: longer than 5 months may be required for convergence of microbial assemblages to occur in some cases. This seems unlikely, however, given the relatively rapid rates of colonization and succession

in microbial communities compared to macroorganisms. Alternatively, the starting conditions of the biofilm may influence microbial succession such that multiple, alternative stable states (rather than just one) are possible, given a set of environmental conditions and host traits. Such concepts have been widely discussed and tested in the field of classical ecology (reviewed by McCook, 1994; Young et al., 2001) and are beginning to feature in the study of microbial ecology as well (Burke et al., 2011a). The fact that there was some degree of host-specificity suggests that a component of the community may be influenced by differences in host traits. Host genetic variation can strongly influence the structure of microbiomes associated with plants (e.g., Bulgarelli et al., 2013) and animals, including mice (Benson et al., 2010) and humans (Arumugam et al., 2011). Our results suggest that *Phyllospora* may also have a core microbiome that can be influenced by local, site-specific conditions, and host traits.

Because crayweed remains absent from the Sydney region, we hypothesized that the environment in Sydney may impact seaweed-associated microbial communities, which may, in turn, have contributed to its failure to recover in the region. We found no consistent evidence that the environment within Sydney had any significant impact on *Phyllospora*-associated microbes. Previously, we reported that individuals transplanted from extant populations to these recipient sites within the Sydney region had survivorship rates comparable to those in natural populations (Campbell et al., 2014). Together with these observations, this suggests that environmental conditions within Sydney are now suitable to support *Phyllospora* again. Furthermore, they suggest that individuals transplanted to or recruiting naturally onto reefs in Sydney will not develop a 'Sydney-specific' biofilm, rather they will adopt a biofilm specific to the local conditions (whether it is within or outside of the Sydney region).

It is possible, however, that at the time of its disappearance, the habitat within Sydney had a negative impact on *Phyllospora*'s interactions with its biofilm. Then, a high volume of poorly treated sewage was released adjacent to the shorelines where this species was dominant (Coleman et al., 2008). Ecotoxicological experiments with *Phyllospora* suggest that it is physiologically sensitive to high levels of nutrients (Burridge et al., 1995). The production of metabolites and other natural products by macroalgae strongly influences the composition and maintenance of biofilms on their surfaces (Egan et al., 2012). Thus an environmentally mediated change in physiology (e.g., due to high levels of pollution) might have indirectly altered *Phyllospora*'s surface-associated microbial community, which may have contributed to is decline. Understanding the role of microorganisms in the health of important, habitat-forming organisms is essential to understand the processes that affect their persistence, and to inform efforts to restore populations of these organisms if they decline. The emergence of molecular techniques to rapidly (and with increasing affordability) assess the composition of microbial assemblages associated with these organisms and even characterize their functional gene profiles, facilitates the inclusion of microbes into more ecological studies.

Most studies on holobionts to date have shown either strong host-specificity of surface-associated microbial communities, suggesting that hosts require a core set of specific taxa, or high variability of surface-associated microbial communities, suggesting that hosts may be colonized by taxonomically distinct bacteria available in the local species pool and where a core set of specific functions is more important for the host than a core set of specific taxa. Our results suggest that a combination of both processes influence bacteria on the *Phyllospora* holobiont. Thus, although some component of the community may vary across sites depending on the available taxa, other components of the community may be driven by specific traits of the host.

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Temporal changes in the diazotrophic bacterial communities associated with Caribbean sponges *Ircinia strobilina* and *Mycale laxissima*

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Sponges that harbor microalgal or, cyanobacterial symbionts may benefit from photosynthetically derived carbohydrates, which are rich in carbon but devoid of nitrogen, and may therefore encounter nitrogen limitation. Diazotrophic communities associated with two Caribbean sponges, *Ircinia strobilina* and *Mycale laxissima* were studied in a time series during which three individuals of each sponge were collected in four time points (5:00 AM, 12:00 noon, 5:00 PM, 10:00 PM). *nifH* genes were successfully amplified from the corresponding gDNA and cDNA pools and sequenced by high throughput 454 amplicon sequencing. In both sponges, over half the *nifH* transcripts were classified as from cyanobacteria and the remainder from heterotrophic bacteria. We found various groups of bacteria actively expressing the *nifH* gene during the entire day-night cycle, an indication that the nitrogen fixation potential was fully exploited by different nitrogen fixing bacteria groups associated with their hosts. This study showed for the first time the dynamic changes in the activity of the diazotrophic bacterial communities in marine sponges. Our study expands understanding of the diazotrophic groups that contribute to the fixed nitrogen pool in the benthic community. Sponge bacterial community-associated diazotrophy may have an important impact on the nitrogen biogeochemical cycle in the coral reef ecosystem.

Keywords: diazotroph, diel cycle, symbiotic microbial community, Caribbean sponge, 454 pyrosequencing

INTRODUCTION

Coral reef ecosystems are well known for their high biodiversity and productivity, despite low ambient nutrient availability. Numerous studies on the nitrogen cycle in coral reefs have linked high local primary production to benthic biological activities, including the efficient recycling of nitrogen between algae and invertebrate hosts and benthic nitrogen fixing communities contributing to a "new" nitrogen source (Dugdale and Goering, 1967; Webb and Wiebe, 1978; O'Neil and Capone, 2009). Marine sponges are important habitat forming organisms in coral reef benthic communities. Their filter-feeding lifestyle can remove large amounts of organic particles in the size range between 0.2 and 10 μm, mainly bacterioplankton and phytoplankton, from the water column (Pile et al., 1996). These microorganisms are consumed as part of the sponge diet. Sponges also serve as hosts to many microbes that live inside the sponge mesohyl as their symbionts. The terms "symbiont" and "symbiosis" are used here consistent with Taylor (Taylor et al., 2007), according to the original definition by de Bary, to refer to two or more organisms found living together for a long period, and do not imply that the organisms benefit or harm each other. The density of microbial symbionts in sponges can reach a billion cells per ml volume, approximately three orders of magnitude higher than in the surrounding seawater (Taylor et al., 2007). Considering these high densities, symbionts are likely to play important roles in the hosts. Molecular tools and high throughput sequencing techniques have helped to overcome the constraints imposed by difficulty in culturing many of these symbionts, and have expanded our knowledge of the sponge microbiome, revealing their connections with host chemical defense, immunity and metabolism (Hentschel et al., 2012). Bacteria are the major driving force in the element biogeochemical cycle (Falkowski et al., 2008). In the sponge mesohyl, frequent water exchange between the sponge and outer environment can create an oxygen gradient and brings in a supply of nutrients (Hoffmann et al., 2005); these conditions may facilitate the essential redox reactions by symbiotic microorganisms (Fiore et al., 2010). The high abundance of microbial cells and suitable conditions are likely to result in significant nutrient flux mediated by the microbial community, which could be important for the local ecosystem.

A classic early study showed the transfer of a photosynthetic carbohydrate from symbiotic cyanobacteria to the sponge hosts (Wilkinson, 1983). The continuous influx of photosynthetic product that is rich in carbon but devoid of nitrogen could trigger the imbalance of C:N ratio in the symbiont-sponge system, leading to a nitrogen source deficiency. Field incubation experiment showed the uptake of ^{15}N labeled ammonium and nitrate by both sponge cells and bacterial fractions and suggested the translocation of labeled nitrogen from bacteria to hosts (Fiore et al., 2013; Freeman et al., 2013). However, coral reef ecosystems are

characterized by low dissolved nitrogen availability in the water column, conditions that might cause the sponge holobiont to seek an alternative nitrogen source to balance their budget. Nitrogen fixation, an anabolic pathway carried out only by prokaryotes, accounts for half of the reactive N supply that sustains ocean primary production (Gruber and Galloway, 2008). This pathway requires an anaerobic microenvironment and significant energy supply for N_2 reduction. To provide suitable conditions for nitrogen fixation, some diazotrophs like *Anabaena* develop heterocysts as a spatial compartment to create the anaerobic condition; other groups like unicellular cyanobacterium *Cyanothece* conduct nitrogen fixation at night, temporally separated from the oxygenic photosynthesis that occurs during the daytime (Dixon and Kahn, 2004; Welsh et al., 2008). In a field study in 2007, we found consistently lower $\delta^{15}N$ values from tissues of the sponge *Ircinia strobilina*, indicating that these sponge individuals obtain their nitrogen from nitrogen fixation, whereas samples from the sponge *Mycale laxissima* showed higher $\delta^{15}N$ ratios, suggesting less reliance on nitrogen fixation. Subsequent molecular studies demonstrated the presence of diverse *nifH* genes from cyanobacteria along with heterotrophic bacteria in both sponges. However, the only *nifH* gene transcripts were those belonging to cyanobacteria (Mohamed et al., 2008). In the current study, we applied a high throughput sequencing method that allowed deeper coverage of the community, and expanded the sampling strategy to monitor the nitrogen fixing activities during a diel cycle. Through our study, we would like to provide more details to major questions regarding the diazotrophic communities associated with sponge hosts. Is the symbiotic community species specific? How stable is the community in the long term? Do active members shift over the diel cycle?

MATERIALS AND METHODS

SAMPLING COLLECTIONS

Tissue samples of *M. laxissima* and *I. strobilina* were collected by SCUBA diving at a depth of 20 m from Sweetings Cay, Bahamas (26° 33.78′N, 77° 52.89′W) in July 2012. Surface water temperature in the collection site was 26.7°C. Prior to collection, three large (1–5 kg) individuals of *M. laxissima* and *I. strobilina* were tagged for recurrent sampling. For each individual, 1 cm^3 piece of tissue was collected with a sterile scalpel at local time 5:00 AM (dawn), 12:00 PM (noon), 5:00 PM (dusk), and 10:00 PM (night) for one diel cycle. To reduce the impact of tissue damage during sampling, small individual samples were taken from distant locations of the same sponge for each time point. During each night dive glow sticks were used instead of dive torches to prevent photosynthetic activity from interfering with nitrogen fixation. Samples for DNA and RNA extraction were preserved in RNAlater stabilization solution (Qiagen, Valencia, CA, USA) on board within 20 min after underwater collection prior to long-term storage at −80°C. Three seawater samples (5–10 L) from the sampling site were collected at noon in close proximity (1 m) to sampled sponges and filtered through 0.22 μm Sterivex filter units (Millipore, Billerica, MA, USA). Seawater samples were collected to compare the diversity of sponge nitrogen fixing bacteria with those found in the surrounding environment.

MEASUREMENT OF STABLE ISOTOPE COMPOSITION

Sponge samples for $\delta^{15}N$ measurement were collected from Sweetings Cay, Bahamas (26° 33.78′N, 77° 52.89′W) in July 2012 and from Conch Reef, Key Largo, Florida, USA, NE Caribbean (24° 57.11′N, 80° 27.57′W) in March 2010 and July 2011, prior to the collection of the sponge samples used in this study for the bacterial community analyses. Sponge samples for this purpose were drained and were rinsed three times with artificial seawater, then frozen at −20°C before processing. Three individuals of each sponge were lyophilized and grounded to fine powder. Samples (c. 1.0 mg) were packed in tin capsules for shipping and analyzed for nitrogen isotope ratios by continuous flow isotope ratio mass spectrometry at the UC Davis Stable Isotope Facility as described previously (Mohamed et al., 2008).

GENOMIC DNA/RNA EXTRACTION AND *nifH* GENE PCR AMPLIFICATION

Total DNA and RNA from the three individuals of each sponge species collected during four time points from Sweetings Cay, Bahamas, July 2012 were extracted using a TissueLyser System (Qiagen), and an AllPrep DNA/RNA Mini Kit (Qiagen), combined with RNAase-free DNase treatment steps (Qiagen) for RNA samples following the manufacturer's protocol. Total DNA from seawater samples was extracted using a Power Water Sterivex DNA isolation kit (Mo Bio, Carlsbad, CA, USA) following the manufacturer's protocol. Nested PCR was used to amplify *nifH* gene fragments from genomic DNA (gDNA), and the cDNA derived from RNA as described below. For gDNA samples, *nifH* gene fragments were amplified by first round primers nifH32F (5′-TGAGACAGATAGCTATYTAYGGHAA-3′) and nifH623R (5′-GATGTTCGCGCGGCACGAADTRNATSA-3′) (Steward et al., 2004) at a concentration of 100 μM each because of the highly degenerate primers used for *nifH* genes covering 128 and 96 different combinations of nucleotide sequences. For RNA samples, the concentration of extracted RNA was measured using a Nanodrop spectrophotometer 2000 (Thermo Scientific, Waltham, MA, USA), and 100 ng of RNA template from each sample was added to RevertAid Reverse Transcriptase mix (Thermo Scientific) with primer nifH3 (5′-ATRTTRTTNGCNGCRTA-3′) as described previously by Zani et al. (2000). After reverse transcription, cDNA was amplified using first round PCR primers nifH3 and nifH4 (5′-TTYTAYGGNAARGGNGG-3′) at a concentration of 100 μM each. RNA samples without the RT step were included as PCR template to check for residual DNA in the RNA samples.

NESTED PCR AMPLIFICATION AND AMPLICON SEQUENCING

A total of 33 PCR product samples (three gDNA from filtered seawater, 24 cDNA samples from four time points of the six individuals and, six gDNA samples for each individual, pooled from four DNA extractions done at each of the four time points) from the first round were sent to Research and Testing Lab (Lubbock, TX, USA) and subject to a second round of PCR targeting a variable region (360 bp) encoding dinitrogenase reductase subunit using barcoded primer sets nifH1 (5′-TGYGAYCCNAARGCNGA-3) and nifH2 (5′-ADNGCCATCATYTCNCC-3′) (Zehr and Mcreynolds, 1989).

Subsequent amplicon pyro-sequencing by 454 Life Science GS FLX + platform (Roche Diagnostics, Branford, CT, USA) generated about 3000 raw sequencing reads from each tagged sample.

SEQUENCE ANALYSIS PIPELINE

Initial data were processed using the mothur software package, following the guidelines and recommendations in the mothur manual (www.mothur.org) (Schloss et al., 2009) for sequence quality trimming, chimeric checking and denoising to generate a single fasta file. Sequence reads less than 300 bp, plus barcoding tag and primer information were subsequently removed using the "trim.seqs" command. The cleaned sequences were pre-clustered using Simultaneous Alignment and Tree Estimation using default setting for nucleotide analysis (Liu et al., 2009). Representative sequences from each cluster were blasted against the GenBank database using the blastn function to confirm sequence identity and non-*nifH* gene sequences were removed. In some of our cDNA samples, non-*nifH* gene sequences accounted for up to half of the total reads, and were classified as either 16S rRNA sequences from bacteria or 23S rRNA sequences from sponges. A possible explanation for this is that the RNA extraction included a large quantity of ribosomal RNA from microbial symbionts and the hosts, therefore the nested PCR used in our study could lead to reverse transcription and amplification of unintended rRNA sequences. After all corrections, we obtained 67,212 *nifH* sequences in 33 samples. Unique *nifH* sequences were translated into amino acid sequences (120 bp) using MEGA, then aligned to the reference *nifH* database from Marine Microbiology, University of California Santa Cruz (http://pmc.ucsc.edu/~wwwzehr/research/database/), built into a phylip-formatted distance matrix and clustered into OTUs at the 90% similarity level in translated amino acid sequences with the nearest neighbor method. Representative *nifH* sequences from each OTU were deposited in the NCBI database under accession numbers KM083066–KM083092 and raw amplicon sequence data were deposited in the NCBI-SRA database under BioSample accession SAMN02869232. 24 cDNA samples were sub-sampled according to the sample with the minimum number of reads to enable diversity comparisons among individuals and time points.

PHYLOGENETIC ANALYSIS OF *nifH* GENES

Translated amino acid sequences from representative OTUs and their top blast hits (GenBank database) were imported into ARB (Ludwig et al., 2004) for *nifH* gene phylogenetic analysis. Multiple sequence alignments were visually checked and improved manually using the ARB editor. The aligned *nifH* sequences (120 bp) were imported into PhyML 3.1 software package to construct a tree based on Maximum Likelihood method (Guindon and Gascuel, 2003). The robustness of the resulting tree topologies was evaluated by 1000 bootstrap replicates.

STATISTICAL ANALYSIS

Diversity metrics (observed OTUs, coverage, Chao1 estimator, Shannon index and, Simpson's inverse) were calculated for sequence data from sponge species and seawater. Diazotrophic communities in sponges were compared by nonmetric multidimensional scaling (nMDS). All analyses were performed using the mothur software package (Schloss et al., 2009). One-Way and multiple factorial ANOVA was performed in the Statistica 7.0 (StatSoft, Tulsa, OK, USA).

RESULTS

δ^{15}N values from *I. strobilina* samples were 1.01 ± 0.97 (*SD*, $n = 24$), consistently lower than the values 3.86 ± 0.92 (*SD*, $n = 24$) from *M. laxissima* (ANOVA between sponge species, $p < 0.01$) and no significant difference (Multiple factorial ANOVA, $p > 0.05$) between the collection years (**Figure 1**). We also measured the δ^{15}N data during the diel cycle. The outcome confirmed the difference in species level, but did not reveal any patterns over a 24 h time span (data not shown).

The diversity of the bacterial diazotrophic communities revealed by *nifH* gene sequences amplified from total DNA was quite similar between the two sponge species. In the current study, deep sequencing of *nifH* amplicons revealed a greater diversity of nitrogen fixing groups in the communities. Based on 90% amino acid sequence similarity, we recovered 22 OTUs from the sponge *I. strobilina*, and a slightly higher diversity (24 OTUs) from *M. laxissima* (**Table 1**). The communities were dominated by cyanobacteria, alpha-proteobacteria and, gamma-proteobacteria. Strict anaerobes belonging to delta-proteobacteria were also found. The diversity of *nifH* genes in the surrounding seawater was lower than in the sponges with 17 OTUs, all from heterotrophic bacteria (**Figure 2**) and, OTU composition was different from those detected in sponge samples (ANOVA, between sponges and seawater $P < 0.001$). Community compositions based on sequence reads from individuals were consistent in gDNA source, with no significant difference in both individuals (ANOVA between individuals, $p > 0.05$) and, species level (ANOVA between sponge species, $p > 0.05$). Detailed individual community compositions are shown in supplemental Figure S1.

The dominant cyanobacterial OTUs were found in all sponges, regardless of location and time of collection. In fact, the representative sequence from OTU1, belonging to the cyanobacterial genus *Leptolyngbya*, shared 100% identity with the DNA

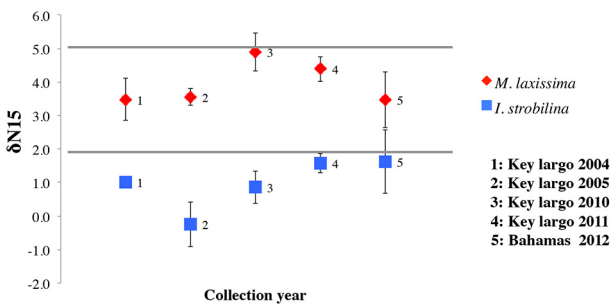

FIGURE 1 | δ15N values of sponges: *I. strobilina* (IS, square in blue), *M. laxissima* (ML, diamond in red). Each point represents the mean δ15N value taken from three sponge individuals of the same species during different years. Error bars indicate the standard deviation of each mean calculation.

sequences found in the same sponge species collected in 2004 and 2005 at Conch Reef, Key Largo.

In the cDNA dataset, transcripts from cyanobacteria, alpha-proteobacteria and gamma-proteobacteria accounted for at least 80% of sequence reads in each sample. The diversity of *nifH* genes from the cDNA libraries was lower than from the gDNA libraries. 13 OTUs were shared between sponge species, although two gamma-proteobacterial OTUs were found exclusively in *M. laxissima* cDNA samples, and one cyanobacterial and one gamma-proteobacterial OTU were found only in *I. strobilina* samples. Cyanobacterial transcripts were found from filamentous cyanobacteria, including heterocyst-forming genera like *Anabaena*, and non-heterocyst forming genera like *Leptolyngbya* and, from unicellular cyanobacteria, closely related to *Cyanothece*. However, no group showed a consistent expression pattern corresponding to particular times in the light/dark cycle. Most transcripts from heterotrophic bacteria were classified either as alpha-proteobacteria closely related to aerobic genus *Xanthobacter* or as gamma-proteobacteria closely related to facultative anaerobe *Klebsiella* (**Table 2**). A complete list of OTUs found in this study is provided (Table S1) and their phylogenetic relationships with cultured nitrogen fixer and closest environmental clones are listed (Figure S2).

We selected sequences from three major groups: cyanobacteria, gamma-proteobacteria and, alpha-proteobacteria. For these groups, we normalized sequence reads by minimum sample reads across all samples and compared the composition of the actively transcribed components of the community during day and night. When we combined transcript reads from six individuals of the two sponge species we found that cyanobacterial transcripts were dominant in the daytime, accounting for $94.1 \pm 10.7\%$ (SD, $n = 6$) of the total sequence reads from both sponge species. The percentage pattern changed significantly at night (One-Way ANOVA, $p < 0.01$), with $72.8 \pm 37.4\%$ (SD, $n = 6$) of transcripts deriving from heterotrophic bacteria (**Figures 3A,B**). In the species level, day/night difference was more significant for *I. strobilina* (ANOVA between symbiont species during day/night, $p = 0.04$) and less significant for *M. laxissma* (ANOVA between symbiont species during day/night, $p = 0.11$), largely due to a relative high proportion of cyanobacterial transcripts found in the nighttime sample of the third *M. laxissma* individual.

Within sponge samples, cyanobacteria transcripts were consistently dominant at dusk and heterotrophic bacteria were more abundant at dawn. In contrast, community structure at noon and, at night showed variation among different individuals at each time point. For example, two *I. strobilina* individuals showed dominance of heterotrophic bacterial transcripts at 10:00 PM, whereas individual 2 at the same time point had transcripts exclusively from cyanobacteria (**Figure 3A**).

In the nMDS plot of community structure, *nifH* gene sequences derived from sponge samples clustered together with no obvious distinction in sequences between the two sponge species, whereas *nifH* gene sequences derived from seawater samples were clearly separated from the sponge-derived sequences (**Figure 4**).

Table 1 | Richness and dominance metrics for diazotrophic communities in sponges and seawater based on *nifH* gene sequences (OTU = 90% amino acid sequence similarity).

Source	Observed OTUs (Sobs)	Number of reads	Expected OTUs (Chao1)	Simpson Inverse Index	Shannon Index
I. STROBILINA					
gDNA	22	8290	26 (17–33)	12.2	3.2
cDNA	16	17855	18 (12–20)	6.9	2.6
M. LAXISSIMA					
gDNA	23	7383	24 (16–33)	12.5	3.2
cDNA	14	18537	17 (11–22)	6.1	2.5
SEAWATER					
gDNA	17	5147	21 (17–30)	9.2	2.9

DISCUSSION

The $\delta^{15}N$ values in the two sponges *M. laxissima* and *I. strobilina* were consistent over two sampling periods 8 years apart, suggesting that the sources of fixed nitrogen for each of these sponge species remain the same over long periods. The fact that *I. strobilina* is considered to be a high microbial abundance (HMA) sponge and *M. laxissima* to be a low microbial abundance (LMA) sponge (Reiswig, 1973, 1974) might translate to a relative higher prokaryote activity like nitrogen fixation in the *I. strobilina*. Based on field observation, adult *I. strobilina* individuals are generally denser than *M. laxissima* and show lower pumping rate, and this could create a less efficient oxygen-penetrated mesohyl for *I. strobilina* thus provide more anaerobic niches for nitrogen fixation by non-heterocyst cyanobacteria and heterotrophic bacteria. The $\delta^{15}N$ values likely reflected the combined effect of biologically available fixed nitrogen input and loss in

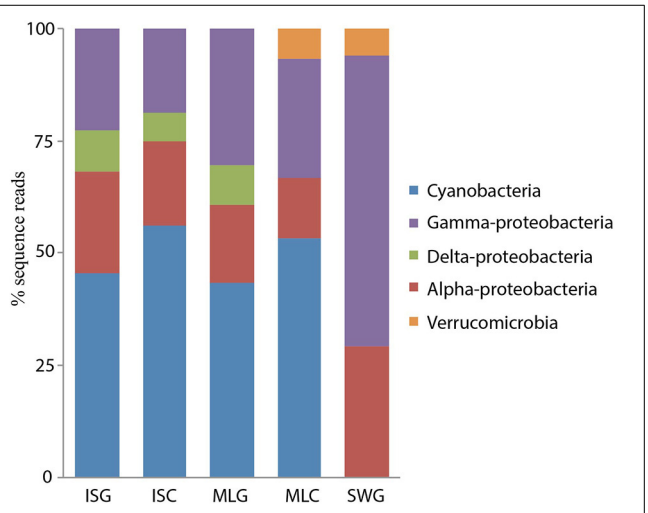

FIGURE 2 | Community structure *I. strobilina, M. laxissima* and seawater based on 90% translated amino acid sequences similarity of *nifH* genes from gDNA and cDNA sources. (ISG: gDNA from *I. strobilina*, ISC: cDNA from *I. strobilina*, MLG: gDNA from *M. laxissima*, MLC: cDNA from *M. laxissima*, SWG: gDNA from seawater).

Table 2 | *nifH* gene OTUs found in high abundance in *I. strobilina* and *M. laxissima* sponge samples and their closest BLAST sequence matches.

Sponge- derived 90%-OTUs	No. of reads in each OTU per source				Closest BLAST match (accession no., % identity, source)	Closest cultivated microorganism (accession no., % identity, source)
	ISG	ISC	MLG	MLC		
OTU01	673	3580	1310	4320	EU594242.1 (100%) Sponge RTMLH02	KC256775.1 (88%) *Leptolyngbya minuta*
OTU02	173	1967	989	2315	HM601491.1 (92%) Florida key reef water	AB264111.1(84%) *Cyanothece sp.*
OTU03	338	2215	282	627	EU594072.1 (96%) Sponge IS15S	HQ906641.1 (99%) *Mastigocladus testaurum*
OTU04	122	855	1688	3152	KF657100.1 (88%) Coral clone	FR669148.1 (84%) *Klebsiella sp.*
OTU05	428	558	155	663	GU594006.1 (95%) Freshwater lake	DQ439648.1 (95%) *Anabaena sphaerica*
OTU06	305	1458	198	1130	EU594012.1 (93%) Sponge IS3H07	CP000781.1 (98%) *Xanthobacter autotrophicus*

ISG, gDNA from I. strobilina; ISC, cDNA from I. strobilina; MLG, gDNA from M. laxissima; MLC, cDNA from M. laxissima.

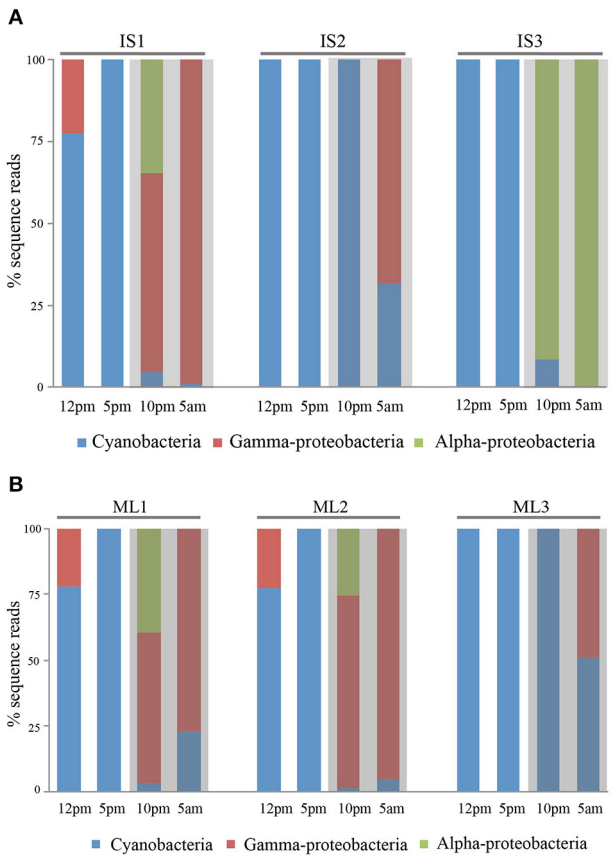

FIGURE 3 | (A) Relative abundance of transcript reads from three individuals of *I. strobilina* in a diel cycle. IS1, IS2, IS3 represent three individual sponges. **(B)** Relative abundance of transcript reads from three individuals of *M. laxissima* in a diel cycle. ML1, ML2, ML3 represent three individual sponges.

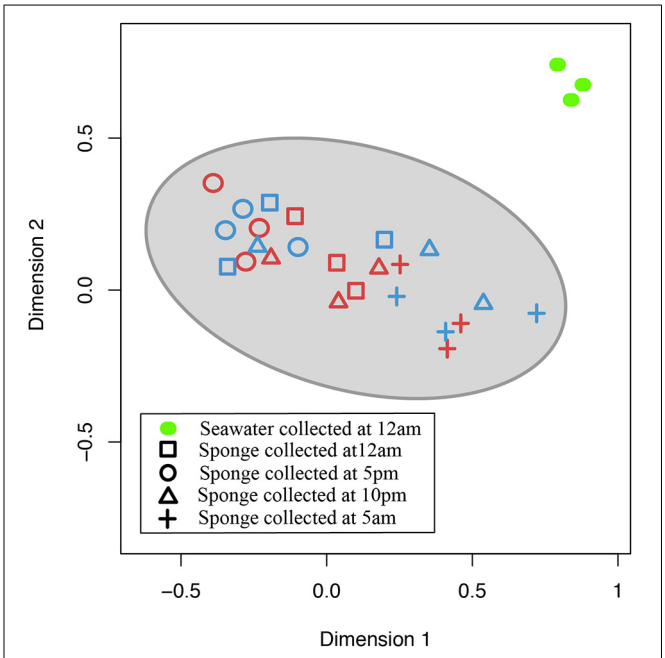

FIGURE 4 | Non-metric multidimensional scaling (nMDS) plot based on Bray-Curtis distances between cDNA samples from 4 different time points: 12 noon (squares), 5:00 p.m. (circles), 10:00 p.m. (triangles), 5:00 a.m. (crosses). Blue color symbols represent samples from *I. strobilina*, red color symbols represent samples from *M. laxissima* and, green color solid oval symbols represent gDNA samples from surrounding seawater (Stress value = 0.10, $R^2 = 0.86$).

the holobiont. Studies on nutrient flux through sponges showed that sponges serve as a net source of nitrate (Jiménez and Ribes, 2007; Hoffmann et al., 2009; Fiore et al., 2013), although not much nitrate and ammonium release were reported for *M. laxissima* and *I. strobilina* (Southwell et al., 2008). This observation is potentially contradictory to the hypothesis that there is nitrogen deficiency in the sponge microbiome community that requires the activity of nitrogen fixing bacteria to provide additional fixed nitrogen. However, the net export of nitrate does not reflect the spatial heterogeneity in the sponge mesohyl (Webster et al., 2001; Radax et al., 2012). Whether the total sponge mesohyl is a nitrogen limited environment or not, carbohydrate input from photosynthesis and inorganic nitrogen species removal by ammonium oxidation and denitrification (Mohamed et al., 2009) likely requires localized replenishment of fixed nitrogen to the bacterial community. Previous research found similar low $\delta^{15}N$ values in spongin fractions derived from sponge tissue, indicated the transfer of nitrogen to the hosts (Weisz, 2006). Measurement of $\delta^{15}N$ values of bacteria and sponge cells separated from the same sample could provide more direct evidences on whether the

hosts benefit more from fixed nitrogen produced by microbial symbionts.

In agreement with our previous study (Mohamed et al., 2009), we found similar nitrogen fixing communities in the two distantly related sponge species, suggesting that the difference in $\delta^{15}N$ values observed in these two species might reflect differences in the rates of nitrogen-fixation rather than being the consequence of different nitrogen-fixing communities. In order to confirm the relatively lower $\delta^{15}N$ data observed in *I. strobilina*, quantitation of *nifH* transcripts using real-time PCR could provide insights into the relative nitrogen fixation activities of the two similar nitrogen-fixing communities found in the two sponge species.

Studies of coral reef ecosystems have shown that nitrogen-fixing bacteria are widely distributed in the water column and corals (Hewson et al., 2007; Lema et al., 2014). Our results show a consistent presence of nitrogen-fixing cyanobacterial groups in marine sponges from two geographic locations of the Caribbean coral reef (Sweetings Cay, Bahamas and Key Largo, Florida). The fact that cyanobacterial-derived *nifH* transcripts are dominant in sponge samples collected during the daytime, suggests that cyanobacteria are key nitrogen-fixing symbionts in the two sponges. The coexistence of heterocyst-forming filamentous cyanobacteria and unicellular diazotrophic cyanobacteria in both sponges also suggests that, in order to protect nitrogenase from an oxidative environment, both spatial and, temporal separation strategies might be adopted by the community. However, when comparing the presence of heterocyst-forming filamentous and, unicellular cyanobacterial transcripts under light/dark conditions, neither group showed a consistent diel pattern in this study.

A previous study on *nifH* gene diversity associated with corals showed a high proportion of heterotrophic bacteria in those communities (Lema et al., 2014). In our study, high throughput sequencing revealed rare phyla like delta-proteobacteria that were not detected by our previous study in which we used a cloning and sequencing method. Unlike the cyanobacteria, nitrogen fixing heterotrophic bacteria seems to be more active in nitrogen fixation at night. The communities were not well conserved between the two sponge species or between sponges of the same species collected at each location, suggesting that the heterotrophic diazotrophic bacteria may not be as closely associated with their host as the cyanobacterial groups. The fact that, based on phylogeny (Figure S2), the closest environmental clones of many heterotrophic bacterial OTUs found in this study are connected with benthic community (i.e., associated either with coral, sea-grass or from the marine sediment) suggests that the heterotrophic nitrogen-fixing bacterial selection may be controlled by a "first come first served" process (Fan et al., 2012), which proposes that local geographic factors matter the most in shaping some bacterial communities.

A recent study on natural community in the Hawaii Ocean Time-Series station found a diel expression pattern in which photosynthesis related transcripts from *Prochlorococcus* peaked at dawn or dusk (Ottesen et al., 2014). Although not directly influenced by sunlight, nitrogen fixation is regulated by local environmental factors include pH, O_2, NH_4^+ and, organic carbon availability which is strongly impacted by a photosynthetic process (Stacey et al., 1992). A study of diazotrophs in the open ocean found temporal patterns of *nifH* transcript abundance in different cyanobacteria phylotypes but no obvious pattern for heterotrophic gamma-proteobacteria (Church et al., 2005). Though the existence of horizontal transfer in *nifH* gene in proteobacteria (Cantera et al., 2004) could potentially complicate the phylogenetic assignment, so far no HGT of *nifH* gene between cyanobacteria and heterotrophs has been reported, thus the multiple taxon *nifH* gene expression in our study likely reflected bacterial responses to sporadic and transient environmental cues inside the hosts. Sponge mesohyl can undergo spatial gradients that fluctuate through active pumping (Hoffmann et al., 2005). The associated diazotrophic community must control N_2 fixation under these oscillating environmental conditions. We detected diurnal patterns in those members of the bacterial community that are actively expressing nitrogen fixation genes. We speculate that this pattern may reflect a combined effect of energy supply from photosynthesis and oxic states in localized regions of the holobiont during the light/dark cycle. The low light intensity at dusk, at the end of the light cycle, may be a time at which energy is still available from the day-time photosynthesis to power nitrogen fixation in cyanobacteria while accumulated oxygen could limit nitrogen fixation in heterotrophic bacteria. Conversely, at dawn, at the end of the dark cycle, the energy gained from photosynthesis by autotrophs may be exhausted, and, bacterial respiration may create an anoxic state by oxygen consumption, favoring nitrogen fixation from heterotrophic bacteria. The other two time points in our dataset may reflect the intermediate state between the two scenarios described above, thus resulting in individual variation in active nitrogen fixers. Meanwhile, some sampling constraints might limit the interpretation of the current results. First, the stress effects incurred by each time of tissue collection on the sponge hosts and associated microbial communities are worth considering. Though the actual stress impacts are difficult to assess, an additional sampling point during the daytime showing the dominance of cyanobacterial *nifH* transcripts immediately after the dark cycle, would strengthen our hypotheses. Alternatively, the detection of the expression of stress gene marker like *hsp70* gene for the host (Lopez-Legentil et al., 2008) or *dnaK* gene (Glatz et al., 1999) for symbiotic cyanobacteria over the time course in future studies might provide insights into stress effects in the sponge during the course of sampling.

The imbalance of the global nitrogen budget indicates a potential underestimation of biological nitrogen fixation (Karl et al., 2002). Nitrogen fixation by non-cyanobacterial groups has been overlooked and could play an important role in marine environments (Moisander et al., 2014). Nitrogen fixation by non-cyanobacterial groups in sponges may also contribute to nitrogen input from the benthic community to nutrient limited coral reef ecosystems.

Our study showed that cyanobacteria are a dominant and consistent group in the diazotrophic community within sponges, and, various heterotrophic bacteria groups can be important components in the community. Composed of "core" cyanobacteria and flexible heterotrophic bacteria, nitrogen fixers in sponges represent an optimal combination to replenish the nitrogen pool.

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SUPPLEMENTARY MATERIAL

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Infectious microbial diseases and host defense responses in Sydney rock oysters

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Aquaculture has long been seen as a sustainable solution to some of the world's growing food shortages. However, experience over the past 50 years indicates that infectious diseases caused by viruses, bacteria, and eukaryotes limit the productivity of aquaculture. In extreme cases, these types of infectious agents threaten the viability of entire aquaculture industries. This article describes the threats from infectious diseases in aquaculture and then focuses on one example (QX disease in Sydney rock oysters) as a case study. QX appears to be typical of many emerging diseases in aquaculture, particularly because environmental factors seem to play a crucial role in disease outbreaks. Evidence is presented that modulation of a generic subcellular stress response pathway in oysters is responsible for both resistance and susceptibility to infectious microbes. Understanding and being able to manipulate this pathway may be the key to sustainable aquaculture.

Keywords: oysters, disease, aquaculture, selective breeding, environmental stress

INTRODUCTION

During the latter part of the last century, aquaculture (the farming of aquatic animals and plants) was seen as a key emergent food source needed to compensate for the world's rapidly growing human population. Aquaculture production increased rapidly during the 1970's and 1980's (FAO, 2012) (**Figure 1**). However, despite continued strong growth in freshwater aquaculture (primarily in Asia), increases in production from farming of marine species, such as oysters, has slowed. In some cases, output has begun to decline. There are a number of reasons for this. Marine aquaculture (mariculture) is susceptible to environmental degradation of coastal and estuarine waters around the world, and the supply of new, accessible farming sites is limited. More importantly, mariculture is particularly prone to infectious diseases, which are the main limiting factors in marine aquaculture production worldwide (Leung and Bates, 2013).

The pathogens and parasites that affect aquaculture production around the world include viruses [e.g., ostreid herpes virus (OsHV1), white spot syndrome virus (WSSV), abalone viral ganglioneuritis], bacteria (e.g., *Vibrio harveyi*, *Flexibacter columnaris*, *Aeromonas salmonicida*), protozoans (e.g., *Perkinsus* species, *Marteilia* species, and *Bonamia* species), and multicellular parasites or pests (e.g., mud worms and platyhelminths) (Renault, 1995; Coelen, 1997). These infectious agents can be highly host specific (e.g., *Marteilia sydneyi* in Sydney rock oysters) or have a broad range of host species (e.g., *Perkinsus olseni*, WSSV, or *Vibrio harveyi*). Disease epizootics caused by these infectious agents are often devastating. For instance, outbreaks of WSSV are responsible for annual losses in the shrimp industry of up to $10 billion worldwide (Flegel and Alday-Sanz, 2007; Sánchez-Martínez et al., 2007; FAO, 2012), whilst OsHV1 microvariant (OsHV-1 μvar) epizootics between 2008 and 2012 caused the production of Pacific oysters (*Crassostrea gigas*) in France to decline by 40% (Segarra et al., 2010).

Factors that affect the susceptibility of mariculture industries to disease epizootics include zoonoses altering the host specificity of pathogens and the ingression of pathogens from wild populations, reliance on high density monocultures, ease of transmission in the aquatic environment, the availability of intermediate hosts, and environmental degradation as a result of human activities (McCallum et al., 2003; Martin et al., 2010; Pulkkinen et al., 2010). This article focuses on the effects of environmental stress on disease susceptibility in aquaculture. It uses a specific example, QX disease in Sydney rock oysters, to highlight the complex relationships that exist between pathogens, their hosts and the environment.

THE SYDNEY ROCK OYSTER INDUSTRY

The Sydney rock oyster, *Saccostrea glomerata* (previously known as *S. commercialis*), has been cultivated in Australian coastal waters since the 1870's. A decline in natural oyster stocks following European settlement led to the establishment of the first cultivation techniques in state of New South Wales (NSW) in 1872 (Nell, 1993). Initial oyster farming techniques were based on the Claire (ponds) method used throughout France. In the 1930's, the Sydney rock oysters industry adopted a new cultivation system, in which oyster larvae were caught on tarred hardwood sticks placed in estuaries where spatfall is reliable (Angell, 1986). Traditionally, sticks were moved around bays or estuaries after spatfall and oysters were grown to maturity in the inter-tidal zone. More recently, farmers implemented a "single seed" culture method, which involves spat being scraped off sticks after

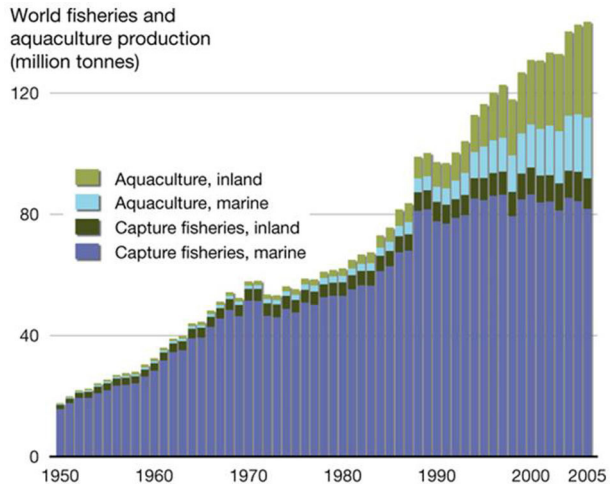

FIGURE 1 | World aquaculture production between 1980 and 2010, showing the contribution from different growing environments (from: http://2011.polarhusky.com/logistics/maps-and-route/data/world-fisheries/).

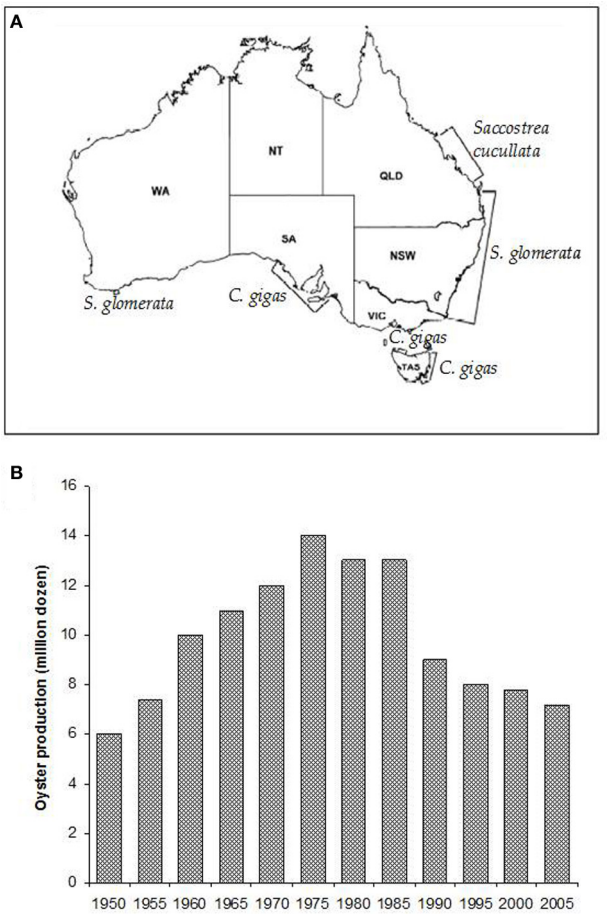

FIGURE 2 | (A) The major oyster growing areas of Australia and the species farmed in those areas (Nell, 2003). **(B)** Sydney rock oyster production grew steadily until the mid-1970's, when pressure from infectious disease epizootics caused a major decline in the industry (Nell, 2003).

settlement. The juvenile oysters are then grown in trays or baskets in sub-tidal and inter-tidal zones (Nell, 1993). The single seed method for capturing spat is now being replaced by hatchery technologies, where spat are produced in hatchery-based selective breeding programs that can control the phenotypic characteristics of oysters.

Sydney rock oysters are produced on the eastern Australian seaboard from the NSW/Victorian border in the south to Moreton Bay in subtropical Queensland (Nell, 1993) (**Figure 2A**). There has also been some success in farming the species on the north coast of Australia and in Western Australia (Nell, 2002).

Sydney rock oyster farming is the fourth biggest oyster aquaculture industry in the world and remains NSW's largest aquaculture industry. However, production levels have fallen by over 40% since the 1970's and remain fragile (**Figure 2B;** Heasman et al., 2000). Three thousand eight hundred and eighty three tonnes of edible oysters were produced in NSW in 2010–2011 (ABARE, 2012). This represents a 22% (1077 tonne, $4.7 million) decrease in edible oyster production compared with 2009–2010. The substantial decline in Sydney rock oyster production since its peak in the 1970's has been due primarily to the impacts of two infectious diseases, Winter Mortality Syndrome, and QX disease.

OYSTER DISEASES

Since the mid-1800's, when overfishing and destruction of natural oyster beds led to the development of oyster farming throughout Europe, disease has been the prominent controlling factor in oyster population dynamics (Roch, 1999). Global oyster production is based almost entirely upon just five species. Hence, oyster industries are usually local monocultures that are subject to inherent disease epizootics. Apart from OsHV1 in Pacific oysters, the majority of oyster diseases are caused by protozoan parasites. The estuarine environments where oysters are cultured provide an ideal medium for the dispersal and survival of parasitic protozoans due to natural currents, stable temperatures, and availability of intermediate hosts. The two main protozoan diseases affecting production of Sydney rock oysters (Winter Mortality and QX disease) only infect *S. glomerata*. The transfer of oysters between estuaries for on-growing during the 1960's was originally thought to have aided the spread of both diseases (Nell, 2003).

The main etiological agent of Winter Mortality is a protist *Bonamia roughleyi* (previously known as *Mikrocytos roughleyi;* Cochennec-Laureau et al., 2003). This includes the parasite in a genus that also commonly afflicts European flat oysters. As its name suggests, Winter Mortality predominantly occurs during colder months from June to August and is restricted to the cooler southern range of Sydney rock oysters. In affected areas, mortalities of up to 80% are common. Oysters in their third winter, just prior to reaching market size, are the most susceptible (Smith et al., 2000). Current methods used to manage the disease include transferring oysters to upstream leases where lower salinities are thought to decrease susceptibility to *B. roughleyi* infection. Many farmers also sell their oysters prior to their third winter when

they are most at risk of infection. However, this option is rarely available to growers in the more southerly regions, because oysters in these colder waters take longer to reach market size (Smith et al., 2000). New information also suggests that other pathogens, in addition to *B. roughleyi*, may be involved in Winter Mortality.

MARTEILIA SYDNEYI AND QX DISEASE

QX (for Queensland Unknown) disease, also known as marteiliosis, is now the most serious disease affecting the Sydney rock oyster industry (Anderson et al., 1995; Adlard and Ogburn, 2003). QX disease is caused by the paramyxean protozoan *Marteilia sydneyi*, which was first described by Perkins and Wolf (1976). The infective season for *M. sydneyi* occurs during the southern hemisphere summer and early autumn, from January to April. Outbreaks of QX disease were first recorded during the late 1970's and were restricted to a small number of estuaries from The Great Sandy Straight of southern Queensland through to the Macleay River in northern NSW (Adlard and Ernst, 1995) (**Figure 3**).

The parasite was later discovered in the Georges River, Sydney, in 1994. Examination of *S. glomerata* digestive tract samples taken from Lime Kiln Bar (33°59′08″S, 151°03′10″E) in the Georges River identified tricellular sporonts that were typical of *M. sydneyi* infections (Adlard and Ernst, 1995). During 1993/1994 over 13 million oysters were produced in the Georges River. However, consistent seasonal outbreaks of QX disease have caused production to steadily decline so that only 750,000 oysters were produced there in 2000/2001. This constitutes a fall in production of 94% over 6 years (Nell and Hand, 2003). Mortality rates from QX disease can reach 95% per year (Peters and Raftos, 2003). Current production in the Georges River is restricted to selectively bred, *M. sydneyi*-resistant strains. Pacific oysters, which are unaffected by QX disease, were introduced as a replacement for Sydney rock oysters in the Georges River. However, these Pacific oysters have since been devastated by outbreaks for OsHV1 µvar, which began in 2010.

The Hawkesbury River, approximately 50 km north of the Georges River in NSW, is the most recent growing area to experience an outbreak of QX disease. This estuary had previously been the second largest producer of Sydney rock oysters in Australia. The disease was first detected in the Hawkesbury River during 2004, when an examination of oysters from upstream oyster leases uncovered sporulating *M. sydneyi* in oyster guts. By the end of 2005, Sydney rock oysters in most growing areas of the estuary had suffered mortality rates of up to 90% (Butt and Raftos, 2007). Again, Pacific oysters were used to replace Sydney rock oysters in the Hawkesbury River, and again they were devastated (up to 100% mortality) by an outbreak of OsHV1 µvar in 2013.

A sensitive, polymerase chain reaction (PCR) diagnostic assay for *M. sydneyi* has been used to test for the presence of the parasite in numerous estuaries along the NSW coastline (Adlard and Worthington-Wilmer, 2003). These tests have shown that *M. sydneyi* is present in the vast majority of *S. glomerata* growing estuaries on the NSW coast, including those that had previously been thought to be parasite free (Adlard and Wesche, 2005). There are approximately 40 estuaries in which Sydney rock oysters are grown, but only 7 of these have experienced QX disease outbreaks. Based on these data, it is now widely believed that *M. sydneyi* is an enzootic parasite of Sydney rock oysters throughout Australia (Adlard and Wesche, 2005).

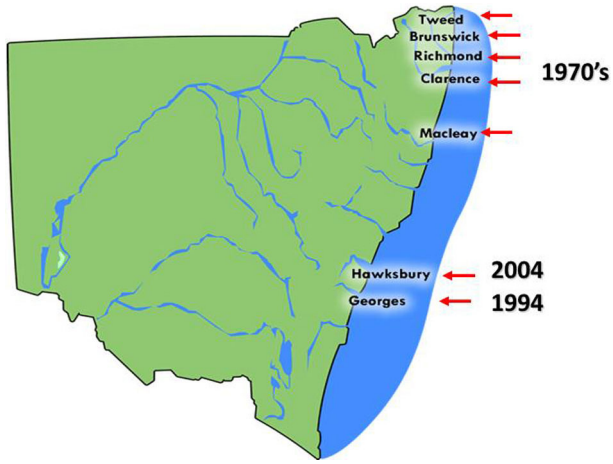

FIGURE 3 | The distribution of Sydney rock oyster farming estuaries on the coast of NSW, Australia that are affected by QX disease and the year in which QX disease outbreaks first occurred.

THE *M. SYDNEYI* LIFE CYCLE

The original description of *M. sydneyi* by Perkins and Wolf (1976) focused on pathogen sporulation in the digestive gland of the oyster host. Traditional laboratory detection techniques limited further elucidation of *M. sydneyi's* early development. However, the use of DNA probes for *in situ* hybridization has allowed the site of initial infection and subsequent development of the parasite within oysters to be defined (**Figure 4**) (Kleeman et al., 2002). The earliest infectious stage of *M. sydneyi* that can be identified in oysters is a uninucleate stem cell. These stem cells were discovered in the palps and gill epithelia of *S. glomerata*. Their presence in these epithelial tissues suggests that infection results from a "free-floating" parasitic stage entering the gills during filter feeding (Kleeman et al., 2002). Stem cells proliferate in the gill epithelium. Once sufficient numbers of stem cells have been generated, they penetrate the basal membrane, entering connective tissues. This enables their dissemination throughout the oyster.

The majority of stem cells converge on the digestive gland, where they enter the digestive tubule epithelium (Kleeman et al., 2002) (**Figure 5**). Once in the digestive gland, replication and further development continues to form a primary 2-celled plasmodium. This plasmodium divides to form between 8 and 16 sporonts. Each sporont then undergoes further internal division to form two spores, each with three concentric cells (Roubal et al., 1988). Spores are shed into the environment via the alimentary canal prior to the death of the oyster (Anderson et al., 1995).

Despite our understanding of *M. sydneyi's* development within oysters, it is only recently that the fate of the parasite has been determined once it is shed from infected oysters. Wesche et al. (1999) assessed spore survival after their release into the environment. They found that spores are relatively short-lived outside their oyster host (7–35 days) when compared to the 3–10 month infection cycle of the pathogen within oysters. Also, no

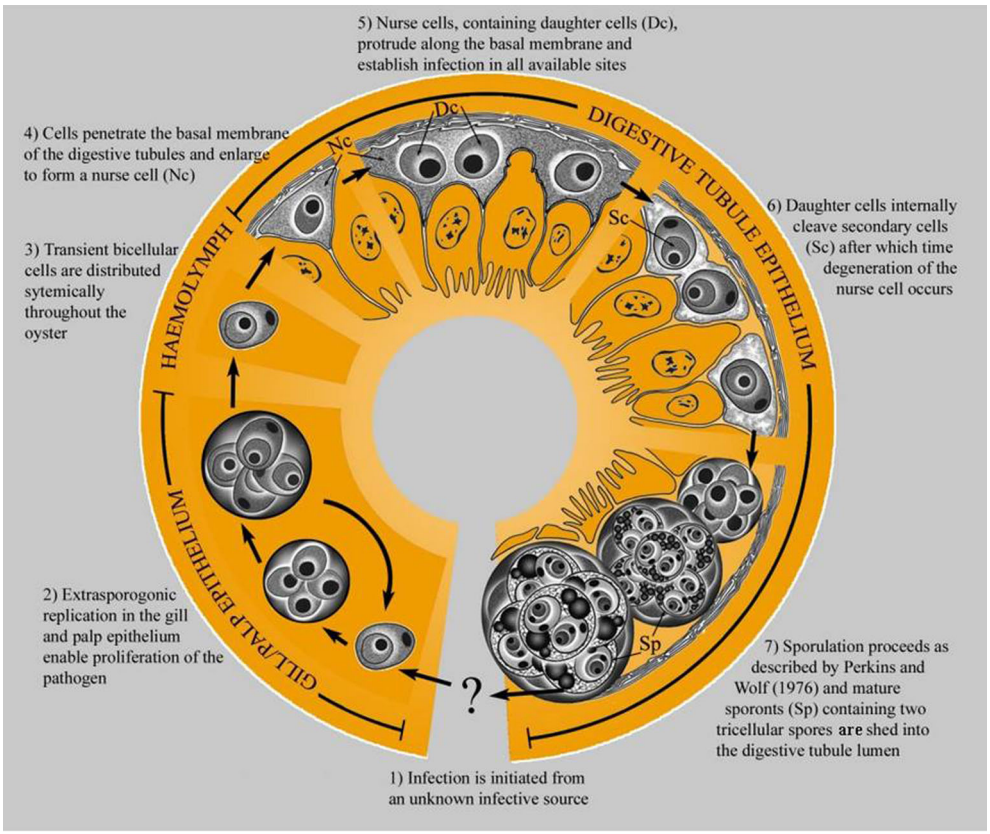

FIGURE 4 | The life cycle of *M. sydneyi* within Sydney rock oysters (Kleeman et al., 2002).

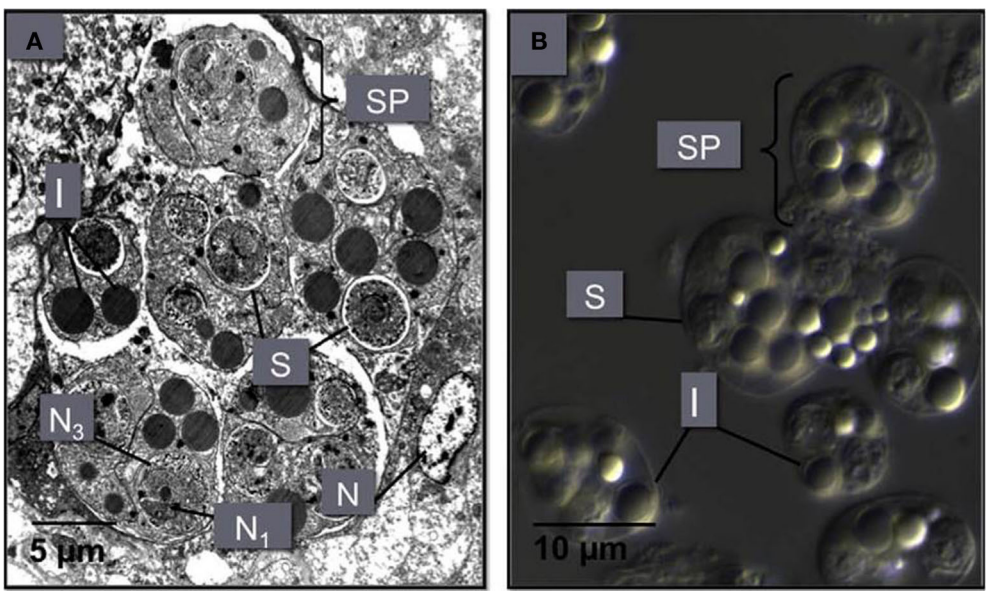

FIGURE 5 | (A) An *M. sydneyi* sporangiosorus in the digestive gland of an infected oyster. This sporangiosorus contains six individual reproductive bodies (sporonts or secondary cells, SP). Cleavage of the sporangium (cytoplasm of the sporont) leads to the development of two to three multinucleated (N1, N3) spores (S), surrounded by inclusion bodies (I). The nucleus (N) of an oyster hemocyte adjacent to the sproangiosorus is also shown. **(B)** A differential interference contrast micrograph of *M. sydneyi* sporonts purified by density gradient centrifugation (Kuchel et al., 2010a,b).

morphological development of the parasite could be detected outside the host and energy reserves were deemed to be insufficient for prolonged survival (Wesche et al., 1999).

These results indicated that there may be an intermediate host for *M. sydneyi*. The existence of an intermediate host was supported by transmission trials on the closely related *Marteilia refringens*, agent of Aber Disease of flat oysters in Europe (Berthe et al., 1998). Healthy European flat oysters did not become infected when inoculated with live *M. refringens* cells, regardless of inoculum strength. Cohabitation trials under laboratory conditions also failed to transmit the parasite between oysters. It was only when uninfected oysters were placed in natural environments within the endemic range of the parasite that infections occurred (Audemard et al., 2001). Further research by Audemard et al. (2002) found that a copepod, *Paracartia grani*, became infected with *M. refringens* after 7 days cohabitation with infected oysters. The population dynamics of *P. grani* in *O. edulis* growing areas also match its potential role as an intermediate host for *M. refringens* (Audemard et al., 2004). However, the full life cycle of *M. refringens* has not been closed because re-infection (*P. grani* to *O. edulis* transmission) experiments have not been successful.

It was originally thought that the intermediate hosts of the QX parasite, *M. sydneyi*, might be scavenging carnivores that ingest spores as they feed on dead oyster tissue. However, the subsequent discovery that *M. sydneyi* spores are released prior to the death of *S. glomerata* suggested that benthic or filter-feeding organisms are more likely intermediate hosts (Roubal et al., 1988). This was further supported by the observation that shed spores have a negative buoyancy and so were likely to sink to the sediment below oyster racks (Wesche et al., 1999). In 2000, Kleeman and Adlard developed PCR and *in situ* hybridization assays to finally resolve the identification of an intermediate host for *M. sydneyi*. Their assays targeted the ribosomal DNA of *M. sydneyi* so that potential hosts could be tested for the presence of the parasite with high precision and specificity. However, definitive evidence for an intermediate host remains unavailable.

OYSTER IMMUNE RESPONSES AGAINST *M. SYDNEYI*

Despite the lethality of *M. sydneyi* infections in some Sydney rock oyster growing areas, there is strong evidence to suggest that oysters can mount effective immune responses against *M. sydneyi*. Butt and Raftos (2007) examined the response of oyster hemocytes (the oyster equivalent of circulating blood cells) to *M. sydneyi in vitro*. They found that both granulocytes and hyalinocytes were able to rapidly ingest parasite sporonts by the process of phagocytosis (**Figure 6**).

In vitro experiments showed that phagocytosis of *M. sydneyi* stimulated intracellular activity of the defensive enzyme, phenoloxidase. This led to the complete melanization of phagosomes containing parasites, and presumably the destruction of the ingested *M. sydneyi* (**Figure 7**). The role of phenoloxidase in phagolysosomal activity against *M. sydneyi* was supported by an electron microscopical study by Kuchel et al. (2010a). They showed that, after ingestion of *M. sydneyi*, granules in *S. glomerata* hemocytes that contain phenoloxidase (Aladaileh et al., 2007a), fuse with phagosome membranes and that the pH of phagosomes decreases in a typical phagolysosomal response (**Figure 8**).

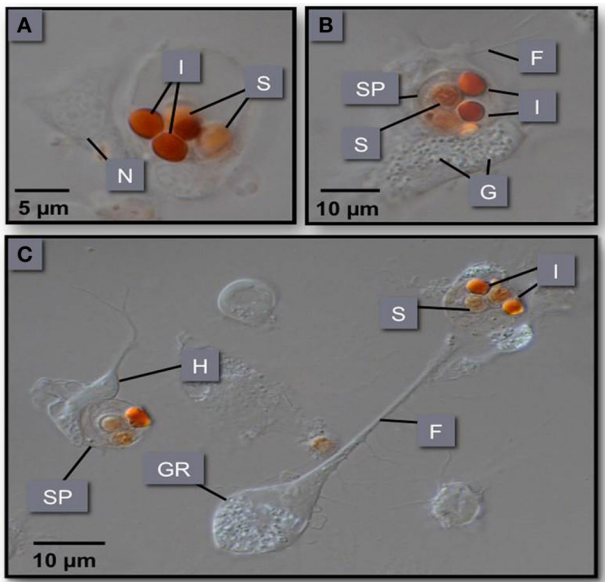

FIGURE 6 | (A–C) Phagocytosis of *M. sydneyi* sporonts by *S. glomerata* hemocytes. H, hyalinocyte; GR, granulocyte; SP, sporont; I, inclusion bodies within sporonts stained with Congo red; F, filopodia; N, nucleus; G, intracellular granules within hemocytes.

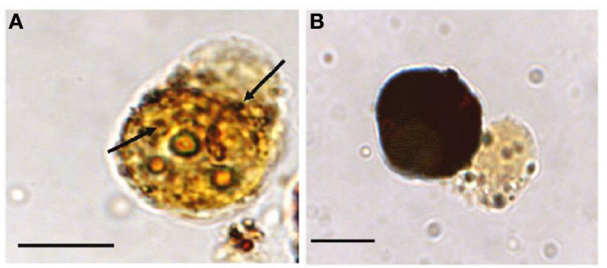

FIGURE 7 | Melanization of *M. sydneyi* by phenoloxidase activity within *S. glomerata* hemocytes. (A) Initially, melanization (brown coloration) is focused around phagocytosed *M. sydneyi* sporonts. Arrows indicate granular melanin deposition around ingested parasite. **(B)** As melanization continues, entire hemocytes, including the internalized sporont(s), become melanized. Bars = 5 µm (Butt and Raftos, 2008).

In addition, Kuchel et al. (2010a) observed the deposition of phenoloxidase metabolites in phagosomes after *in vitro* phagocytosis of *M. sydneyi* sporonts by *S. glomerata* hemocytes. Most importantly, ingested and melanized *M. sydneyi* have also been detected *in vivo* among hemocytes from infected oysters (Butt and Raftos, 2008). All of these data suggest that Sydney rock oyster hemocytes can recognize and phagocytose *M. sydneyi*, and that phenoloxidase is a critical intracellular effector mechanism that acts against ingested *M. sydneyi* as part of the phagolysosomal process.

Phenoloxidase is a key enzyme in the immunological defenses of invertebrates (Söderhäll and Cerenius, 1998). The role of phenoloxidase in host defense is best characterized in arthropods, although numerous studies have also demonstrated its

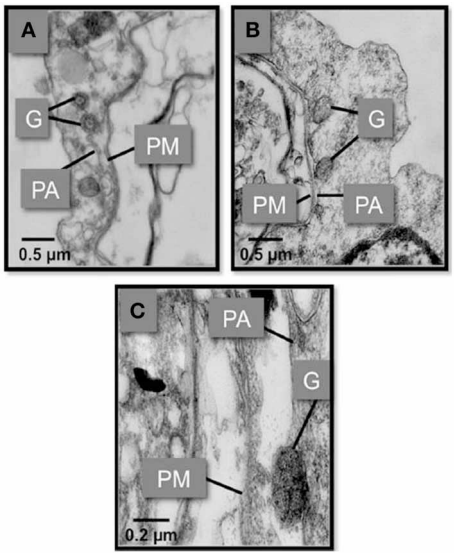

FIGURE 8 | Transmission electron micrographs showing the fusion of *S. glomarata* hemocyte granules with phagosomes containing ingested *M. sydneyi*. (A) Hemocyte granules (G) approach and fuse to the phagosomal membrane (PA) that surrounds a *M. sydneyi* sporont. PM, parasite membrane. **(B)** Granules fuse to the phagosome containing *M. sydneyi* sporonts. **(C)** High resolution image of a granule fusing with the phagosome membrane around an *M. sydneyi* sporont (Kuchel et al., 2010a,b).

importance in bivalve molluscs (Deaton et al., 1999; Jordan and Deaton, 2005; Munoz et al., 2006; Aladaileh et al., 2007a; Hellio et al., 2007; Butt and Raftos, 2008). The enzyme facilitates the formation of the pigment melanin, which is important in the sequestration of foreign material during defensive encapsulation (Söderhäll and Cerenius, 1998). Melanin and its intermediate metabolites in the phenoloxidase pathway also have direct antimicrobial activities in both extracelullular fluids and intracellular phagolysosomes (Asokan et al., 1997).

We have made similar observations on the roles of phenoloxidase in the immune system of Sydney rock oysters. Aladaileh et al. (2007a) demonstrated that phenoloxidase is a key component of intracellular granules within *S. glomerata* phagocytes. It is also evident that intracellular phenoloxidase in *S. glomerata* can produce metabolites that are associated with antimicrobial activity (Aladaileh et al., 2007b), and that challenging Sydney rock oyster hemocytes with pathogen-associate molecules increases phenoloxidase activity (Aladaileh et al., 2007c).

ENVIRONMENTAL STRESS AND DISEASE IN MOLLUSCS

Given that Sydney rock oysters clearly have effective immunological defenses against *M. sydneyi*, the outstanding question about QX disease is, why do oysters lose control of the parasite leading to outbreaks of lethal disease? Our work, described in detail below, suggests that environmental stress results in the suppression of the Sydney rock oyster immune system, and that this immunosuppression contributes to QX disease epizootics.

The survival of all organisms depends upon their ability to maintain homeostasis in highly variable environments. This balance is perhaps most difficult to maintain in sessile poikilothermic osmoconformers, such as bivalve molluscs. External stressors constantly threaten the physiological steady state of these organisms (Lacoste et al., 2002). Stressors to which oysters are exposed vary widely, but include extremes of temperature, salinity, and pH, as well as anthropogenic factors (Kuchel et al., 2011). The estuarine environment, which is home to commercial and wild oyster populations, is prone to extreme hydrological changes. These changes are associated with tidal fluctuations and rainfall events, and can lead to the introduction of effluent (nutrient loading and chemical contamination) and increased sediment loads resulting from upstream runoff. Oysters require a range of adaptive responses to counteract these stressors. Such responses, or the stressors themselves, often affect the effective function of physiological mechanisms, including the immune system. Immunosuppression, which can result from environmental stress, leaves organisms more susceptible to disease epizootics (Chu et al., 2002).

The link between aquaculture species, pathogens, and the environment has been acknowledged for some time (Kuchel et al., 2011). As long ago as 1974, it was recognized that disease outbreaks in fish only occurred when environmental conditions were suitable (Snieszko, 1974). However, mechanistic links between host immunological defense and environmental change has only been established far more recently. Lacoste et al. (2001a,b,c, 2002) have shown that oysters possess a form of catecholamine-based neuroendocrine response similar to the adrenergic system that is activated in vertebrates during acute stress responses. Their work demonstrated that, as environmental conditions change, immunological function in marine invertebrates can be inhibited by adaptive changes to their own physiology mediated by the catecholamine hormone, noradrenaline. Once released into the hemolymph, noradrenaline was shown to decrease hemocyte phagocytic activity and the production of reactive oxygen species (ROS) in phagolysosomes (Lacoste et al., 2001a,c).

Other studies have investigated the types of environmental or anthropogenic stressors that affect immunological activity in molluscs. Pipe et al. (1999) examined the effects of copper on various immunological parameters in the marine mussel, *Mytilus edulis*. They identified dose-dependent changes in hemocyte numbers, particularly a decrease in the frequency of circulating eosinophilic granulocytes, after copper exposure. These eosinophilic cells are responsible for most peroxidase, phenoloxidase, and phagocytic activity in *M. edulis* (Pipe et al., 1997). However, contrary to expectations, decreases in phenoloxidase and peroxidase activity in copper-affected mussels were not statistically significant. This could be explained in part by the large variability in the activity of these enzymes between individual mussels (Pipe et al., 1999).

Further work on the effects of anthropogenic pollutants on the *M. edulis* immune system showed that polycyclic aromatic hydrocarbons (PAH), such as fluoranthene and phenanthrene, inhibit phagocytic activity and damage lysosomes. PAH's accumulate within lysosomes so that damage is caused by direct physical disturbance of the lysosomal membrane. Similar disruption inhibits phagocytosis (Grundy et al., 1996). The combined stress of high temperature and copper exposure was also shown

to have deleterious effects on *M. edulis* hemocytes. Both total and differential hemocyte counts were affected, as were superoxide production and phagocytic activities (Parry and Pipe, 2004).

Pacific oysters (*C. gigas*) have also been tested to determine the effects of mechanical disturbance on immunological function. The effects of mechanical agitation were investigated because oyster culture techniques require individuals to be continually sorted and redistributed throughout their development using mechanical grading machines. Lacoste et al. (2002) tested whether such disturbance made oysters more susceptible to disease outbreaks. They found that hemocyte chemotaxis, phagocytosis and ROS production were all inhibited after continuous shaking for 15 min. These immunological parameters rapidly recovered in the 60–90 min after shaking, indicative of an acute hormonal stress response. Similar results were reported when abalone (*Haliotus tuberculata*) were exposed to mechanical disturbance. Immediately after agitation, hemocyte numbers, as well as amoeboid, phagocytic, and superoxide activities, were significantly decreased. This was followed by a compensatory increase in most parameters 4 h after the stress (Malham et al., 2003). In both of these studies, noradrenaline and dopamine concentrations were measured as stress indicators. The concentrations of both catecholamine hormones increased immediately after the onset of the mechanical disturbance. Such results suggest a direct link between physical stress, hormonal responses, and immunological impairment (Lacoste et al., 2002; Malham et al., 2003).

However, other studies on oysters have demonstrated very different effects depending upon the type of stress applied. In Eastern oysters (*C. virginica*) exposure to various concentrations of tributyltin was found to have negligible effects on immunological activity (Anderson et al., 1996). Similarly, exposure to different salinity levels had no detectable effect on lysozyme or respiratory burst activities in the European flat oyster, *O. edulis* (Hauton et al., 2000). This contrasted studies looking at dietary effects on immunological activity in *C. gigas*. Zhang and Li (2006) found that starving oysters for 42 days reduced condition indices and lysosomal membrane integrity. Alternatively, improving nutrition in Pacific oysters increased oxidative activity and phagocytic clearance rates. These results were also replicated in similar trials using the Manila clam, *Ruditapes philippinarum* (Delaporte et al., 2003).

Normal physiological variables have also been shown to impair immunological activity. For instance, gametogenesis in Pacific oysters reduces phagocytic activity and hemocyte function (Delaporte et al., 2006). It is thought that these physiological changes associated with broadcast spawning could explain some of the seasonal variability in immunological activity observed in many molluscan species and their susceptibility to disease (Duchemin et al., 2007).

Even though the role of environmental stress in the impairment of immune responses has been established in a variety of aquaculture species, the effects that this immunological suppression has on disease susceptibility has only been investigated in a small number of host-pathogen relationships (Lafferty and Kuris, 1999). One study investigated the collapse of the Black abalone (*Haliotis cracherodii*) fishery on the Californian coast. Field studies concluded that the combination of numerous stressors, including increased water temperatures, pollutants, over-fishing, and competition from sea urchins left the abalone susceptible to the etiological agent of withered foot syndrome, *Xenohaliotis americanus* (Davis et al., 1992; Lafferty and Kuris, 1999). Laboratory studies using another abalone species, *Haliotis diversicolor supertexta*, found that exposures to ammonia, high temperatures and low dissolved oxygen concentrations all impaired cellular immunological responses. As a result, the abalone in all treatments showed increased susceptibility to infection by *Vibrio parahaemolyticus* (Cheng et al., 2004a,b,c).

Disease susceptibility in the Eastern oyster, *C. virginica,* has also been shown to increase in response to anthropogenic pollutants (Chu and Hale, 1994; Fisher et al., 1999). However, Chu et al. (2002) concluded that this increased susceptibility may not have been caused by immunological inhibition. Despite testing a range of cellular and humoral immunological parameters, no differences could be detected between control oysters and those exposed to contaminated sediments.

THE ROLE OF ENVIRONMENTAL STRESS IN QX DISEASE

Similar associations between environmental stress, the immune system and disease have been identified in QX disease outbreaks. Peters and Raftos (2003) found that inhibition of oyster immunological function (reflected by phenoloxidase activity) is associated with infective periods for *M. sydneyi*. The fact that phenoloxidase activity was suppressed in oysters that were not actively infected with *M. sydneyi* suggests that external influences, and not *M. sydneyi* itself, were responsible for this inhibition of the immune system.

Anecdotal evidence from oyster farmers had long pointed to a link between QX disease and environmental factors. Initial examination of QX disease in southern Queensland suggested that outbreaks occurred after heavy summer rainfall (Haysom, 1978; Lester, 1986). This prompted studies to determine whether epizootics of *M. sydneyi* were triggered by a drop in environmental pH associated with runoff from acid sulfate soils. Early work by Anderson et al. (1995) found that *M. sydneyi* infection still occurred during periods when no major pH fluctuations were observed. However, a subsequent study by Wesche (1995) found that infection outbreaks did occur soon after a major drop in environmental pH, even though no causal relationship between pH and QX disease outbreaks could be established. It was also demonstrated that substantial drops in pH could occur without resulting in a QX disease epizootic.

A breakthrough in linking QX disease to environmental stress came from a study by Peters and Raftos (2003), which again tested a relationship between rainfall and disease, this time focusing on low salinity rather than altered pH. They used field trials to show that the activity of the key defensive enzyme, phenoloxidase, was consistently lower in oysters held in QX disease prone areas, relative to those in QX disease free locations. This suggested that suppression of phenoloxidase activity may be responsible for QX disease outbreaks. Reanalysis of their data to include the salinity of the water at the different locations over time identified a strict relationship between low salinity associated with summer rainfall, suppression of phenoloxidase activity and increasing intensity of *M. sydneyi* infection. This work (Peters and Raftos, 2003) also provided an explanation for temporal variation in QX

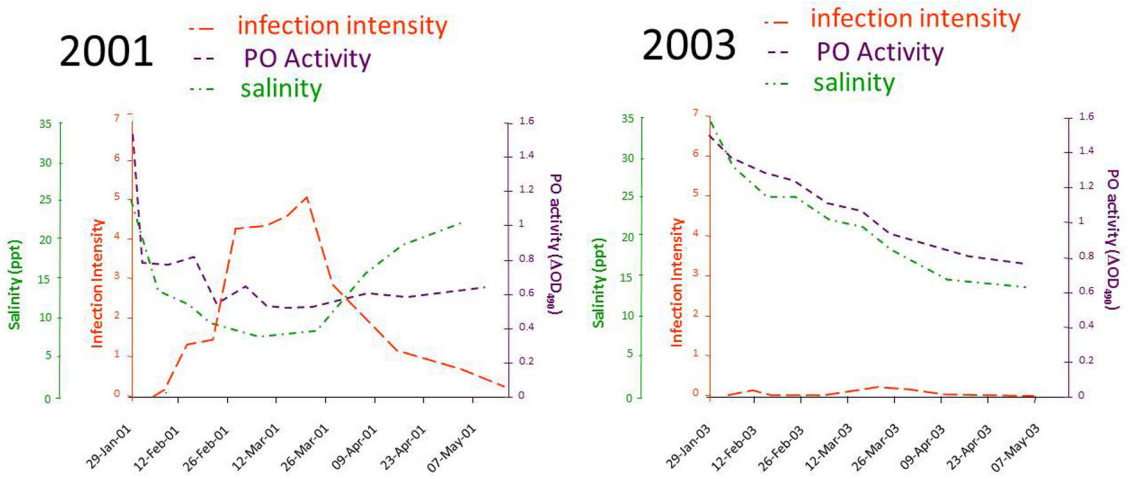

FIGURE 9 | Results of a field trial conducted in the Georges River, Sydney, between 2001 and 2004. Data were recorded for the salinity of water in the river, the phenoloxidase (PO) activities in hemolymph from oysters held in the river, and the intensity of *M. sydneyi* infections within those oysters. Data were recalculated from those reported in Peters and Raftos (2003).

disease, wherein severe disease outbreaks can occur within an oyster growing area one year, but not the next. Their field studies in the Georges River showed substantial differences in salinity, phenoloxidase activity, and the intensity of *M. sydneyi* infection between 2001, a year in which seasonally average levels of rainfall were recorded in the Georges River catchment during summer, and 2003, which was the beginning of a major drought on the east coast of Australia (**Figure 9**).

The link between low salinity, immune suppression, and QX disease was later supported by Butt et al. (2006), who demonstrated that phenoloxidase activities were proportionally lower in oysters exposed under laboratory conditions to low (7 ppt) and intermediate (13.5 ppt) salinities, relative to those held under "normal" (34 ppt) conditions. This correlation between low salinity and low phenoloxidase activity was also evident in experiments in which oysters were held in water from different sites within the same river system that had different levels of salinity (Butt et al., 2006). Significantly, these data also matched historical observations that QX disease outbreaks are more severe in the low saline, upper reaches of estuaries. It may also explain why more estuaries are affected by QX disease in sub-tropical regions of Australia (northern NSW and southern Queensland) where average annual rainfall can be more than twice that of temperate (southern) areas.

Further investigation revealed that a range of environmental stressors, not just low salinity, have substantial effects on the Sydney rock oyster immune system that might be associated with disease susceptibility. For instance, Butt et al. (2008) showed that the muscle relaxant, magnesium chloride, which was being trialed for use in Sydney rock oyster hatcheries, significantly affected a range of parameters associated with immune function. Total hemocyte frequencies, acid phosphatase activities, and superoxide production were all found to increase within 48 h of exposing oysters to magnesium chloride. In contrast, the phenoloxidase activities of oysters exposed to magnesium chloride declined significantly relative controls. All of these responses were relatively short term (96 h), again indicating an acute stress response.

Starvation also has a modulatory effect on Sydney rock oysters. Butt et al. (2007) demonstrated that the frequency of hemocytes and phenoloxidase activity in oyster hemolymph decreased by up to 25% in oysters whose diet had been halved relative to fully fed controls. Superoxide and peroxidase production also decreased significantly when oysters were starved (no food) for 2–4 weeks. All of these parameters returned to normal when starved oysters were fed. The recovery of phenoloxidase activities over-compensated during the recovery (full feeding) phase of the experiment to the extent that phenoloxidase activities post-recovery were substantially higher than those before starvation.

These data suggest that a range of well-defined stressors can affect the Sydney rock oyster immune system and may be associated with disease susceptibility. However, in some cases, the nature of the environmental perturbation associated with immune suppression and disease remains unknown. Butt and Raftos (2007) investigated a QX disease outbreak during 2005 in the Hawkesbury River, Sydney, in an effort to identify environmental variables associated with the disease in that river system. As in the Georges River, they found that phenoloxidase (and antimicrobial) activity was significantly inhibited during a key period of *M. sydneyi* infectivity (January–March 2005) and that phenoloxidase inhibition was strictly correlated with the intensity of *M. sydneyi* infection in oysters. The data indicated that some transient environmental stressor may have affected phenoloxidase activity during the critical infection window, increasing susceptibility of oysters to disease. However, the simultaneous analysis of a broad range of environmental variables (salinity, temperature, pH, algal density, chlorophyll a concentration, and dissolved oxygen) failed to identify any single factor that was associated with decreased phenoloxidase activity or disease intensity.

IMMUNOSUPPRESSION AND DISEASE SUSCEPTIBILITY MAY BE ASSOCIATED WITH PROGRAMMED CELL DEATH

Even though there is strong evidence for a link between environmental stress and disease susceptibility, information is only now emerging to indicate a mechanistic basis for this association. A number of studies have shown that environmental stressors affect oyster hemocytes, which are the main mediators of immune responses. For instance, Kuchel et al. (2010b), Kuchel and Raftos (2011) investigated the effects of mechanical agitation, hypo-saline conditions, and exposure to the air on the hemocytes of Akoya pearl oysters (*Pinctada imbricata*). They found that both phagocytosis and phenoloxidase activity

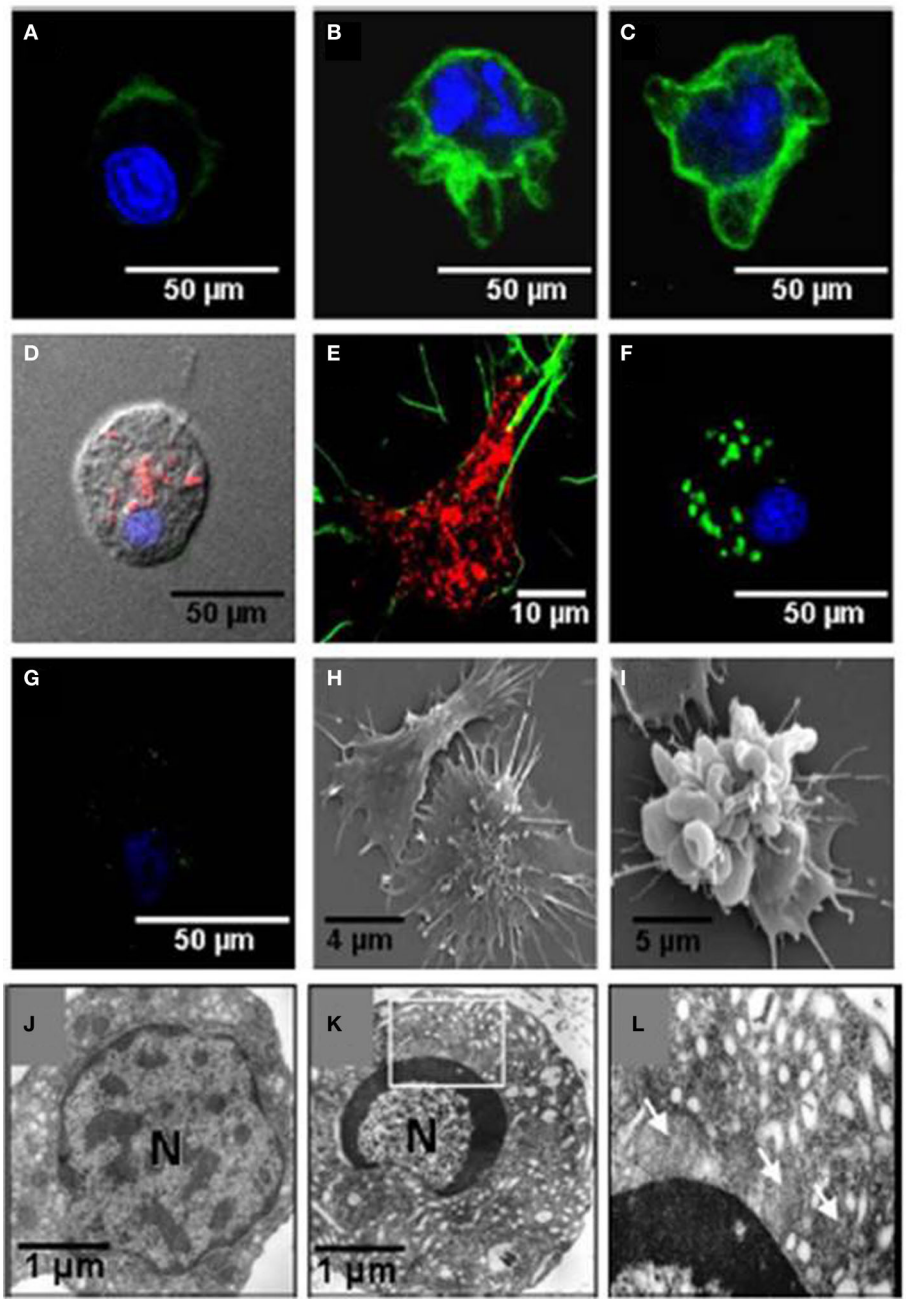

FIGURE 10 | Effects of noradrenaline on hemocytes morphology. Hemocytes were stained for mitochondrial membrane potential (MitoTracker, red), F-actin (phalloidin-Alexa Fluor 488, green), and nuclear DNA (TO-PRO-3, blue). **(A–C)** Show stained hemocytes after different periods of noradrenaline treatment (10, 20, and 30 min, respectively). **(D,E)** are untreated hemocytes. **(F,G)** Show noradrenaline-untreated and treated hemocytes, respectively, stained for mitochondrial membrane potential with DiOC6(3) (green) and nuclear DNA with TO-PRO-3 (blue). **(H,I)** Show scanning electron micrographs of untreated hemocytes and noradrenaline-treated hemocytes, respectively. **(J,K)** Show transmission electron micrographs of untreated hemocytes, and noradrenaline-treated hemocytes, respectively. The box in **(K)** is enlarged in **(L)**. Mitochondria are shown by arrows. (Aladaileh et al., 2008b).

decreased significantly when oysters were exposed to all three stressors. Transient decreases were also evident in total hemocyte counts after mechanical stress and exposure to air, while significant increases in total hemocyte counts occurred after exposure to low salinity. Most significantly, the frequency of different hemocyte sub-populations in the hemolymph of *P. imbricata* was significantly altered when oysters were subjected hypo-saline conditions.

Lacoste et al. (2001a,b,c, 2002) began to uncover the mechanistic basis of these cellular responses to stress by demonstrating clear links between adrenergic stress responses, the suppression of cell-mediated immune responses, and disease susceptibility in Pacific oysters. This early work was then taken forward by Aladaileh et al. (2008a,b) and Kuchel and Raftos (2011). Aladaileh et al. (2008a) showed that noradrenaline secretion in Sydney rock oysters was stimulated by altered salinity, extremes of temperature, and physical agitation. This suggested that environmental factors that are commonly associated with oyster farming lead to adrenergic stress responses. The same study demonstrated that injecting noradrenaline into *S. glomerata* inhibits the phenoloxidase activities of both whole hemolymph and serum. It also decreases the frequency of hemocytes in hemolymph, alters differential hemocyte frequencies (including the frequency of phenoloxidase-positive cells), and inhibits phagocytic activity. Additional *in vitro* studies showed that the production of reactive oxygen intermediates, such as superoxide and peroxide, by hemocytes increased in the presence of noradrenaline.

The effects of noradrenaline on the function of the Sydney rock oyster immune system were linked to changes in the composition of the defensive hemocyte population by Aladaileh et al. (2008b). Noradrenaline was shown to induce some of the typical features of programmed cell death (apoptosis) in *S. glomerata* hemocytes. These features included the loss of mitochondrial membrane potential, DNA fragmentation, and plasma membrane "blebbing." Restructuring of the F-actin cytoskeleton was associated with these changes, which could explain why hemocyte adhesion and pseudopodia formation by hemocytes were inhibited by noradrenaline (**Figure 10**).

Similar observations have been made in the Akoya pearl oyster (*P. imbricata*) (Kuchel and Raftos, 2011; Kuchel et al., 2011). They found that treating *P. imbricata* hemocytes *in vitro* with noradrenaline resulted in enhanced DNA fragmentation relative to controls. Annexin V-FITC staining, a marker of early apoptotic events, and hemocyte adhesion were also significantly affected by exposure to noradrenaline. In addition, morphological and ultrastructural alterations that are typical of apoptosis were identified in noradrenaline treated hemocytes using transmission and scanning electron microscopy. These changes included chromatin and cytoplasmic condensation, the formation of apoptotic bodies, vacuolization, and blebbing. Polymerization of F-actin was also observed around the periphery of the cytoplasm.

All of these data support a model in which the apoptotic cell death caused by hormonal responses to environmental stress cause a depletion of critical hemocytes populations in oysters, resulting in immunosuppression and disease susceptibility.

CONCLUSIONS AND FUTURE DIRECTIONS

Infectious diseases are the main factors that limit the production of food and other products by aquaculture industries worldwide. Substantial evidence suggests that disease susceptibility in at least some aquaculture species, notably *S. glomerata*, is closely linked to the immunosuppressive effects of environmental stress. These effects seem to be mediated by hormonal stress responses that result in the apoptotic death of crucial hemocytes populations involved in immunological defense. Our next challenge is to understand the subcellular processes that result in apoptosis, and how these may be controlled to develop disease resistant populations for aquaculture. We are currently investigating whether environmental stress leads to increased metabolic activity with the consequent production of harmful ROS within oyster hemocytes that damages the cytoskeleton and initiates apoptosis. At a broader level, aquaculture industries worldwide need to start integrating our growing understanding of the cellular and genetic basis of disease resistance into effective management practices, such as marker assisted selection for disease resistance.

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A comprehensive analysis of the microbial communities of healthy and diseased marine macroalgae and the detection of known and potential bacterial pathogens

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Microorganisms are increasingly being recognized as the causative agents in the diseases of marine higher organisms, such as corals, sponges, and macroalgae. *Delisea pulchra* is a common, temperate red macroalga, which suffers from a bleaching disease. Two bacterial strains, *Nautella italica* R11 and *Phaeobacter gallaeciensis* LSS9, have been shown *in vitro* to cause bleaching symptoms, but previous work has failed to detect them during a natural bleaching event. To provide a link between *in vitro* observations and natural occurrences of the disease, we employ here deep-sequencing of the 16S rRNA gene to comprehensively analyze the community composition of healthy and diseased *D. pulchra* samples from two separate locations. We observed operational taxonomic units (OTUs) with 100% identity and coverage to the 16S RNA gene sequence of both *in vitro* pathogens, but only the OTU with similarity to strain LSS9 showed a statistically significant higher abundance in diseased samples. Our analysis also reveals the existence of other bacterial groups within the families *Rhodobacteraceae* and *Flavobacteriaceae* that strongly contribute to difference between diseased and healthy samples and thus these groups potentially contain novel macroalgal pathogens and/or saprophytes. Together our results provide evidence for the ecological relevance of one kind of *in vitro* pathogen, but also highlight the possibility that multiple opportunistic pathogens are involved in the bleaching disease of *D. pulchra*.

Keywords: delisea pulchra, bacterial disease, macroalgae, bleaching, opportunistic pathogens

INTRODUCTION

Marine sessile macroorganisms, such as seaweed, sponges, and corals, are often colonized by a large number and diversity of bacteria, with which they can have either positive, neutral or negative interactions (Ainsworth et al., 2010; Egan et al., 2012; Hollants et al., 2013). In recent years, disease caused by microorganisms, have been increasingly recognized as a major negative interaction that influences the composition and function of benthic community members (Bourne et al., 2009; Egan et al., 2013). Disease of marine invertebrates and macroalgae has also been clearly linked to changes in the marine environment, such as anthropogenic stressors (pollutants, urbanization etc.) and climate change (e.g., Campbell et al., 2011). However, linking bacterial pathogens to particular diseases in the marine environment is often challenging (Rosenberg et al., 2009; Egan et al., 2013). This can be due to many factors, including the opportunistic nature of pathogens, the existence of multiple pathogens causing the same disease and/or the inability to sensitively detect a particular pathogen in complex environmental samples.

Delisea pulchra is a marine red macroalgae commonly found across the temperate Eastern coast of Australia, but is also found as widespread as Japan and Antarctica (Papenfuss, 1964). *D. pulchra* suffers from a bleaching disease, which is characterized by a loss of pigments and which occurs more frequently during summer months, when ultra-violet light radiation and temperature are elevated (Campbell et al., 2011). The bleaching disease has also been shown to have a significant impact on fecundity and survival of the red algae (Campbell et al., 2014). The involvement of bacteria in the bleaching disease is implied by a significant difference in the bacterial community between healthy and diseased *D. pulchra* individuals (Campbell et al., 2011; Fernandes et al., 2012). Furthermore, two pathogens, *Nautella italica* R11 and *Phaeobacter gallaeciensis* LSS9, have been isolated that can cause the bleaching disease *in vitro* (Case et al., 2011; Fernandes et al., 2011). In the laboratory, these two pathogens can invade the tissue of *D. pulchra* under conditions of elevated temperature and when the alga's chemical defense based on UV-sensitive molecules called furanones is reduced. These *in vitro* experiments link pathogen function with the relevant ecological stressors or changes. However, previous studies using denaturing gradient gel electrophoresis (DGGE) from multiple bleaching events (Campbell et al., 2011) and 16S rRNA gene sequencing of clone libraries from a single bleaching event could not detect the two *in vitro* pathogens (Fernandes et al., 2012). Therefore, a link between the *in vitro* bleaching observations (Case et al., 2011; Fernandes et al., 2011) and environmental disease events (Campbell et al., 2014) remains to be determined.

The previous inability of Fernandes et al. (2012) to detect *N. italica* R11 and *P. gallaeciensis* LSS9 *in vivo* may be due to the limited number of samples analyzed and/ or the relatively shallow sequencing analysis of the microbial community of *D. pulchra*. To address this issue, we use here deep-sequencing of the 16S rRNA gene to investigate the microbial community on healthy and bleached individuals from two, natural disease events.

RESULTS

DEEP 16S rRNA GENE SEQUENCING OF MICROBIAL COMMUNITIES SHOWS OTUs MATCHING *IN VITRO* PATHOGENS IN ENVIRONMENTAL SAMPLES

In this study we analyzed replicate samples for two separate bleaching events of natural *D. pulchra* populations in two locations (Bare Island and Long Bay) that occurred during the austral summer of 2008 (see Table S1) (Campbell et al., 2011). Using deep-sequencing of the 16S rRNA gene V4 region with the Illumina HiSeq 2000 platform, we obtained 100 bp reads ranging from 64,151, to 142,324 sequences per microbial community sample after stringent quality filtering (see Experimental Procedures and Table S1). These reads were clustered into operational taxonomic units (OTUs) at both 97% and 100% identity. After removal of spurious OTUs and adjusting for the variation in the 16S rRNA gene copy number (see Experimental Procedures) the sequence abundances per sample ranged from 27,815 to 68,051 for the 97% identity OTUs and from 24,995 to 64,048 for 100% identity OTUs. Rarefaction analysis indicated that the sequencing effort started to saturate the diversity of the 16S rRNA gene fragment for all samples (see **Figure 1**) and this was further supported by Good's coverage estimates of greater than 97% for both OTU definitions (see Table S1). At the level of phylogenetic resolution provided by the 16S rRNA gene fragment sequenced, our results show an almost complete sampling of the bacterial diversity present on the surface of *D. pulchra*, which was not achieved with previous Sanger-based sequencing efforts (Fernandes et al., 2012, see thick, dashed line in **Figure 1**).

The extent of the sampling effort should detect OTUs with similarity to the previously described *in vitro* pathogens *N. italica* R11 and *P. gallaeciensis* LSS9, if they are present. In order to match the *in vitro* pathogens to OTUs with the highest phylogenetic resolution that can be obtained with our data, only here we used the OTUs defined at a 100% identity cutoff. Using a 100% query coverage and identity cut-off, a single OTU could be found to match the 16S rRNA gene sequence for each pathogen. These two OTUs occurred with abundances between 0.004% (which is the lowest possible given the sequence depth achieved) and 0.196% (**Figure 2**). Statistical analysis of the relative abundances in healthy and bleached samples of the two different bleaching events showed that only the OTU that matched to *P. gallaeciensis* LSS9 was significantly more abundant in the bleached samples ($p = 0.006$) overall, and in particular in Long Bay ($p = 0.012$) (**Figure 2**, Table S2). The putative *N. italica* R11 OTU was only detected in one sample (a bleached individual from Long Bay).

DIFFERENCE ON BACTERIAL COMMUNITIES OF HEALTHY AND BLEACHED *D. PULCHRA*

Given that the two OTUs assigned to the *in vitro* pathogens only made up a small fraction of the overall community, we investigated, if other changes occur in the community that correlated with the bleaching of *D. pulchra*. Based on OTUs defined at a 97% identity cut-off, *D. pulchra* communities had a richness (Chao1) that ranged between 1940 and 3034 and diversity (inverse Simpson index) were between 10.5 and 99.4 (Table S1), but were not significantly different between healthy and bleached samples overall or in either location (**Table 1**).

In contrast, comparisons of community composition (presence/absence data) and structure (abundance data) using a Bray-Curtis similarity revealed clear differences between healthy and bleached algae (see **Figures 3**, S1). Hypothesis testing based on a multivariate generalized linear model (MGLM) analysis of variance (MGLM-ANOVA) showed a significant interaction between condition and location (**Table 1**). This indicates that the

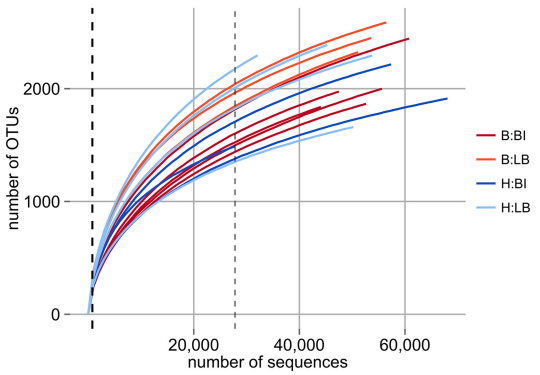

FIGURE 1 | Rarefaction analysis of *D. pulchra* microbial community samples with OTUs defined at a 16S rRNA V4 region sequence identity of 97%. Bleached (B) and healthy (H) samples from Bare Island (BI) and Long Bay (LB) are shown. The thick dashed line, which essentially overlaps with the y-axis, shows the sequencing depth of a previous study based on 16S rRNA gene clone libraries (Fernandes et al., 2011). The thin dashed line shows the minimum number of sequences for any sample analyzed here and to which level all samples were subsampled for subsequent community comparison.

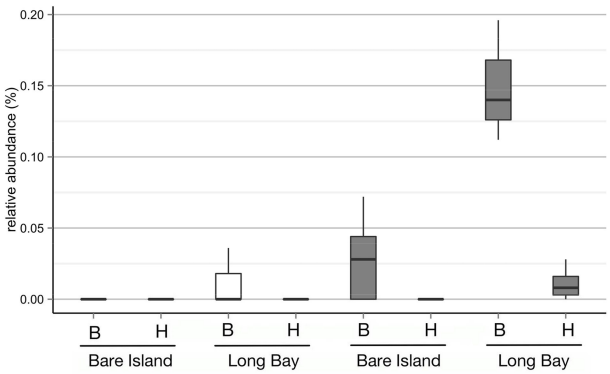

FIGURE 2 | Relative abundances of OTUs defined at a sequence identity of 100% for the 16S rRNA V4 region and with 100% identity to the 16S rRNA gene of *N. italica* R11 (white boxes) and *P. gallaeciensis* LSS9 (gray boxes) on *D. pulchra* collected at different locations (Bare Island and Long Bay) and with different health status (B, bleached; H, healthy).

Table 1 | Hypothesis tests of different measurements for microbial communities of *Delisea pulchra* from Bare Island (BI) and Long Bay (LB).

	Chao		InvSimpson		Composition		Structure	
	F	***p*-value**	***F***	***p*-value**	**Deviance**	***p*-value**	**Deviance**	***p*-value**
Location	4.8	0.05	10.67	0.008*	7755	0.029*	10145	0.015*
Condition	1.89	0.2	0.054	0.82	1220	0.002*	16441	0.001*
Location × condition	0	0.99	0.06	0.81	3534	0.004*	5517	0.014*
Condition BI	1.980	0.21	0.19	0.68	6864	0.047*	9189	0.022*
Condition LB	0.56	0.49	0.0002	0.990	7977	0.043*	12239.75	0.029*

*For richness (Chao) and diversity (InvSimpson) a conventional ANOVA was used, while for composition and structure a MGLM-ANOVA was employed. *denotes p-values below significance level of 0.05.*

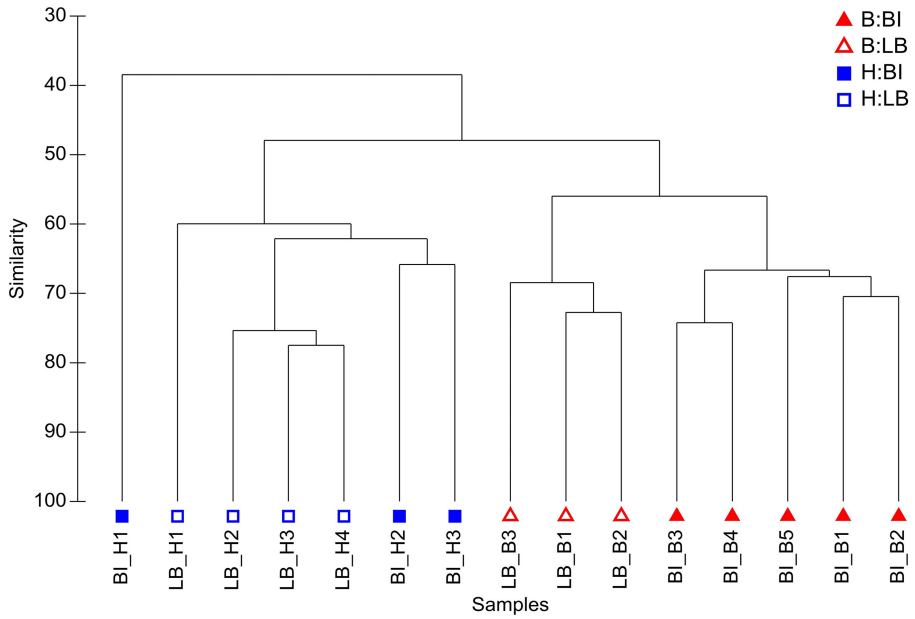

FIGURE 3 | Hierarchical agglomerative clustering based on Bray Curtis similarity of microbial community structure on *D. pulchra*. Bleached (B) and healthy (H) samples from Bare Island (BI) and Long Bay (LB) are shown. For this analysis OTUs were defined at a sequence identity of 97% for the 16S rRNA V4 region.

magnitude and/or direction of the differences between microbial communities of healthy and bleached samples can be affected by the location of the samples. Therefore, further comparisons were made within each sampling site to appropriately assess whether there are significant differences between healthy and bleached samples. Comparisons within each site showed significant difference in the community composition and structure between healthy and bleached samples from both sampling sites (**Table 1**), which is consistent with observations made in two previous studies (Campbell et al., 2011; Fernandes et al., 2012).

Having established general differences in the communities, we next asked which OTUs contribute in a statistically significant way to the observed changes. Univariate (i.e., OTU-by-OTU) results of the MGLM-ANOVA test using a conservative adjustment for multiple testing, showed that 31 OTUs are statistically significant different between healthy and bleached samples across the two bleaching events (**Figure 4**, Table S3). These OTUs, which represent 0.8% of all OTUs, contribute to the top 5% of differences (as measured by the deviance test statistic) between healthy and bleached samples and 27 of these OTUs were more abundant in bleached samples. These abundant OTUs in bleached samples had a wide taxonomic distribution, belonging to three different phyla, six classes, and nine families (**Figure 4**). At the phylum level, the majority of the OTUs belong to the Proteobacteria (52%) and Bacteroidetes (44%), with one OTU assigned to the Verrucomicrobia (4%). At the lowest taxonomy level, at which still most of the OTUs could be classified (93% at family level), these OTUs are classified as *Rhodobacteraceae* (41%) and *Flavobacteriaceae* (26%). The remaining OTUs belong to the *Rhodospirillaceae* (4%), *Bacteriovoracaceae* (4%), *Saprospiraceae* (7%), *Cytophagaceae* (4%), *Flammeovirgaceae* (4%), and *Verrumicrobiaceae* (4%). The four OTUs that were more abundant in healthy samples can be assigned to the family *Rhodobacteraceae*, the genera *Schleiferia*

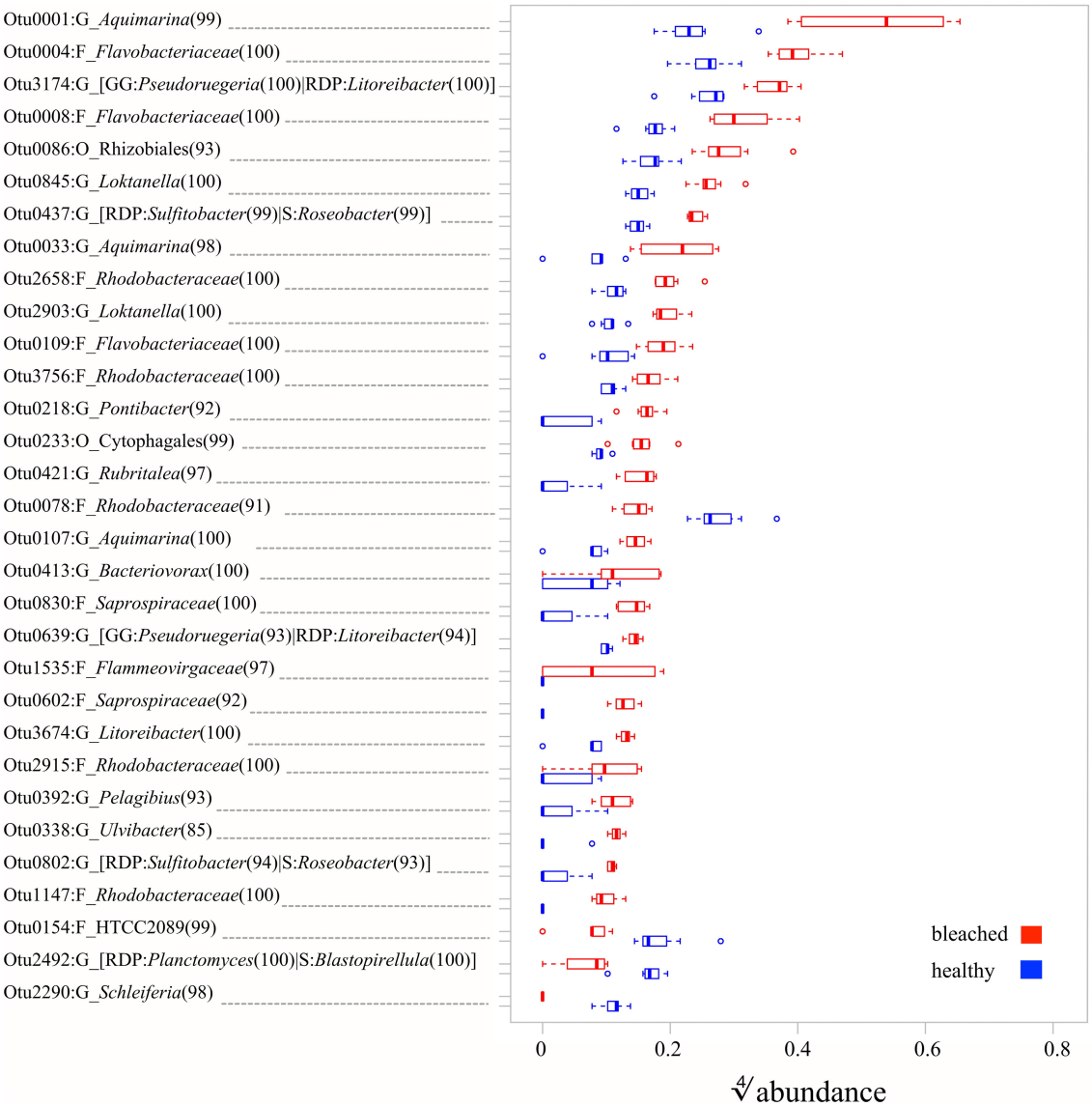

FIGURE 4 | Relative abundances of OTUs (defined at a sequence identity of 97% for the 16S rRNA V4 region) with statistically significant (MGLM-ANOVA with $p_{adjusted} < 0.05$) difference between healthy and bleached *D. pulchra* samples. On the y-axis, the letter after the colon indicates the taxonomic level, at which the OTU could be classified (O, Order; F, Family; G, Genus). Alternative taxonomic assignment by different databases (RDP, Ribosomal Database Project; GG, Greengenes; S, Silva) are shown in square brackets. The consensus confidence of the OTU classifications are shown in round brackets. The OTUs are ordered in decreasing order from the top by their relative abundance in bleached samples.

(phylum Flavobacteria), *Planctomyces* or *Blastopirellula* (as classified with RDP (Cole et al., 2014) or SILVA (Quast et al., 2013), respectively), and the class Gammaproteobacteria.

DISCUSSION

THE PRESENCE OF *IN VITRO* PATHOGENS ON *D. PULCHRA* AND THEIR CONTRIBUTION TO CHANGES IN THE MICROBIAL COMMUNITY OF DISEASED INDIVIDUALS

Detection of known pathogens in natural disease events is often limited by sensitivity i.e., the lack of appropriate sequencing depth resulting in incomplete description of the microbial diversity present in a sample. A comprehensive analysis of microbial communities can be achieved by deep-sequencing of part of the 16S rRNA gene with current sequencing technologies (Caporaso et al., 2012; Werner et al., 2012), however as with any PCR-based technology bias due to primer binding and amplification efficiencies can potentially misrepresent the relative abundance of certain microbial groups (e.g., Engelbrektson et al., 2010).

After the complete sequence processing, which includes OTU clustering at 100% identity, removal of potentially spurious OTUs and 16S rRNA gene copy number correction, we obtained an average of 46,255 sequence reads per sample (or a 60 times increase to the per sample sequence average of previous study by Fernandes et al., 2011), which resulted in an

estimated coverage of the community diversity of greater than 97% (with the caveats of bias, mentioned above) and a detection limited for any given OTU at a relative abundance of 0.004%.

This sequence analysis allowed us to detect an OTU with similarity to *N. italica* R11 only in one bleached sample and not in any other of the 14 samples analyzed. Considering the high sampling coverage and the low detection limit reached, this would suggest that *N. italica* R11 is unlikely to play a major role in the bleaching events observed here and that it constitutes a rare member of the microbial community of the *D. pulchra* individuals analysed here.

An OTU matching to *P. gallaeciensis* LSS9 was detected in 9 out of 15 samples (six bleached samples and three healthy ones) and this OTU showed a statistically significant higher abundance in bleached samples in general and in Long Bay when assessing the difference within sites (Table S2). The limited phylogenetic resolution of the V4 region used here however did not allow us to unambiguously assign the OTU to strain LSS9 as BLAST analysis also showed 100% identity to 16S rRNA genes from other members of the *Rhodobacteraceae* family. Nevertheless, the statistically significant increase of the OTU in one bleaching event would indicate that *P. gallaeciensis* LSS9 or closely-related organisms might contribute to the disease in natural settings.

The inability to consistently detect higher relative abundance for the OTUs matching to either *in vitro* pathogen on bleached samples could be due to two alternative, but not mutually exclusive scenarios. Firstly, the bleaching disease of *D. pulchra* could progress through multiple stages, in which different sets of pathogens colonize and proliferate at any one time. In this scenario, the inability to consistently detect *N. italica* R11 or *P. gallaeciensis* LSS9 could be simply because they were not abundant in the disease stages in which the samples were taken. A well-understood example for "staged diseases" is given by the Black Band Disease of corals, which involves distinct morphological stages (e.g., cyanobacterial patches and microbial lesions) and defined microbial succession patterns (Sato et al., 2013). Further temporal studies of the bleaching disease of *D. pulchra* would be required to define potential disease stages, however field observations have so far failed to observe distinct morphological states prior to bleaching.

The second scenario is that there is a pool of pathogens naturally present on healthy *D. pulchra* living as commensals. When the natural host defenses are compromised due to detrimental environmental factors (e.g., UV stress etc.), then these pathogens proliferate in an opportunistic fashion (Egan et al., 2012; Fernandes et al., 2012). Under this scenario, each time a bleaching event occurs, a different subset of opportunists dominate based on chance, similar to what has been postulated for the colonization and proliferation of planktonic bacteria on the surface of the green alga *Ulva australis* (Burke et al., 2011). Support for the second scenario is given by the fact that the majority (80%) of OTUs (defined at a 97% identity cut-off) that contribute most to the overall community difference between healthy and bleached samples (top 20% of the deviance) are in fact different in each bleaching event. Moreover, the majority of OTUs that are abundant on diseased samples were also present in healthy samples (71% and 88% of the OTUs that contributed most to the difference in Bare Island and Long Bay, respectively), which would be consistent with their commensal role in healthy macroalgae.

DETECTION OF MULTIPLE POTENTIAL PATHOGENS OF *D. PULCHRA*

In addition of attempting to detect *in vitro* pathogens, we also investigated if and which other OTUs (97% identity cut-off) make a significant contribution to the differences between microbial communities of bleached and healthy *D. pulchra* (**Figure 4**). Most OTUs with significant difference in relative abundance where enriched in bleached samples and of those the majority were classified as belonging to the families *Rhodobacteraceae* (41%) and *Flavobacteriaceae* (26%).

Within the *Rhodobacteraceae*, all OTUs that could be classified to the genus level (7 out of 11) belong to the Roseobacter clade. The Roseobacter clade is one of nine major marine clades (Giovannoni and Rappe, 2000) and its members have been found in practically every marine niche, including in associations with marine eukaryotes, such as corals, sponges, cephalopods, scallop larvae, seagrasses as well as micro- and macroalgae (Buchan et al., 2005; Wagner-Döbler and Biebl, 2006). Roseobacter clade bacteria are in fact frequently isolated from macroalgae (Brinkhoff et al., 2008) and have the capacity to utilize algal osmolytes, like putrescine, taurine, creatine, sarcosine, and dimethylsulfoniopropionate (DMSP) (Wagner-Döbler and Biebl, 2006; Kalhoefer et al., 2011; Thole et al., 2012). Of particular interest is DMSP, as several marine micro- and macroalage contain it in high concentrations (Yoch, 2002). Therefore, degrading tissue of bleached *D. pulchra* could possibly be a source of DMSP (and other osmolytes) for saprophytic behavior of members of the Roseobacter clade.

Alternatively, the Roseobacter-clade OTUs enriched on bleached samples could constitute opportunistic pathogens, with similar properties to *N. italica* R11 and *P. gallaeciensis* LSS9, which also belong to this group. The Roseobacter clade contains many other known or putative pathogens, such as *Roseovarius crassostreae*, which causes the Roseovarius Oyster Disease (Maloy et al., 2007); the strains that are consistently detected in corals affected with white plague-like disease and black band disease (Cooney et al., 2002; Pantos et al., 2003); and diseased individuals of the sponge *Rhopaloides odorabile* (Webster et al., 2002). As for algal disease, the bacterium *Ruegeria atlantica* has been shown *in vitro* to synthesize compounds that lyse the dinoflagellate *Alexandrium catenella* (Amaro et al., 2005) and Roseobacter strains can cause a tumor-like gall disease in the red alga *Prionitis lanceolata* (Ashen and Goff, 1998).

Within the *Flavobacteriaceae*, 3 out of 7 OTUs were assigned to the genus *Aquimarina*, with one of them (OTU1) representing the most abundant OTU in the community of bleached *D. pulchra* (relative abundance of 9.3%; see in **Figure 4**). Members of the *Aquimarina* have previously been reported to be associated with diseases of marine eukaryotes. For example, *Aquimarina homaria* dominates (jointly with another *Flavobacteriaceae* species) the microbial communities associated with shell lesions in American lobster (Chistoserdov et al., 2012; Quinn et al., 2012). In addition, *Aquimarina agaralytic*, which was isolated from a red macroalga, was found to posses a large number of diverse agarases (Lin

et al., 2012a,b) that may function to degrade host tissue, and *Aquimarina salinaria* was shown to produce metabolites with algicidal activity (Chen et al., 2011). Other OTUs that contributed to the difference in bleached *D. pulchra* were assigned to the order Cytophagales, the family *Saprospiraceae,* and the genus *Saprospira*. These taxa along with the *Flavobacteriaceae* OTUs, belong to the Cytophaga/Flavobacterium/Bacteroidetes (CFB) group, which contains most of the algicidal bacteria isolated from marine and coastal environments (Fandino et al., 2001; Furusawa et al., 2003; Mayali and Azam, 2004; Roth et al., 2008; Chen et al., 2011). The potential role of such algicidal bacteria as macroalgal pathogens is thus worth further investigation. Additionally, members of the CFB group have been show to contribute to secondary infections of red alga *Chondrus crispus* after initial infection by an endophytic green algae (Correa and McLachlan, 1994; Craigie and Correa, 1996; Goecke et al., 2010).

In conclusions, the contribution that specific OTUs of the *Rhodobacteraceae* and *Flavobacteriaceae*/CFB make to the community differences between healthy and diseased samples, together with the fact that members of these groups have previously been implicated in marine diseases, suggests that these groups of organisms contain prime candidates for alternative pathogens of *D. pulchra*. While the environmental observation made here shows a correlation with disease, isolates from these groups need to be obtained in the future in order to clearly demonstrate them as causative agents in either *in vitro* or *in vivo* settings. If more strains with pathogenic properties can be demonstrated to exist, then this would further support the model that environmental diseases, such as the bleaching disease of *D. pulchra,* result from the action and activities of widespread opportunistic pathogens (Egan et al., 2013). How these potential pathogens respond to environmental conditions and interact with other community members is likely important for the development of disease. Complex shifts in the microbial community have also been observed in diseases affecting other marine organisms, such as corals (Rosenberg et al., 2007; Thurber et al., 2009; Ainsworth et al., 2010; Mouchka et al., 2010; Littman et al., 2011) and sponges (Webster et al., 2008; Angermeier et al., 2011; Fan et al., 2013; Olson et al., 2014), and for those diseases a model involving multiple pathogens might also be applicable.

EXPERIMENTAL PROCEDURES

SAMPLING AND MICROBIAL COMMUNITY DNA ISOLATION

Replicate samples of healthy ($n = 7$) and bleached ($n = 8$) *D. pulchra* individuals were collected by SCUBA at depths of around 9 m at Bare Island (S33°59′30.80″, E151°13′53.60″) and Long Bay (S 33°57′59.79″, E151°15′26.11″), off the coast of Sydney, Australia, during the austral summer (12 February 2008) (see Table S1). Each algal sample was enclosed individually in clip-sealed plastic bags *in situ* and transported to the laboratory, where they were rinsed in filtered seawater three times to remove any loosely associated epibionts. Algae were then gently patted with sterile paper tissue to remove excess seawater and then freeze-dried. DNA was extracted from freeze-dried algal samples (50–100 mg) using a ZR Soil Microbe DNA extraction kit (Zymo) following the manufacturer's protocol as previously described (Campbell et al., 2011).

16S rRNA GENE SEQUENCING AND PROCESSING

DNA samples were processed through the Earth Microbiome Project (EMP) (Gilbert et al., 2010) to generate amplicons for the variable region 4 (V4) of the 16S rRNA gene using the universal bacterial/archaeal primers 515F/806R. The amplified samples were sequenced on a HiSeq Illumina 2000 platform with 100 bps from the 515F primer. Reads were demultiplexed and quality trimmed using the QIIME software. For a more detailed description of these methods see www.earthmicrobiome.org. The sequencing data have been deposited in the Short Reads Archive under accession SRX824554 (Bare Island) and SRX824555 (Long Bay).

Sequences were further processed in Mothur (Schloss et al., 2009) using the guidelines of the MiSeq standard operational procedure (http://www.mothur.org/wiki/MiSeq_SOP) with the following modifications: sequences were trimmed with parameters qwindowaverage = 30, qwindowsize = 5, maxambig = 0, maxhomop = 8, minlength = 100. The alignment was done using the reference alignment of Silva release 102 cut to the V4 region of the 16S rRNA gene. Aligned sequences were pre-clustered using diffs = 1. Chimeras were removed using chimera.uchime with dereplicate = t and contaminants (i.e., sequences from chloroplasts, mitochondria, eukaryotes or with unknown taxonomical affiliation) were filtered after classifying the sequences with the RDP version 9 reference taxonomy. Sequences were clustered into OTUs at 97% or 100% using the cluster.split command (splitmethod = classify, taxlevel = 4) and the same reference as with the alignment.

OTU-BASED COMMUNITY ANALYSIS

Due to the large number of reads produced by the HiSeq Illumina platform, sequences that have been filtered for high quality can still produce spurious OTUs. However, an additional abundance-based OTU filtering can produce an OTU collection that better reflects the true diversity of the microbial sample (Bokulich et al., 2013). Here, OTUs were removed that had an absolute abundance across samples lower than 15 (or relative abundance of 0.0009% or lower across samples), which is the total number of samples analyzed here.

The number of reads per OTU was further corrected for the known or inferred 16S rRNA gene copy number of the taxon that the OTU was assigned to. For this, the Greengenes database (version of October 2012) (DeSantis et al., 2006) was downloaded and its taxonomy file clustered at 0.99 identity were formatted according to Mothur specifications. This reference dataset was used in Mothur to classify sequences and obtain a majority consensus taxonomy using the classify.otu command with default settings. This taxonomic classification was used to create a QIIME-formatted OTU table, which was then employed in Copyrighter (Angly et al., 2014) using the default trait estimates file (ssu_img40_gg201210.txt) and option –t. The OTU table with corrected absolute abundances was converted back into the Mothur format and used for all subsequent analysis. After this adjustment, the minimum relative abundance of any OTU in any sample was 0.004%.

The number of OTUs, coverage, Chao1 and the Inverse Simpson diversity index were calculated with Mothur for 1000

random subsamples using the smallest 16S rRNA gene abundance in any sample (i.e., sample BI_H1 with an abundance after 16S rRNA gene copy number correction of 27,815). The average of the subsamples were used to perform Analysis of Variance (ANOVA) tests between samples types. Bray-Curtis similarities were calculated for the communities' composition (presence/absence data) and structure (abundance data) and hierarchical agglomerative clustering dendograms of samples were generated using Primer-E v6 (Clarke and Gorley, 2006). To calculate the Bray-Curtis similarity of community structure, the OTU abundances were square-root transformed. To test whether there were statistically significant differences between healthy and bleached samples in both composition and structure a two-factor design (Location with levels "Bare Island" and "Long Bay" and Condition with levels "healthy" and "bleached") was used to adjust the data to a MGLM using the mvabund package (Wang et al., 2012). In this approach, each OTU is treated as a variable that is fitted to a separate generalized linear model (GLM) using a negative binomial distribution for the analysis of community structure and a binomial distribution for composition analysis. For multivariate hypothesis testing, the ANOVA function (which implements an analysis of deviance) was applied to the MGLM using the p.uni argument set to return univariate OTU-by-OTU results adjusted to control the family-wise error rate across OTUs. Additionally, these univariate ANOVA-like tests were ordered by deviance to identify the OTUs that contribute more strongly to the overall difference between healthy and bleached samples.

Using the databases Silva (release 119), RDP (PDS version 10) and Greengenes (release of August 2013) in Mothur, three separate majority-based consensus taxonomic classifications (classify.otu command with default parameters) were obtained for the OTUs that had a statistically significant effect by Condition. A consensus of the three classifications was manually built by reporting only the deepest taxonomic assignment, using the highest consensus confidence results observed and showing alternative taxa when different classifications were obtained with the different reference database.

DEFINITION OF OTUs THAT MATCH THE 16S rRNA GENE OF KNOWN, *IN VITRO* PATHOGENS

To investigate the presence of the strains *N. italica* R11 and *P. gallaeciensis* LSS9 their 16S rRNA gene were searched against the 100% OTU clusters using blastn and a cutoff for coverage and identify of 100% for the OTU sequence. Using this criteria one OTU cluster was found for each pathogen. Because the OTU cluster assigned to *N. italica* R11 was present in only one sample, no further analysis were done on it. For the OTU assigned to *P. gallaeciensis* LSS9, the mvabund software package (Wang et al., 2012) was used to fit the by-sample standardized OTU abundance to a GLM using again a negative binomial distribution. This GLM was then used to test the significance of the difference between healthy and bleached samples using the ANOVA function (analysis of deviance).

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SUPPLEMENTARY MATERIAL

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Effects of sample handling and cultivation bias on the specificity of bacterial communities in keratose marine sponges

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Complex and distinct bacterial communities inhabit marine sponges and are believed to be essential to host survival, but our present-day inability to domesticate sponge symbionts in the laboratory hinders our access to the full metabolic breadth of these microbial consortia. We address bacterial cultivation bias in marine sponges using a procedure that enables direct comparison between cultivated and uncultivated symbiont community structures. Bacterial community profiling of the sympatric keratose species *Sarcotragus spinosulus* and *Ircinia variabilis* (Dictyoceratida, Irciniidae) was performed by polymerase chain reaction-denaturing gradient gel electrophoresis and 454-pyrosequecing of 16S rRNA gene fragments. Whereas cultivation-independent methods revealed species-specific bacterial community structures in these hosts, cultivation-dependent methods resulted in equivalent community assemblages from both species. Between 15 and 18 bacterial phyla were found in *S. spinosulus* and *I. variabilis* using cultivation-independent methods. However, *Alphaproteobacteria* and *Gammaproteobacteria* dominated the cultivation-dependent bacterial community. While cultivation-independent methods revealed about 200 and 220 operational taxonomic units (OTUs, 97% gene similarity) in *S. spinosulus* and *I. variabilis*, respectively, only 33 and 39 OTUs were found in these species via culturing. Nevertheless, around 50% of all cultured OTUs escaped detection by cultivation-independent methods, indicating that standard cultivation makes otherwise host-specific bacterial communities similar by selectively enriching for rarer and generalist symbionts. This study sheds new light on the diversity spectrum encompassed by cultivated and uncultivated sponge-associated bacteria. Moreover, it highlights the need to develop alternative culturing technologies to capture the dominant sponge symbiont fraction that currently remains recalcitrant to laboratory manipulation.

Keywords: holobiont, microbial cultivation, microbial diversity, pyrosequencing, symbiosis

INTRODUCTION

"The great plate count anomaly," as introduced by Staley and Konopka (1985), describes the difference observed between the number of bacterial colony forming units (CFUs) grown on a culture medium and that of cells detected by microscopy for a given sample. This way, it was estimated that only 0.1–1.0% of the total bacterial cells in the environment could be accessed via standard cultivation approaches (Staley and Konopka, 1985). This result was supported by several studies of free-living (Kogure et al., 1979; Staley and Konopka, 1985; Amann et al., 1995) and host-associated bacterial communities (Friedrich et al., 2001; Webster and Hill, 2001). However, the plate count anomaly as originally described disregards the phylogenetic diversity of those CFUs grown on plates and uncultivated cells observed under the microscope (Donachie et al., 2007): the ratio CFU/microscopy cell counts, when directly used to describe "cultivation bias," assumes even relative abundances between all microbial species or phylotypes that constitute the community. Despite this assumption, natural communities more often display uneven species abundances in which a few members dominate the assemblage followed by a majority of diverse, but rarer, species or phylotypes (Sogin et al., 2006; Gomes et al., 2010; Webster et al., 2010; Hardoim et al., 2013). Unfortunately, microbial ecologists have tended to overlook this pattern in the past when referring to our capacity to cultivate microbial species from the environment (Donachie et al., 2007). Several questions remain poorly understood, for instance, how does the diversity of readily cultivatable microorganisms compare with that resulting from cultivation-independent methods? Are we culturing the few dominant members of a community or preferentially the rare ones? Do communities resulting from cultivation-dependent and cultivation-independent methods share a majority of microbial

phylotypes or are these communities divergent? Few studies have addressed these questions in detail (Donachie et al., 2007; Li et al., 2011; Sipkema et al., 2011; Montalvo et al., 2014), a situation which severely restricts our knowledge of the extent of the cultivatable microbiome diversity from natural habitats.

Marine sponges are reservoirs of microbial genetic and metabolic novelties. Up to 28 bacterial phyla, including candidate phyla, have been found in association with these animals (Simister et al., 2012). This conspicuous taxonomic diversification suggests functional variety, and several possible roles have been proposed for sponge symbionts upon interaction with their hosts (Taylor et al., 2007b; Webster and Taylor, 2012). Among them, the putative chemical defense enabled by biologically active compounds produced by sponge-associated bacteria is receiving considerable attention (Piel et al., 2004; Hochmuth and Piel, 2009; Siegl and Hentschel, 2010; Thomas et al., 2010b; Hentschel et al., 2012). Consequently, cultivating marine sponge bacteria is important from both a phylogenetic and biotechnological standpoint. Several attempts have been made to domesticate sponge-associated bacteria (Muscholl-Silberhorn et al., 2008; Esteves et al., 2013). Nevertheless, few studies assessed cultivation-dependent and -independent discrepancies in diversity surveys of the sponge microbiome (Sipkema et al., 2011; Montalvo et al., 2014).

The present study uses polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and 454-pyrosequencing profiling to address the extent to which culturing and specific sample handling procedures affect the structure of bacterial communities in the keratose marine sponges (i.e., lacking mineral spicules and possessing an organic fibers skeleton instead) *Sarcotragus spinosulus* Schmidt 1862 and *Ircinia variabilis* Schmidt 1862. Previous studies suggest that these species harbor divergent bacterial communities and are the source of cultivatable bacteria with antimicrobial capacities (Hardoim et al., 2012; Esteves et al., 2013). However, the full diversity breadth of these microbial consortia and of their corresponding cultivatable fraction remains to be determined. We employed a strategy that circumvents the need to isolate single colonies during diversity surveys of cultivated bacteria and instead enables the direct comparison between bacterial community structures retrieved with cultivation-dependent and cultivation-independent methods. Although fewer bacterial species are usually recovered by cultivation than by cultivation-independent approaches, we hypothesized that cultivation would nevertheless represent the phylogenetic diversity of the communities assessed here to a higher extent than expected from CFU/microscopy count ratios. We also applied in-tube fluorescent *in situ* hybridization (FISH) of dominant sponge-associated bacteria to obtain the first insights into their localization and distribution in the two sponge species, and to compare the relative abundances of the bacterial symbionts estimated using sequencing and cell imaging technologies.

MATERIALS AND METHODS

SPONGE AND SEAWATER SAMPLING

Sampling took place at Galé Alta, Armação de Pêra (37° 04' 09.6"N and 8° 19' 52.1"W) off the coast of the Algarve, southern Portugal, in June 2010. Four specimens of *S. spinosulus* and *I. variabilis* (Demospongiae, Dictyoceratida, Irciniidae), and four samples of surrounding seawater (1 L each, about 1 m above sponge specimens), were collected in sterile Ziploc® bags by scuba diving at about 15 m depth. Samples were placed in cooling boxes, transported to the laboratory (*c.* 2 h) and immediately processed as described below. Sponge species were identified based on macro- and microscopic morphological criteria coupled to molecular phylogenetic inference (Hardoim et al., 2012).

CULTIVATION-INDEPENDENT AND -DEPENDENT TOTAL COMMUNITY DNA EXTRACTION

For each collected sponge specimen, three procedures of sample processing were undertaken prior to total community DNA (TC-DNA) extraction, hereafter called "direct," "indirect," and "plate washing" methods of sponge sample processing. The first two methods lead to cultivation-independent analysis of TC-DNA directly extracted from the sponge body ("direct" method) or from sponge-derived microbial cell pellets ("indirect" method). "Plate washing" involves extracting TC-DNA from washes of Marine Agar culture plates and is therefore a culture-dependent methodology for assessing the sponge-associated microbiome without having to purify and singularize colonies. Details of each procedure are given in Appendix S1 (Supplementary Material). TC-DNA extraction was performed with the UltraClean® Soil DNA isolation kit (MO BIO, Carlsbad, CA, USA), according to the manufacturer's protocol. The same kit was used for TC-DNA extraction from bulk seawater samples following Hardoim et al. (2012). Thus, 28 metagenome samples (four seawater replicates; four *I. variabilis* replicates handled with "direct," "indirect," and "plate washing" methods; and four *S. spinosulus* replicates handled with "direct," "indirect," and "plate washing" methods) were subjected to PCR-DGGE and 454-pyrosequencing bacterial community profiling as explained below.

PCR-DGGE BACTERIAL COMMUNITY PROFILING

A nested PCR-DGGE approach targeting the V6 hypervariable region of the 16S rRNA gene was used to fingerprint the bacterial communities associated with both sponge species under the three methods of sample processing. The reaction mixture and thermal cycling for both reactions were as described by Hardoim et al. (2012), except for the concentration of primers (0.6 μM, **Table 1**). PCR-DGGE profiling was then performed using a PhorU-2 gradient system (Ingeny International, Goes, The Netherlands). Gel gradient, marker constituents, electrophoresis and staining procedures were described previously (Hardoim et al., 2012). Multivariate statistical analysis of PCR-DGGE fingerprints followed methods described elsewhere (Costa et al., 2006; Hardoim et al., 2009) and is detailed in Appendix S1.

454-PYROSEQUENCING BACTERIAL COMMUNITY PROFILING

A barcoded pyrosequencing approach was employed for in-depth analysis of bacterial community composition and diversity. A thorough description of (i) pyrosequencing sample preparation, (ii) data processing and (iii) analyses is provided in Appendix S1. Briefly, the V4 hypervariable region of the 16S rRNA gene

Table 1 | Polymerase chain reaction-denaturing gradient gel electrophoresis and 454-pyrosequencing primers used in this study.

Name	Sequence (5′–3′)	Usage	Reference
F27	AGAGTTTGATCMTGGCTCAG	First DGGE PCR	Weisburg et al. (1991)
R1492	TACGGYTACCTTGTTACGACTT	First DGGE PCR	Weisburg et al. (1991)
F984-GC	CGCCCGGGGCGCGCCCCGGGCGGGG CGGGGGCACGGGGGGAACGCGAAGAACCTTAC	Second DGGE PCR	Heuer et al. (1997)
R1378	CGGTGTGTACAAGGCCCGGGAACG	Second DGGE PCR	Heuer et al. (1997)
V4_titF	AYTGGGYDTAAAGNG	454-Pyrosequencing	http://pyro.cme.msu.edu/pyro/help.jsp#intro
V4_tit_R	TACNVRRGTHTCTAATYC	454-Pyrosequencing	http://pyro.cme.msu.edu/pyro/help.jsp#intro

was PCR-amplified using the Ribosomal Database Project primer set (**Table 1**), which generates amplicons of around 248 bp in length. Two PCR mixtures of 25 μL were prepared per sample, each containing ∼20 ng of template DNA. Each sample was tagged by unique 8-mer barcodes attached to the reverse primer. The amplicons were delivered for pyrosequencing on a 454 Genome Sequencer GS FLX Titanium platform (Roche Diagnostics Ltd, West Sussex, UK) at BIOCANT (Biotechnology Innovation Center, Cantanhede, Portugal). Raw data were processed using AmpliconNoise (Quince et al., 2011) and Galaxy (https://main.g2.bx.psu.edu/; Taylor et al., 2007a), enabling noise filtering (e.g., homopolymers), chimera removal, sequence sorting, and trimming. The Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso et al., 2010) was then applied to the filtered data set for operational taxonomic units (OTUs) determination and taxonomic assignment, followed by the generation of a samples-OTUs table using customized scripts (Appendix S1). Data analyses encompassed (i) phylum- and class-level bacterial composition in individual and pooled samples, (ii) assessment of specific and shared bacterial symbionts across sample groups via OTU networks and Venn diagrams, (iii) estimates of symbiont richness (Chao1) and diversity (Shannon's index) and (iv) multivariate analysis of OTU data. The latter was performed via (a) principal coordinate analysis (PCoA) of OTU profiles using the Unifrac metric within QIIME and (b) constrained ordination of OTU profiles and independent variables (i.e., seawater, sponge, sponge species, and sample processing methods) with the software package Canoco for Windows 4.5 using Hellinger-transformed OTU abundance data. Analyses (i) to (iv) were carried out using both full size (whole data set exploration) and size-normalized sample libraries. The analysis of full size libraries was used to determine the absolute number (and the identity) of all OTUs shared by and specific to each sample category ($n = 7$: seawater; *I. variabilis* under "direct," "indirect," and "plate washing" methods; and *S. spinosulus* under "direct," "indirect," and "plate washing" methods). The analysis of normalized libraries was applied in the quantitative comparison of bacterial richness, diversity and community structure between the sample categories. For size-normalized analyses, two depth thresholds were defined, 1236 and 3688 sequence reads per sample, which allowed the comparison of (i) all four replicate samples of both sponge species under the three methods of sample processing and triplicate seawater samples, and (ii) all sponge-derived libraries (seawater samples excluded), respectively. Similar analyses were performed for the unfiltered data set, disregarding chimera and noise removal procedures. Pyrosequencing data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP021445.

IN-TUBE FLUORESCENT *IN SITU* HYBRIDIZATION AND CONFOCAL LASER SCANNING MICROSCOPY (FISH-CLSM)

To determine the spatial distribution and infer the abundance of sponge-associated bacteria at the micro-scale, in-tube FISH-CLSM was performed as described by Cardinale et al. (2008) with slight modifications (Appendix S1). For the detection of all bacteria, an equimolar mixture of Cy3-labeled EUB338, EUB338II, and EUB338III probes was used (Amann et al., 1990, **Table 2**). Samples were further hybridized with ALEXA488- or Cy5-labeled FISH probes specific for *Acidobacteria* (SS_HOl1400; Meisinger et al., 2007), *Alphaproteobacteria* (ALF968; Neef, 1997), and *Gammaproteobacteria* (Gam42a; Manz et al., 1992; **Table 2**). These taxa were selected based on their predominance revealed by 454-pyrosequencing. Taxon-specific abundances relative to total bacterial cell density were calculated by averaging the fraction of specifically stained cells from at least 15 randomly selected fields (confocal stacks) retrieved from three independent FISH experiments per specific probe.

TESTS OF SIGNIFICANCE

Homogeneity of variance tests were used to inspect the normal distribution of the richness and diversity measurements from PCR-DGGE fingerprints and 454-pyrosequencing. Analysis of variance (ANOVA) tested whether the mean values obtained for all sample groups ($n = 7$: seawater; *I. variabilis* under "direct," "indirect," and "plate washing" methods; and *S. spinosulus* under "direct," "indirect," and "plate washing" methods) were equal. A pairwise *t*-test which analyses the significance between groups was then carried out. Homogeneity of variance and ANOVA were also employed to compare 454-pyrosequencing relative abundances of the most dominant bacterial phyla and classes found across groups. The analyses were performed with the stat package in R programming (R Development Core Team, 2012). For both PCR-DGGE and 454-pyrosequencing data, Monte-Carlo permutations were performed to test whether the generated

Table 2 | Fluorescent *in situ* hybridization probes used in this study.

Name	Sequence (5′–3′)	Target	Formamide (%)[1]	Reference
EUB338[2]	gctgcctcccgtaggagt	Most bacteria	20	Amann et al. (1990)
EUB338II[2]	gcagccacccgtaggtgt	*Planctomycetales*	20	Daims et al. (1999)
EUB338III[2]	gctgccacccgtaggtgt	*Verrucomicrobiales*	20	Daims et al. (1999)
ALF968	ggtaaggttctgcgcgtt	*Alphaproteobacteria*	40	Neef (1997)
Gam42a	gccttcccacatcgttt	*Gammaproteobacteria*	40	Manz et al. (1992)
Gam42a competitor	gccttcccacttcgttt	*Betaproteobacteria*	40	Manz et al. (1992)
SS_HOL1400	ttcgtgatgtgacgggc	*Acidobacteria*	20	Meisinger et al. (2007)
NONEUB	actcctacgggaggcagc	–	[3]	Wallner et al. (1993)

[1] *Concentration of formamide for hybridizations at 43° C.*
[2] *Used in a mixture of equimolar concentrations.*
[3] *Used as negative control with the same formamide concentration as used for positive FISH.*

sponge symbiont profiles clustered according to the sample groups.

RESULTS

PCR-DGGE FINGERPRINTING OF BACTERIAL COMMUNITIES

The PCR-DGGE profiles of *S. spinosulus* obtained with cultivation-independent "direct" and "indirect" sample processing methods were visually very similar whereas much larger band variation was observed between *I. variabilis* fingerprints generated using the same methods (**Figure 1**). Profiles obtained for both sponge species via "plate washing" were different from those generated by cultivation-independent methods (**Figure 1A**). Ordination analysis of PCR-DGGE banding patterns suggested that both the host species and processing methods determined the structures of the surveyed symbiont communities (**Figure 1B**). A detailed description of PCR-DGGE results and a discussion on how they compare with 454-pyrosequencing data are given as Supplementary Material (Table S1, Appendix S2).

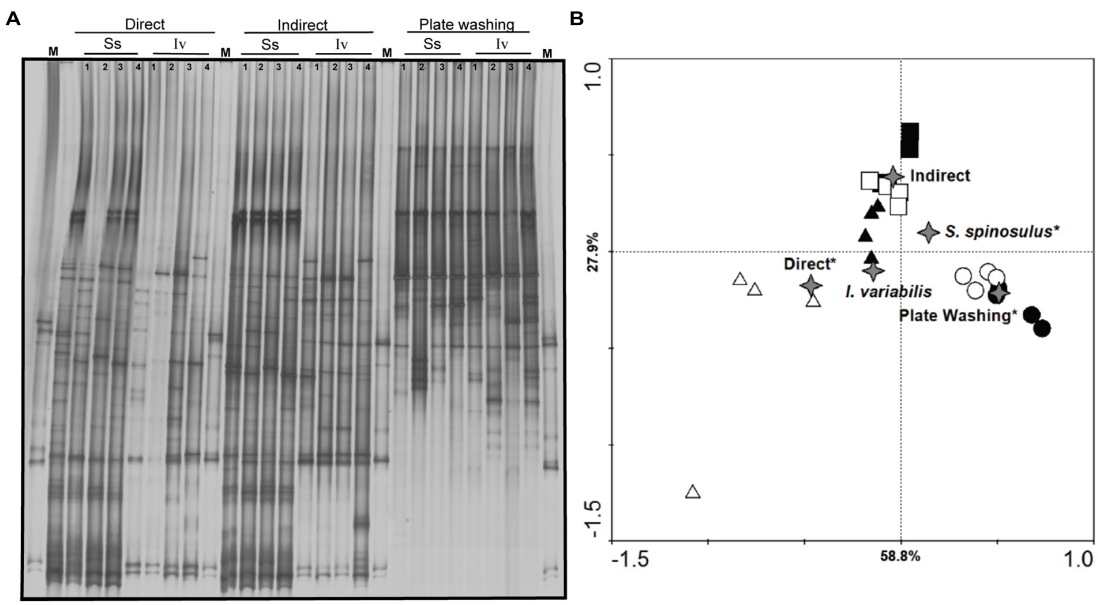


FIGURE 1 | Polymerase chain reaction-denaturing gradient gel electrophoresis 16S rRNA gene fingerprinting of bacterial communities associated with the marine sponges *Sarcotragus spinosulus (Ss)* and *Ircinia variabilis* (Iv). Profiles obtained under the three methods of sample processing ("direct," "indirect," and "plate washing") are shown **(A)** along with their corresponding ordination by redundancy analysis **(B)**. M: 16S rRNA gene marker used to control the DGGE run. Samples on the ordination diagram: ▲ – *S. spinosulus* "direct" processing, ■ – *S. spinosulus* "indirect" processing, ● – *S. spinosulus* "plate washing" processing, △ – *I. variabilis* "direct" processing, □ – *I. variabilis* "indirect" processing, ○ – *I. variabilis* "plate washing" processing. Labels displayed on the diagram axes refer to the percentage variations of PCR-DGGE ribotypes – environment correlation accounted for the respective axis. The "star" symbol represents the centroid positions of the canonical variables (i.e., sponge species and sample processing methods) in the diagram. Variables that significantly ($P < 0.05$) influenced bacterial community composition are highlighted with an asterisk.

454-PYROSEQUENCING

Bacterial richness and diversity

A total of 237,773 16S rRNA V4-tag sequences passed preliminary filtering on the 454 apparatus. After sequence trimming and further quality filtering, 166,442 bacterial 16S rRNA gene V4-tag sequences were obtained and constituted the analytical data set. Sequences were assigned to 639 OTUs at a 97% similarity cut-off (**Table 3**). Considering all normalized sequence libraries (depth = 1236 sequences/sample), about twofold higher bacterial richness was observed in seawater than in sponge samples (**Figures 2A,E**). Bacterial richness increased significantly in both sponge species – and across all processing methods – when analyses were made with larger libraries (depth = 3668 sequences/sample). In this case, *S. spinosulus* and *I. variabilis* hosted from *c.* 80 to 95 bacterial OTUs/specimen under cultivation-independent methods (**Figures 2B,E**). Regardless of the sequence depth, two major trends were found across the data. First, a large reduction in bacterial symbiont richness and diversity was observed for both sponge species because of culturing (**Figures 2A–E**). Specifically, the number of bacterial OTUs detected using the "plate washing" method represented only 11.9 and 15.25% of the OTU richness recorded for *S. spinosulus* and *I. variabilis*, respectively, when using the "direct" method. Second, when cultivation-independent methods were compared, contrasting results were obtained for each sponge host. Whereas no difference in richness and diversity values was found for *S. spinosulus* treated with both the "direct" and "indirect" methods, handling of *I. variabilis* with the "indirect" method resulted in significant reduction of such estimates in this host (**Figures 2A–E**). Overall, Shannon diversity indices were not affected by the size of libraries used in the comparisons (**Figures 2C,D**), and seawater and *S. spinosulus* bacterial diversities obtained with culture-independent methods were of comparable magnitude (**Figure 2C**) in spite of the significantly higher bacterial richness detected in seawater (**Figure 2A**). Our sequencing effort (see **Table 3** for details) was found to encompass about 65, 81, and 84% of the estimated bacterial diversity in seawater, *S. spinosulus* and *I. variabilis* samples ("direct" method), respectively, while the diversity found in the cultivatable bacterial fraction was fully covered. Richness and diversity estimates for the unfiltered sequencing data are given in Figure S1 (Supplementary Material).

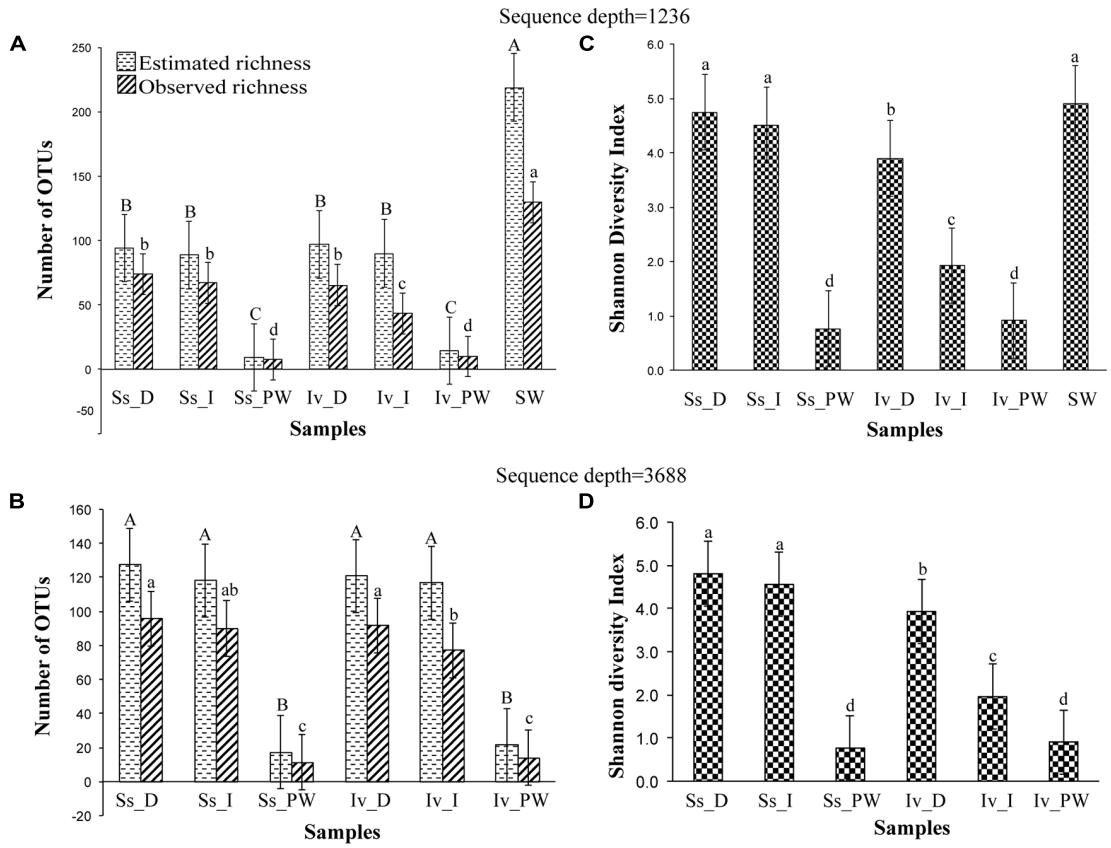
Community composition at the phylum level

All major bacterial phyla detected in this study displayed significant shifts in relative abundance either as a function of the surveyed microenvironment (seawater *vs.* sponge), the host species (*S. spinosulus vs. I. variabilis*) or the sample processing method ("direct" *vs.* "indirect" *vs.* "plate washing"). Trends observed for the full quality-filtered data set (**Figure 3**), described below, were consistently reproduced when analyses made with size-normalized libraries were performed (Appendix S3, Supplementary Material). Seawater presented much lower bacterial richness at the phylum level than sponges (**Figure 3A**), it being largely dominated by *Bacteroidetes* (90 OTUs in 4918 sequences) and *Proteobacteria* (156 OTUs in 3401 sequences), which accounted for 57 and 40% of all sequences, respectively (**Figure 3A**, **Table 4**). By contrast, up to 21 bacterial phyla (438 OTUs in 157,786 sequences across all methods) could be detected in the sponge samples (**Figure 3A**), with each individual specimen usually hosting between 14 and 16 phyla (Figure S2, Supplementary Material). Cultivation-dependent bacterial communities from both sponges consisted mainly of *Proteobacteria* (>97% of all sequence reads) and were much reduced in phylum diversity when compared to cultivation-independent communities (**Figure 3A**). The most abundant sponge-associated phyla found using cultivation-independent methods were *Proteobacteria*, *Actinobacteria* and *Acidobacteria* (**Figure 3A**, Figure S2A). Considering only these methods, *Proteobacteria* was the most diverse phylum in sponges (188 OTUs in 16,127 sequences) followed by *Actinobacteria* (29 OTUs in 26,931 sequences) and *Acidobacteria* (27 OTUs in 30,346 sequences; **Table 4**). Differences in phylum relative abundances, without changes to within-phylum OTU richness, were observed among *I. variabilis* communities depending on which cultivation-independent method was used. For instance, higher abundance of *Acidobacteria* at the expense of much lower proportions of *Proteobacteria* and *Bacteroidetes* were retrieved with the "indirect" method when compared to the

Table 3 | Sequence data summary.

Sample type	Sample processing	N	454 filtering		454+AmpliconNoise filtering	
			Sequences	OTUs 97	Sequences	OTUs 97
Sarcotragus spinosulus	Direct	4	35,198	739	29,174	199
	Indirect	4	34,988	705	29,227	184
	Plate washing	4	27,497	247	24,547	33
Ircinia variabilis	Direct	4	29,503	671	24,222	215
	Indirect	4	25,899	541	22,686	225
	Plate washing	4	31,983	257	27,930	39
Seawater	n.a.	4	15,567	598	8656	329
Total	n.a.	28	200,635	1974	166,442	639

N, number of replicate samples; n.a., not applicable.



E

Samples	Observed Richness ± SD (n=1236 sequences)	Observed Richness ± SD (n=3688 sequences)
Ss_D	73.82 ± 4.93	95.80 ± 5.75
Ss_I	66.95 ± 7.03	89.85 ± 9.55
Ss_PW	7.57 ± 2.20	11.37 ± 2.53
Iv_D	65.32 ± 8.23	91.80 ± 8.00
Iv_I	43.25 ± 8.54	77.17 ± 15.90
Iv_PW	10.1 ± 4.90	14.00 ± 5.62
SW	129.5 ± 23.0	-

FIGURE 2 | Quantitative analysis of bacterial richness and diversity in marine sponges and seawater. Observed and estimated (Chao1) richness measures **(A,B)**, Shannon diversity indices **(C,D)** and observed richness ± standard deviation **(E)** of bacterial OTUs are displayed using normalized depths of 1236 [includes seawater libraries, **(A,C,E)**] and 3866 [sponge libraries only, **(B,D,E)**] sequences per library. OTUs were determined at 97% 16S rRNA gene similarity. Values on bars are mean ± standard deviation of four replicates within each sample category, except for seawater where three replicates were used. Bars labeled with different letters represent statistically distinct sample categories in terms of richness and/or diversity values. In **(A,B)**, uppercase letters define differences in estimated richness across sample categories, whereas lowercase letters define differences in observed richness. Ss_D, *Sarcotragus spinosulus* under the "direct" processing method; Ss_I, *S. spinosulus* under the "indirect" processing method; Ss_PW, *S. spinosulus* under the "plate washing" processing method; Iv_D, *I. variabilis* under the "direct" processing method; Iv_I, *I. variabilis* under the "indirect" processing method; Iv_PW, *I. variabilis* under the "plate washing" processing method; SW, seawater.

"direct" method (**Figure 3A**, Figure S2A, **Table 4**). In contrast, differences in relative abundances of phyla because of cultivation-independent, sample handling methods were negligible in *S. spinosulus* (**Table 4**).

Taxonomic classes within the most abundant phyla were assigned to OTUs when possible (**Figure 3B**). In seawater, *Flavobacteriia* (*Bacteroidetes*, 67 OTUs in 4576 sequences), *Alphaproteobacteria* (64 OTUs in 1803 sequences) and *Gammaproteobacteria* (62 OTUs in 1511 sequences) were the dominant classes (**Figure 3B**, Figure S2B). Comprising 113 OTUs in 14,270 sequences and 72 OTUs in 50,256 sequences across all processing methods, *Gammaproteobacteria* and *Alphaproteobacteria*, respectively, were the most abundant classes in the culture-dependent sponge bacterial communities. Using cultivation-independent procedures, however, their dominance was shared with several other classes, namely *Sphingobacteriia* (*Bacteroidetes*, 23 OTUs in 6478 sequences), *Acidimicrobiia* (*Actinobacteria*, 21 OTUs in 26,820 sequences), *Deltaproteobacteria* (20 OTUs in 2788 sequences), *Anaerolinea* (*Chloroflexi*, 14 OTUs in 2994 sequences) and Sva075 (*Acidobacteria*, 8 OTUs in 26,599 sequences; **Figure 3B**, Figure S2B).

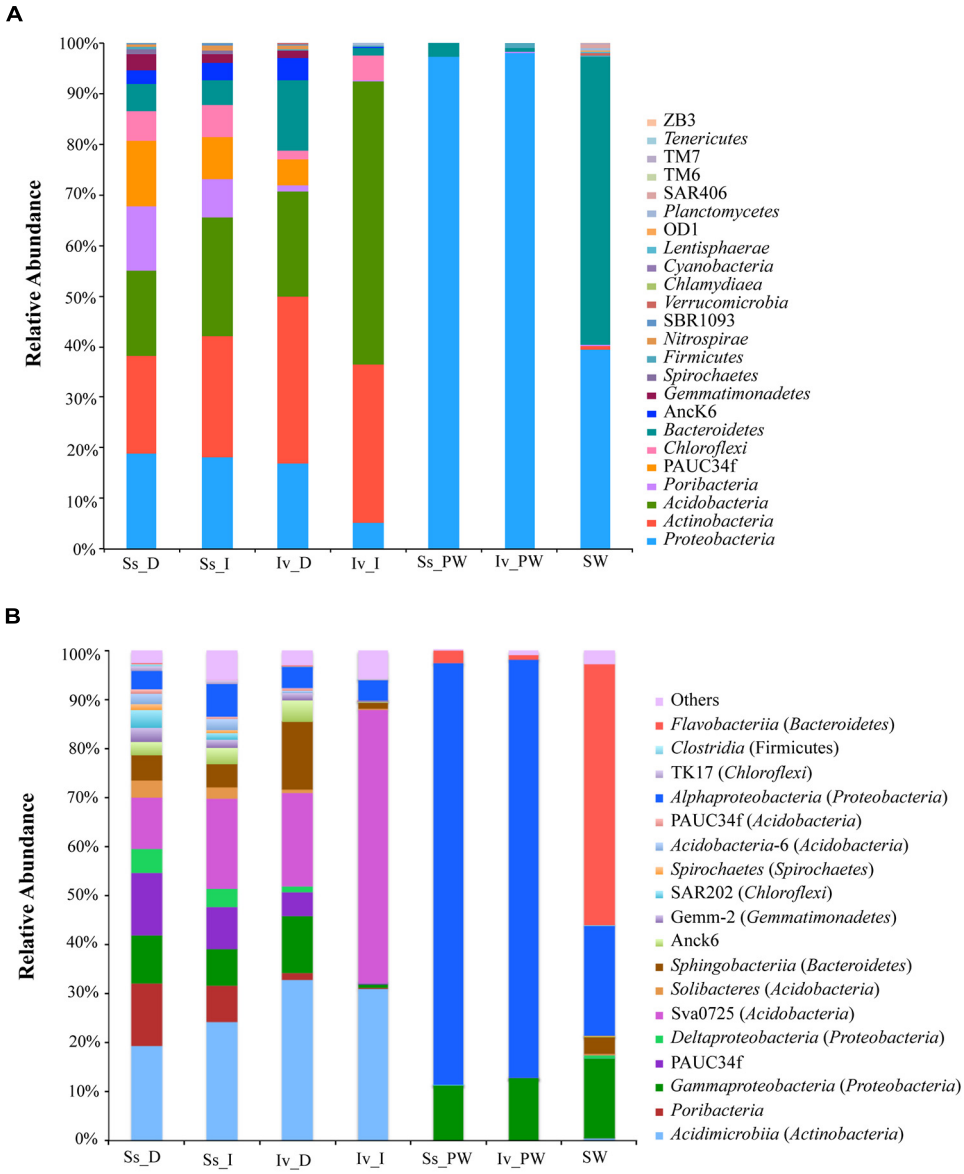


FIGURE 3 | Phylum- (A) and class-level (B) bacterial community composition in marine sponges and seawater. Compositional data for *S. spinosulus* and *I. variabilis* handled with the "direct," "indirect," and "plate washing" processing methods are shown. Results obtained using pooled replicate samples ($n = 4$) within each sample category are displayed. Bacterial community composition in each replicate sample is shown as Supplementary Material (Figure S2). In **(B)** the top 18 bacterial classes are listed and all remaining taxa are labeled as "others." Labeling of sample categories is as described in legend to **Figure 2**.

Specificities and commonalities: shared and exclusive OTUs

Operational taxonomic units network analysis revealed that most bacterial OTUs found in seawater were specific to this environment, placing the bacterioplankton far apart from symbiotic communities (**Figure 4A**). Several bacterial OTUs exclusive to the "direct" and "indirect" methods were detected in *I. variabilis* community profiles, positioning *I. variabilis* samples processed with these methods farther apart from one another than the corresponding *S. spinosulus* samples in the network diagram (**Figure 4A**). Cultivation-dependent methods resulted in similar community compositions in both sponges, with only a few OTUs specific to each sponge species (**Figure 4A**). These trends were quantified using Venn diagrams (**Figures 4B–G**). Strikingly, only 4 and 13 bacterial OTUs were common to all three methods of sample processing in *S. spinosulus* and *I. variabilis*, respectively (**Figures 4B,C**). The proportion of OTUs specific to either of the cultivation-independent ("direct" and "indirect") methods was indeed higher in *I. variabilis* than in *S. spinosulus* (**Figures 4B,C**). Despite the reduced bacterial diversity retrieved from the cultivation-dependent "plate washing" method, this approach led to the detection of several "cultivation-specific" OTUs not observed using cultivation-independent methods

Table 4 | Number of OTUs and sequences per bacterial phylum across sample categories.

Phylum	Ss_D		Ss_I		Ss_PW		Iv_D		Iv_I		Iv_PW		Seawater	
	OTUs	seqs	OTUs	seqs	OTUs	seqs	OTUs	seqs	OTUs	seqs	OTUs	seqs	OTUs	seqs
Acidobacteria	15	4947	15	6806	0	0	15	5041	17	13,552	1	1	5	17
Actinobacteria	10	5674	10	7141	1	1	20	7976	21	6140	2	3	13	51
AncK6	1	802	1	896	0	0	2	1051	1	61	0	0	1	2
Bacteroidetes	16	1487	15	1305	3	678	21	3424	21	330	2	308	90	4918
Chlamydiae	0	0	0	0	0	0	0	0	3	8	0	0	0	0
Chloroflexi	35	1763	34	1902	1	1	18	411	19	1140	1	1	6	10
Cyanobacteria	0	0	0	0	0	0	3	6	3	8	0	0	1	1
Firmicutes	7	120	1	1	1	1	5	12	4	19	2	308	5	8
Gemmatimonadetes	8	879	7	503	0	0	7	364	5	17	0	0	1	7
Lentisphaerae	0	0	0	0	0	0	0	0	1	1	0	0	4	20
Nitrospirae	2	163	1	272	0	0	2	205	2	8	0	0	1	2
OD1	0	0	0	0	0	0	0	0	0	0	0	0	1	2
PAUC34f	4	3759	4	2634	0	0	4	1198	2	41	0	0	2	8
Planctomycetes	5	13	4	15	0	0	8	20	11	91	0	0	9	45
Poribacteria	6	3726	5	2137	0	0	5	318	4	14	1	1	2	11
Proteobacteria	80	5474	76	5312	27	23,866	101	4102	103	1239	30	27,308	156	3401
SAR406	0	0	0	0	0	0	0	0	0	0	0	0	14	82
SBR1093	1	32	1	120	0	0	1	84	1	2	0	0	0	0
Spirochaetes	2	319	3	162	0	0	1	2	1	2	0	0	1	1
Tenericutes	0	0	0	0	0	0	0	0	0	0	0	0	1	5
TM6	1	2	1	2	0	0	1	3	1	2	0	0	1	3
TM7	0	0	0	0	0	0	0	0	0	0	0	0	1	3
Verrucomicrobia	6	14	6	19	0	0	1	5	5	11	0	0	13	57
ZB3	0	0	0	0	0	0	0	0	0	0	0	0	1	2
Total	**199**	**29,174**	**184**	**29,227**	**33**	**24,547**	**215**	**24,222**	**225**	**22,686**	**39**	**27,930**	**329**	**8656**

Values correspond to quality-filtered OTUs and sequences across the full data set. Ss, Sarcotragus spinosulus; Iv, Ircinia variabilis; D, "direct" method; I, "indirect" method; PW, "plate washing" method; seqs, sequence.

(**Figures 4B,C**). Dominant OTUs in the cultivatable community included the genera *Pseudovibrio*, *Vibrio*, *Shewanella*, *Aquimarina*, *Ruegeria,* and *Microbulbifer*. However, none of these taxa were ranked among the dominant OTUs captured by cultivation-independent methods. Unexpectedly, *Poribacteria*, *Chloroflexi* (SAR 202) and *Acidobacteria* (Sva0725) lineages were found in the cultivatable sponge community, however, each taxon was represented by one single OTU consisting of only one sequence read.

Irrespective of sample processing methods, 167 bacterial OTUs were found common to *S. spinosulus* and *I. variabilis*, while 103 and 168 bacterial OTUs were exclusively associated with each species, respectively (**Figure 4D**). For each sponge species, the most abundant "species-specific" OTU was affiliated with the *Bacteroidetes* phylum (class *Sphingobacteriia*). Bacterial OTUs found common to seawater and sponge communities from all processing methods numbered just 4 OTUs shared between *S. spinosulus* and seawater and five OTUs shared between *I. variabilis* and seawater (**Figures 4E,F**). These numbers rose to 32 bacterial OTUs shared by both sponge species with seawater when only cultivation-independent methods were considered (**Figure 4G**).

Analyses performed only with OTUs containing at least 50 sequences (i.e., "rare" symbionts discarded) showed that the number of otherwise considered "species-specific" or "method-specific" OTUs dramatically decreased (Figure S3, Supplementary Material). Notably, the community of "rare" OTUs was highly diverse in both sponge species, encompassing 163 and 236 OTUs found in *S. spinosulus* and *I. variabilis*, respectively, of which 86 and 160 OTUs were exclusive to each sponge species (Table S2, Supplementary Material). These OTUs comprised typical sponge-associated phyla encountered in the dominant symbiont pool such as *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Proteobacteria* (*Alpha* and *Gamma* classes) and *Poribacteria* (Tables S2A,B). A thorough overview of the taxonomic affiliation of OTUs shared by or specific to the host species and processing

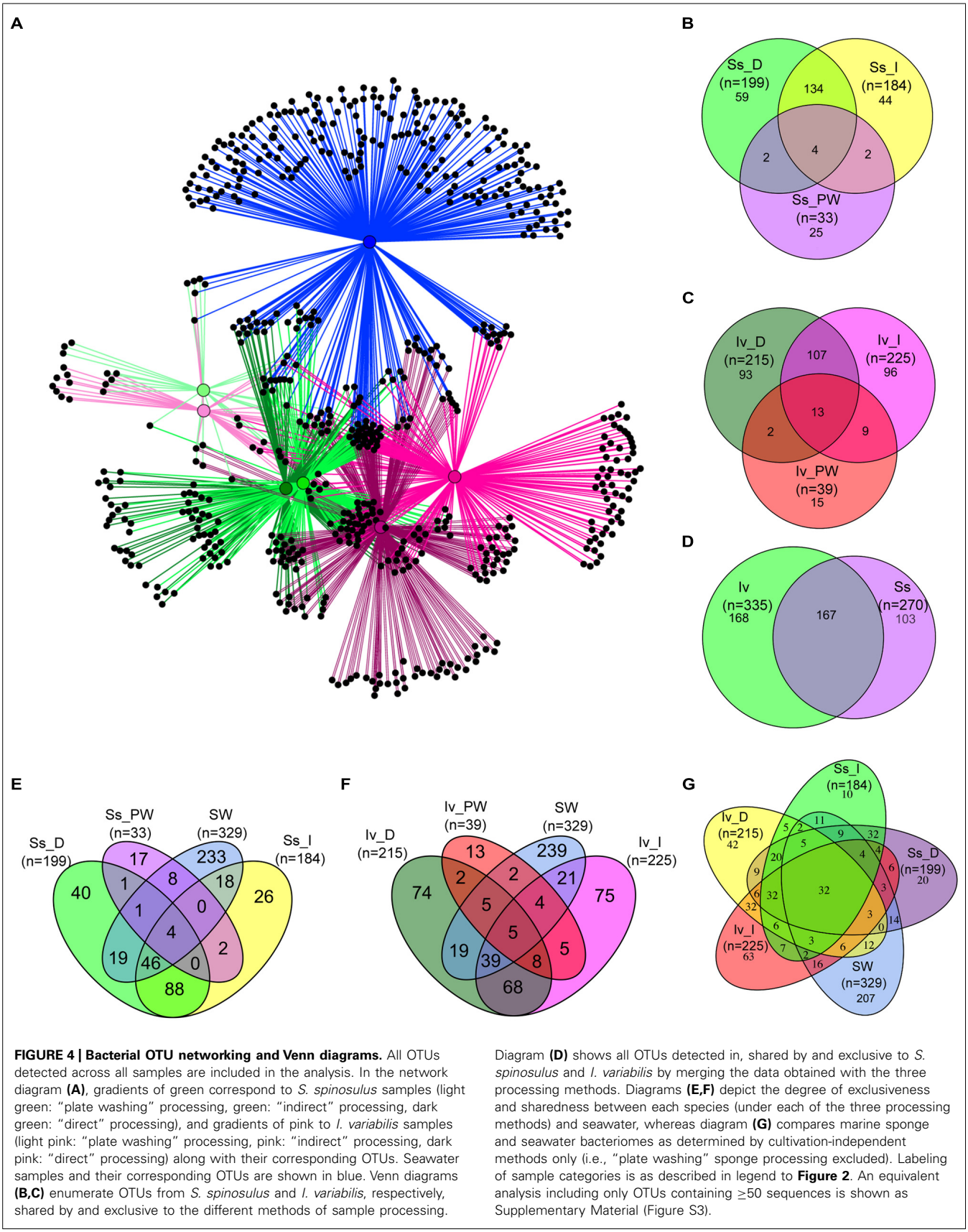


FIGURE 4 | Bacterial OTU networking and Venn diagrams. All OTUs detected across all samples are included in the analysis. In the network diagram **(A)**, gradients of green correspond to *S. spinosulus* samples (light green: "plate washing" processing, green: "indirect" processing, dark green: "direct" processing), and gradients of pink to *I. variabilis* samples (light pink: "plate washing" processing, pink: "indirect" processing, dark pink: "direct" processing) along with their corresponding OTUs. Seawater samples and their corresponding OTUs are shown in blue. Venn diagrams **(B,C)** enumerate OTUs from *S. spinosulus* and *I. variabilis*, respectively, shared by and exclusive to the different methods of sample processing. Diagram **(D)** shows all OTUs detected in, shared by and exclusive to *S. spinosulus* and *I. variabilis* by merging the data obtained with the three processing methods. Diagrams **(E,F)** depict the degree of exclusiveness and sharedness between each species (under each of the three processing methods) and seawater, whereas diagram **(G)** compares marine sponge and seawater bacteriomes as determined by cultivation-independent methods only (i.e., "plate washing" sponge processing excluded). Labeling of sample categories is as described in legend to **Figure 2**. An equivalent analysis including only OTUs containing ≥50 sequences is shown as Supplementary Material (Figure S3).

methods surveyed in this study is provided in Appendix S4 (Supplementary Material). Highly congruent results with those reported above were obtained when network analysis was applied to size-normalized data sets (Appendix S5, Supplementary Material).

Ordination of bacterial OTUs

For the first sequence threshold comparison (1236 sequences/sample), two concise sample clusters could be visualized by PCoA: (i) all seawater replicates, and (ii) all sponge specimens processed with the "plate washing" method (**Figure 5A**). The remaining samples comprised all sponge replicates processed via cultivation-independent methods. The PCoA analyses showed a high similarity within *S. spinosulus* replicates treated with both cultivation-independent methods, while there was a lower correspondence between methods and higher individual-to-individual variability detected for *I. variabilis* samples (**Figure 5A**). After increasing the sequence depth by removing the seawater samples from the analysis (3688 sequences/sample, **Figure 5B**), the sharp dichotomy between sponge samples handled with cultivation-dependent and cultivation-independent methods persisted, whereas the divergence between *I. variabilis* specimens treated with cultivation-independent procedures became more apparent. For both sequence thresholds, the 3D plots were helpful in demonstrating correspondences between the samples and some of the most abundant bacterial phyla and/or classes, such as the prevalence of the *Alphaproteobacteria* in the culturable sponge fraction and the shift between *Flavobacteriia* and *Sphingobacteriia* as the prevalent *Bacteroidetes* class in seawater and sponge samples, respectively (**Figure 5**).

Canonical correspondence analysis (CCA) of the whole OTU data revealed that, collectively, independent variables (i.e., seawater, processing methods, and sponge species) could explain 46.8% of the total data set variation. The discrepancies between (i) cultivation-dependent and cultivation-independent methods, and (ii) sponge (all handling methods included) and seawater samples accounted for 41.8 and 41.4% of the explained variability, respectively (Figure S4, Supplementary Material). These values were considerably larger than the individual effects of the sponge species (*I. variabilis vs. S. spinosulus*, 12%) and cultivation-independent procedures ("direct" *vs.* "indirect," 4.8%) on community data variation. The resulting CCA diagram distinguished seawater, sponges processed with cultivation-independent methods, and sponges processed with the cultivation-dependent method into three sample clusters (Figure S4A). Patterns of host species-specificity became evident when only *I. variabilis* and *S. spinosulus* specimens characterized by cultivation-independent methods were contrasted (Figure S4B), revealing that each sponge species held its own unique bacterial community. Finally, the shape of *I. variabilis* communities was significantly influenced by the cultivation-independent methods used (Figure S4C), however no such clustering was observed for *S. spinosulus* (Figure S4D).

IN TUBE FISH-CLSM

The bacterial groups targeted by FISH were chosen to represent distinct patterns of abundance within the sequencing data set. Whereas *Proteobacteria* can be regarded as a "generalist" phylum dominant in seawater and sponge communities (both cultured and uncultured), *Acidobacteria* constitutes a "specialist" phylum with greater abundance within the unculturable sponge microbiome. The detected bacterial cells were mainly cocci found in between sponge cells, with no evidence for a taxon-dependent aggregation of bacteria within the sponge body. In all analyzed samples, bacterial cells were seldom found on spongin filaments. The high abundance of cells precluded discrete counting of cell numbers, and taxon abundance data relative to total bacterial coverage was

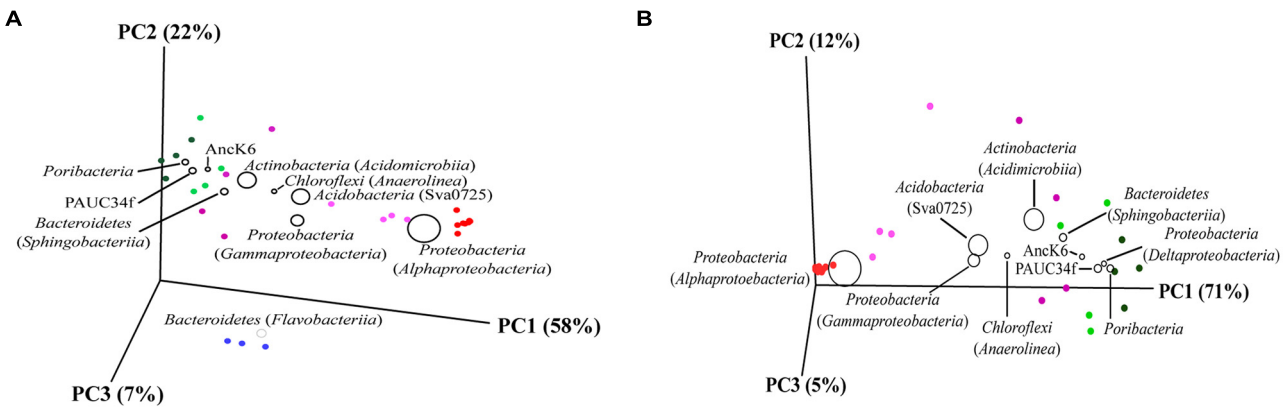


FIGURE 5 | Principal coordinates analysis of bacterial community profiles at the phylotype (OTUs) level. Analyses including sponge and seawater samples **(A)** and sponge samples only **(B)** are shown, and were performed with normalized sequencing depths [1236 seqs/sample in **(A)** and 3668 seqs/sample in **(B)**] using the Unifrac metric. The ten most dominant bacterial taxa (at the phylum or class level) are plotted on both diagrams. Symbol sizes of bacterial taxa correspond to their respective, mean relative abundances across the whole data set. The position of bacterial taxa in the ordination space is determined by the correlation between their relative abundances and the sample categories defined in this study. Colored circles represent replicates within each sample category. Labeling of sample categories is as described in legend to **Figure 4**, except for both sponge species under the "plate washing" method, which are colored in red. Alternatively, constrained canonical ordination was performed using normalization of abundance data (and not of library size) to determine whether sample categories significantly influence variation in 16S rRNA gene profiling by 454-pyrosequencing. These analyses are shown as Supplementary Material (Figure S4).

retrieved instead. The relative abundance of *Alphaproteobacteria* in both sponge species was similar: 27.14% in *S. spinosulus* (**Figures 6A,E**) and 22.85% in *I. variabilis* (**Figures 6B,F**), whereas the *Gammaproteobacteria* were more abundant in *S. spinosulus* (21.41%, **Figures 6C,E**) than in *I. variabilis* (9.55%, **Figures 6D,F**). Both *Alphaproteobacteria* and *Gammaproteobacteria* were more abundant than *Acidobacteria* in *S. spinosulus* (9.09%, **Figure 6G**). In *I. variabilis*, the abundance of *Acidobacteria* (6.20%, **Figure 6H**) was similar to that of *Gammaproteobacteria* and lower than that of *Alphaproteobacteria*. Instead, *Acidobacteria* 16S rRNA gene tags accounted for 16.91 and 20.68% of the total communities in *S. spinosulus* and *I. variabilis*, respectively, surpassing numbers obtained for *Alpha-* (3.90% for *S. spinosulus* and 4.27% for *I. variabilis*) and *Gammaproteobacteria* (*S. spinosulus* 9.78% and *I. variabilis* 11.57%) in both sponge species according with 454-pyrosequencing.

DISCUSSION

SAMPLE HANDLING EFFECTS ON SPONGE BACTERIAL COMMUNITIES DEPEND ON THE HOST SPECIES

The extent to which existing methods of sample preservation, processing and DNA extraction may affect the structure of sponge symbiont communities is currently under-appreciated. Indeed, detailed analyses comparing sponge microbial community data retrieved with different methodologies are very scarce (see e.g., Hardoim et al., 2009; Simister et al., 2011). The "direct" processing method has been widely used to evaluate the diversity of microbial communities in marine sponges (Hentschel et al., 2002; Taylor et al., 2005; Lee et al., 2011; Hardoim et al., 2012) and is, moreover, the fundamental technique used in metatranscriptomic studies (Radax et al., 2012). By contrast, the "indirect" processing method, through which most of the sponge cells are eliminated, is used in metagenomic, metaproteomic,

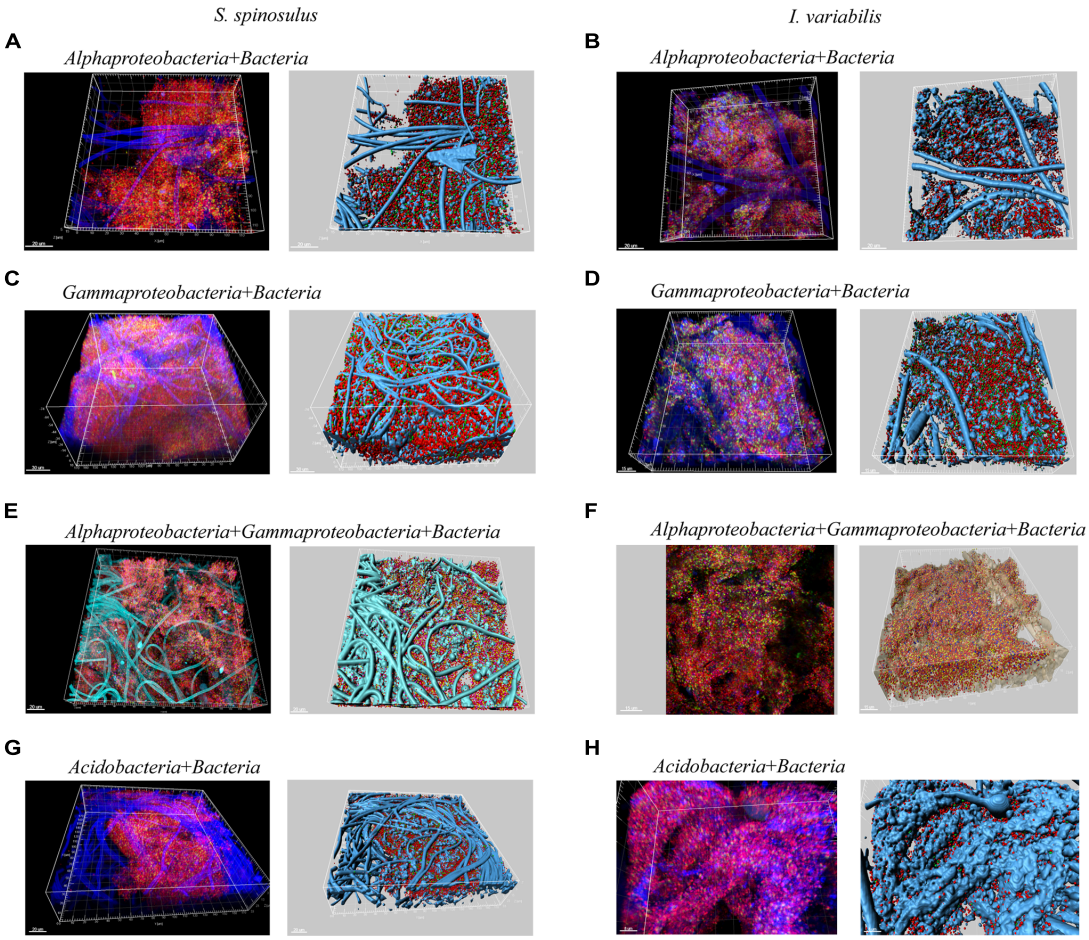


FIGURE 6 | Confocal laser scanning microscopy images of fluorescent *in situ* hybridization-stained bacteria in *S. spinosulus* and *I. variabilis*. Volume rendering images (left in each panel) and their corresponding 3D reconstructions (right in each panel) are shown for hybridizations with the Cy3-labeled universal bacterial probe (red cells) coupled to ALEXA488- or Cy5-labeled group-specific probes targeting the *Alpha-* and *Gammaproteobacteria* classes and the phylum *Acidobacteria*. When solely used in combination with the universal probe **(A–D,G,H)**, cells of these taxonomic groups appear as yellowish cells in the volume rendering images and as green objects in the 3D reconstructions. For co-hybridizations including bacterial, alpha and gammaproteobacterial probes **(E,F)**, the latter two groups are represented by yellowish and pink cells, respectively, except in the 3D reconstruction of *I. variabilis* **(F)**, in which gammaproteobacterial cells appear in purple. Sponge background structure, vastly dominated by profuse spongin filaments, is shown overall in cyan or blue, except in **(F)** where it is displayed in semi-transparent brown.

and single cell genomic surveys that target the sponge microbiota (Fieseler et al., 2006; Siegl and Hentschel, 2010; Thomas et al., 2010a; Siegl et al., 2011; Fan et al., 2012; Liu et al., 2012; Bayer et al., 2013). In the present study, both approaches led to similar bacterial diversity and composition results for *S. spinosulus*, but not for *I. variabilis*. In fact, we found that several OTUs consistently lost abundance while one OTU classified as *Acidobacteria* showed increased abundance in *I. variabilis* replicates processed with the "indirect" as compared to the "direct" method (see Appendix S6 for details), resulting in higher methodologically dependent symbiont community variability in this species than in *S. spinosulus*. This contrast may relate to the density of the collagenous filaments present in the mesohyl of these sponges, which is higher in *I. variabilis* making this species exceptionally tough and more difficult to tear or cut in comparison with other species in the Irciniidae family (Cook and Bergquist, 2002). Consequently, variability in the mechanical easy of bacterial cell detachment and disruption from the *I. variabilis* endosome matrix may have resulted in methodology-dependent, non-corresponding community structures retrieved from the same host. Rapid fingerprinting of sponge microbial communities by traditional methods such as PCR-DGGE or T-RFLP may still be an adequate means of assessing whether microbial cell enrichments usually prepared for metagenomics are representative of the community directly determined from the sponge body. Overall, the ability of each cultivation-independent processing method to accurately estimate the actual *in situ* sponge bacterial community may differ from species to species; therefore preliminary data acquisition to aid in the choice of methodology is advisable prior to in-depth analyses of sponge microbiome diversity and function.

CONTRIBUTION OF ABUNDANT AND RARE OTUs TO HOST-SPECIFIC BACTERIAL COMMUNITY PROFILES

The degree of conservation of bacterial communities in marine sponges across host species, habitats and oceans has important implications for the management of marine genetic and metabolic resources given the status of these holobionts as the most prolific source of biologically active compounds in the oceans (Piel, 2004). Recent studies have suggested that these communities are host species-specific (Webster et al., 2010; Lee et al., 2011; Schmitt et al., 2012), contrary, in principle, to earlier studies which indicated microbiome conservation across sponge hosts and geographical locations (Hentschel et al., 2002). Through a detailed analysis of sympatric and co-familial sponges this study supports the increasing evidence for host-dependent symbiont community structures among phylogenetically related sponge species, acquired recently with the use of more traditional techniques (Erwin et al., 2012; Hardoim et al., 2012). Qualitatively, the pool of OTUs found exclusively in each species was largely circumscribed by "rare" symbionts: when OTUs deemed as less dominant were discarded from the analysis, the number of "host species-specific" bacterial phylotypes was drastically reduced whereas a strong signal for conservation of the more prevalent symbionts remained. We therefore propose that the views of host-conserved and host-specific bacterial communities in marine sponges are not mutually exclusive, but rather complementary facets of one single biological process. Indeed, the quantitative contribution of the so-called "rare" and often "species-specific" OTUs to the total bacteriome of the surveyed sponges was small (*c.* 1.9% of all analyzed sequence reads) despite their diversity. Host specificity was therefore found to be chiefly determined by full quantitative profiles including all community members, their corresponding abundance ranks and differences in abundance between host species. Interestingly, the phylum-level taxonomic composition of the uncultivated, "rare" sponge symbionts resembled that of the dominant symbionts. This could suggest maintenance of core, functional attributes across phylogenetically related microbes with varying abundances within the community, a mechanism that could confer functional stability to the marine sponge holobiont in face of changing conditions, be they host-induced or not. However, closely related bacterial species, and even strains within species, may also have widely differing functions that can result from the acquisition of traits via horizontal gene transfer, a well-documented phenomenon within the *Proteobacteria* (Costa et al., 2009; van Elsas et al., 2011). In light of the current evidence for abundant horizontal gene transfer potential (Thomas et al., 2010a) and whole genome differentiation among close bacterial relatives within the marine sponge microbiome (Esteves et al., 2013), the net contribution of intra- and inter-species bacterial diversity to the spectrum of functions in these communities remains to be understood.

CULTURABLE SYMBIONTS ESCAPE THE MOLECULAR RADAR

It has been demonstrated that cultivation-independent methods often fail to detect bacteria isolated with culture-dependent procedures, raising concerns about the validity of cultivation-independent studies (Donachie et al., 2007). In our assay, cultivation of sponge-associated bacteria detected about 35–40 bacterial OTUs per species in comparison with 180–220 OTUs identified by cultivation-independent methods (see **Table 3** for details). These ratios surpass typical cultivability estimates based on CFU/microscopy cell count ratios, which in the case of our sponge specimens was in the range of 0.01–0.1% (Hardoim et al., 2012). However, about half of the OTUs recovered with the "plate washing" method escaped detection via cultivation-independent approaches, while the remainder was usually detected by the latter procedures in much lower numbers. These results indicate that marine agar culturing enriches for lower abundance symbionts that remain elusive to cultivation-independent methods. Because our methodology enabled direct comparisons between cultured and uncultured microbiomes through a standardized metagenomic DNA analysis pipeline, biases induced by DNA extraction, PCR amplification and colony picking-and-purification procedures are less likely to explain the abrupt differences observed between these communities in our survey. The hypothesis that the cultivatable sponge-associated bacteriome encompasses less abundant phylotypes that are considerably enriched during cultivation, by likely outcompeting more prevalent but less competent and/or slow-growing bacteria, must be assessed with protocols designed to detect rarer populations in the sponge community (Montalvo et al., 2014).

In this regard, the in-tube FISH-CLSM approach used here to image highly ranked bacterial taxa may be a powerful tool to localize specific and less abundant symbionts in keratose sponges. In this study, FISH-CLSM and 454-pyrosequencing estimates of bacterial abundance were not always congruent. Variable target specificity and coverage between the techniques may have accounted for these differences, although the additional steps required for 454-pyrosequencing (cell detachment and lysis, DNA extraction and PCR) are expected to add more analytical biases compared to the direct FISH-CLSM method. Copy numbers are unlikely to explain the observed discrepancies. *Alphaproteobacteria* (more abundant in FISH-CLSM than in 454-pyrosequencing results) have an average of 2.4 copies of the ribosomal operon, whereas *Acidobacteria* (more abundant in 454-pyrosequencing) have an average of only 1.6 copies (Klappenbach et al., 2001). These results highlight the importance to use multiple methods for a more comprehensive analysis of symbiont abundance in marine sponges, as proposed elsewhere for other dense microbial settings such as soils (Ushio et al., 2014). Coupling nucleic acid sequencing (especially primer-less approaches, e.g., direct metagenome sequencing) and advanced imaging technologies seems an adequate strategy to be used in upcoming studies.

Although we did not anticipate the detection of *Acidobacteria*, *Chloroflexi,* and *Poribacteria* on culture plates, recent research supports their aerobic and heterotrophic growth capacities (Davis et al., 2011; Siegl et al., 2011; Kamke et al., 2013; see Appendix S7 – Supplementary Material – for a Discussion on *Poribacteria* results). Due to the very low number of sequences recovered, marine agar showed to be inappropriate for the future isolation of these organisms. Intriguingly, these and other lineages, such as *Kiloniella* sp. and *Ferrimonas* sp., could not be detected by a colony picking-and-isolation effort previously applied to our samples (Esteves et al., 2013). Beyond the comprehensiveness of the "plate washing-deep sequencing" procedure used here to characterize the cultivation-dependent communities, this outcome could be explained by the further possibility, enabled by this procedure, of uncovering strictly syntrophic microorganisms or micro-colonies that elude detection by the naked eye (Davis et al., 2011). Finally, the direct comparison between cultivation-dependent and -independent communities made in this study shows that traditional cultivation fails at capturing the prevalent bacterial associates of marine sponges. In this context, not only alternative medium recipes and incubation strategies are needed to enhance the cultivability of sponge symbionts in the laboratory, but improvements to our current methods to sample and analyze cultivated sponge microbiomes are desirable to extend our ability to assess the diversity and metabolic capacities of these and other symbiont cohorts in the future.

AUTHOR CONTRIBUTIONS

Cristiane C. P. Hardoim, Ana I. S. Esteves, and Rodrigo Costa conceived and designed the study. Cristiane C. P. Hardoim, Ana I. S. Esteves, Massimiliano Cardinale, and Rodrigo Costa performed the laboratory experiments. Cristiane C. P. Hardoim, Ana C. B. Cúcio, Massimiliano Cardinale, Cymon J. Cox, and Rodrigo Costa analyzed the data. Rodrigo Costa, Gabriele Berg, Joana R. Xavier, and Cymon J. Cox contributed reagents, materials and analysis tools. Cristiane C. P. Hardoim, Massimiliano Cardinale, and Rodrigo Costa wrote the manuscript draft. All authors revised the draft, approved the final manuscript version and are accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00611/abstract

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An ortholog of the *Leptospira interrogans* lipoprotein LipL32 aids in the colonization of *Pseudoalteromonas tunicata* to host surfaces

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The bacterium *Pseudoalteromonas tunicata* is a common surface colonizer of marine eukaryotes, including the macroalga *Ulva australis*. Genomic analysis of *P. tunicata* identified genes potentially involved in surface colonization, including genes with homology to bacterial virulence factors that mediate attachment. Of particular interest is the presence of a gene, designated *ptlL32*, encoding an ortholog to the *Leptospira* lipoprotein LipL32, which has been shown to facilitate the interaction of *Leptospira* sp. with host extracellular matrix (ECM) structures and is thought to be an important virulence trait for pathogenic *Leptospira*. To investigate the role of PtlL32 in the colonization by *P. tunicata* we constructed and characterized a Δ*ptlL32* mutant strain. Whilst *P. tunicata* Δ*ptlL32* bound to an abiotic surface with the same capacity as the wild type strain, it had a marked effect on the ability of *P. tunicata* to bind to ECM, suggesting a specific role in attachment to biological surfaces. Loss of PtlL32 also significantly reduced the capacity for *P. tunciata* to colonize the host algal surface demonstrating a clear role for this protein as a host-colonization factor. PtlL32 appears to have a patchy distribution across specific groups of environmental bacteria and phylogenetic analysis of PtlL32 orthologous proteins from non-*Leptospira* species suggests it may have been acquired via horizontal gene transfer between distantly related lineages. This study provides the first evidence for an attachment function for a LipL32-like protein outside the *Leptospira* and thereby contributes to the understanding of host colonization in ecologically distinct bacterial species.

Keywords: LipL32, seaweed/s, marine bacteria, *Pseudoalteromonas*, host-microbe interaction, bacterial attachment, algae

INTRODUCTION

Macroalgae, or seaweeds, are important ecosystem engineers in temperate marine environments and are a rich source of biologically active compounds (Egan et al., 2008, 2013b). The surface-associated microbial community (SAMC) that rapidly colonize the alga have important roles for normal morphological development, nutrient supply, and defense against unwanted colonizers (Goecke et al., 2010; Wahl et al., 2012; Hollants et al., 2013). Furthermore, the SAMC can influence the health of the host alga and members of this community have the potential to function as opportunistic pathogens (Gachon et al., 2010; Egan et al., 2013a). The composition of the SAMC for many macroalgal species has been well studied and includes both generalist epibionts and host-specific taxa that are distinct from the surrounding seawater (Longford et al., 2007; Tujula et al., 2010; Burke et al., 2011; Lachnit et al., 2011). In contrast, there remains a paucity of knowledge regarding the specific mechanisms that facilitate interaction of the SAMC with the host alga (Egan et al., 2013a).

Attachment is considered a key stage in host colonization, and facilitates the progression of both commensal and pathogenic bacterial-host interactions (Kline et al., 2009; Petrova and Sauer, 2012; Bogino et al., 2013). The abundance of specialized adhesins and pili encoded in the genomes of seaweed-associated bacteria indicates this is similarly an important aspect of macroalgal epibiosis (Thomas et al., 2008; Fernandes et al., 2011; Thole et al., 2012). The marine bacterium *Pseudoalteromonas tunicata* was originally isolated from the tunicate *Ciona intestinalis* and has since been well studied as a surface colonizer of the alga *Ulva australis* (Holmström et al., 1998; Rao et al., 2005, 2006). Attachment of *P. tunicata* to biotic and abiotic surfaces occurs within 2 h of contact, and cells proceed into biofilm formation within 24 h through the production of differentiated mushroom-shaped microcolonies (Mai-Prochnow et al., 2004; Dalisay et al., 2006). With the exception of a MSHA-like pili that has been demonstrated to play a role in the attachment of the bacterium to both abiotic and host surfaces (Dalisay et al., 2006), there is a lack of experimental data on the specific factors that mediate host colonization in this bacterium. Genome analysis of *P. tunicata* has identified genes with homology to putative colonization factors, lipoproteins, pili and outer membrane proteins

(OMP) (Thomas et al., 2008). A number of these genes have homology to factors that mediate specific interactions with host cells in other bacteria. One example, designated *ptlL32* (locus tag PTD2_05920) encodes for a protein with 47% identity to the *Leptospira* MSCRAMM (microbial surface components recognizing adhesive matrix molecules) lipoprotein, LipL32.

Leptospira species are the causative agent of the endemic zoonotic infection, leptospirosis (reviewed in Levett, 2001; Adler and de la Pena Moctezuma, 2010). LipL32 is highly conserved in pathogenic *Leptospira* species (with an average 98% amino acid identity) where it is the most abundantly expressed lipoprotein (Haake et al., 2004; Dey et al., 2007; Adler et al., 2011). The absence of LipL32 in saprophytic *Leptospira* strains and its ability to bind to extracellular matrix (ECM) structures suggest that LipL32 plays a major role in host-cell attachment during mammalian infections (Hauk et al., 2008; Hoke et al., 2008; reviewed in Murray, 2013). However the precise involvement of LipL32 in *Leptospira* pathogenesis remains unclear as recent studies using a *L. interrogans lipL32* transposon mutant failed to demonstrate a direct role for this protein in infection models (Murray et al., 2009).

P. tunicata rapidly attaches to ECM structures (Hoke et al., 2011) and the tunicate, *C. intestinalis,* a natural host of *P. tunicata*, possesses the genes necessary for ECM synthesis, including those encoding for collagen type IV, fibronectin, laminin, and nidogen (Huxley-Jones et al., 2007). In addition, *Ulva liza*, a close relative of *U. australis,* possesses the genes encoding ECM-like proteins, including collagen (Stanley et al., 2005). Interestingly, recombinantly produced PtlL32 bind ECM structures in a manner analogous to LipL32 from *Leptospira* sp. (Hoke et al., 2008). Moreover heterologously expressed PtlL32 is immunologically cross reactive with *Leptospira* LipL32 antibodies (Hoke et al., 2008) and the amino acid sequence across the characteristic calcium-binding and putative polypeptide binding regions of LipL32 is conserved in the two proteins (Hauk et al., 2009). This biochemical information suggests that PtlL32 is an ECM-binding protein, however the biological function and importance for *P. tunicata* is not established. Moreover prior to the identification of this LipL32 ortholog in *P. tunciata*, it was widely believe that LipL32 proteins were unique to pathogenic *Leptospira* spp. (Murray, 2013), raising the question as to the origin of PtlL32 and its' prevalence in other environmental bacteria. Here we use a combination of allelic exchange mutagenesis, attachment assays, colonization experiments, and phylogenetic analysis to demonstrate a role for this conserved lipoprotein in facilitating bacterial interaction with host surfaces and provide evidence that suggests LipL32-proteins may have been acquired via horizontal gene transfer from an environmental origin. This study provides the first experimental evidence for a function of LipL32-like proteins outside of the *Leptospira* genus and builds upon our current understanding of the traits that drive host colonization in marine bacteria.

MATERIALS AND METHODS

ALLELIC EXCHANGE AND COMPLEMENTATION OF *P. TUNICATA ptlL32*

A *P. tunicata ptlL32* allelic replacement mutant strain was generated using the Gene Splicing by Overlap Extension (SOE) PCR strategy (Horton, 1995) coupled with bi-parental conjugation and homologous recombination as described previously for the mutagenesis of *P. tunicata* (Egan et al., 2002; Mai-Prochnow et al., 2004). Briefly, the first section of the *ptlL32* gene was amplified from wild type (WT) genomic DNA with primers, first-forward, 5′ ATG AAA ATC AAA CTG GTC GTG G 3′; and first-reverse, 5′ CTG GTT TCG CTA AAT CAC CCA C 3′. In a separate PCR reaction, the second section was amplified with primers, second-forward, 5′ CAG ACA AAT TAA AAG CCG ATA AAG; and second-reverse, 5′ TTA TTT ATT GAC TGC TTT ATG TAA C 3′. A kanamycin resistance (kan^R) cassette was amplified from the plasmid pACYC177 (**Table 1**) using the following primers: kan^R forward, 5′ GAT TTA TTC AAC AAA GCC ACG 3′; kan^R reverse, 5′ ATT TAT TCA ACA AAG CCG CC 3′. A recombinant *ptlL32* knockout fragment (*ptlL32*::kan^R) was constructed using SOE-PCR (Horton, 1995) by "PCR splicing" the overhangs on the first and second PCR products onto the 5′ and 3′ ends of the kan^R cassette, respectively. Following PCR amplification of *ptlL32*::kan^R using the first-forward and second-reverse primers, the fragment was introduced into the *EcoRI* site of the suicide vector pGP704 (**Table 1**) (Miller and Mekalanos, 1988) to generated the vector pLP704. Standard electroporation techniques (Ausubel et al., 1994) were used to transfer pLP704 into *E. coli* SM10. The *P. tunicata* Δ*ptlL32* strain was constructed using allelic exchange by conjugation of the recombinant *E. coli* SM10 pLP704 strain with *P. tunicata* WT (str^R) (**Table 1**) (according to the method described in Egan et al., 2002). Exconjugants with the *ptlL32*::kan^R fragment inserted into the chromosome by homologous recombination were selected using VNSS (Marden et al., 1985) agar plates supplemented with streptomycin (200 $\mu g\ ml^{-1}$) and kanamycin (85 $\mu g\ ml^{-1}$). PCR confirmation of the recombination event was performed with a forward primer that target a region upstream of *ptlL32* in the WT chromosome, 5′ AAG CAT CCA GTG TGC AGT CG 3′, and the kan^R reverse primer.

To complement the Δ*ptlL32* strain, the *ptlL32* gene and surrounding potential promoter and terminator regions was amplified from WT genomic DNA with primers: WT forward, 5′ GCA ATA GCT TTC TTT GTT CCT C 3′; WT reverse, 5′ GAC ACA TCA GCA TCA CTC AC 3′. The *ptlL32* PCR product was ligated into the *SmaI* site of the broad host range plasmid, pBBR1 MCS5 (Kovach et al., 1995) to generate the complementation plasmid, pBBR1L. Standard electroporation was used to transfer pBBR1L into an *E. coli* SM10 donor strain (Ausubel et al., 1994), before bi-parental conjugation of the donor strain with *P. tunicata* Δ*ptlL32* as described previously (Egan et al., 2002). *P. tunicata* Δ*ptlL32* ex-conjugants with *ptlL32* complemented *in trans* were selected for on VNSS agar plates supplemented with gentamycin (50 $\mu g\ ml^{-1}$), kanamycin and streptomycin, and verified by PCR using the WT forward primer, and the gen resistance cassette reverse primer 5′ GCGGCGTTGTGACAATTT 3′. The confirmed complemented Δ*ptlL32* strain was named CΔ*ptlL32*.

BACTERIAL ATTACHMENT TO POLYSTYRENE AND MATRIGEL™ BASEMENT MEMBRANE MATRIX

The attachment of *P. tunicata* Δ*ptlL32* to polystyrene was compared to the WT using a cell adhesion assay. The bacterial strains were grown at 28°C for 24 h shaking in VNSS supplemented with the appropriate antibiotics (**Table 1**). The cell suspension was

Table 1 | Plasmids and bacterial strains used in this study.

Strain or plasmid	Relevant genotype[#]	References
E. coli		
SM10 λ pir	RP4-2-Tc::Mu, π replicase (pir); str^s kan^s	Simon et al., 1983
SM10 pLP704	pGP704:: *ptlL32*::kan^R *amp*	This study
SM10 pBBR1L	pBBR1 MCS5::*ptlL32 gen*	This study
SM10 pCJS10	pCJS10 *cat*	Dalisay et al., 2006
***P. tunicata* D2**		
WT str^R	Spontaneously resistant to streptomycin	Egan et al., 2002
GFP-labeled WT str^R	pCJS10 *cat* str^R	Dalisay et al., 2006
Δ*ptlL32*	*ptlL32* knockout mutant; str^R, *ptlL32*::kan^R *amp*	This study
CΔ*ptlL32*	Complemented *ptlL32* knockout mutant; *str, ptlL32*::kan^R *amp*; pBBR1 MCS5*ptlL32 gen*	This study
GFP-labeled Δ*ptlL32*	str^R, *ptlL32* knockout mutant; *ptlL32*::kan^R *amp;* pCJS10 *cat*	This study
PLASMIDS		
pBBR1 MCS5	Broad host range mobilizable vector, *gen*	Kovach et al., 1995
pBBR1 MCS5::*ptlL32*	Complementation vector containing intact *ptlL32*	This study
pCJS10-GFP	RSF1010 broad host range backbone, *gfp*mut3, *cat*	Rao et al., 2005
pGP704	Suicide vector, R6K ori, mob, *amp*	Miller and Mekalanos, 1988
pLP704	pGP704 with *ptlL32*::kan^R knockout fragment	This study
pACYC177	Cloning vector, *amp* kan^R	Chang and Cohen, 1978

[#] *str^S, streptomycin sensitive; kan^S, kanamycin sensitive; str^R, streptomycin resistance; kan^R, kanamycin resistance; tet, tetracyclin resistance; amp, ampicillin resistance; cat chloramphenicol resistance; gen, gentamycin resistance.*

centrifuged (6000 × g, 5 min), washed twice and resuspended in 1 ml of sterile NSS (Marden et al., 1985) at $Abs_{600\,nm} = 1$ ($\sim 10^9$ CFU ml^{-1}). Fifty microliters of cell suspension for each strain was added to triplicate wells of a polystyrene Costar® 96 well plate (Corning™). The plate was incubated for 6 h at 28°C with gentle shaking before non-adherent cells were removed by rinsing the wells six times with sterile PBS. Twenty-five microliters of a 200 μg ml^{-1} solution of trypsin were added to each well and the plate incubated at 37°C for 5 min to allow for cell detachment. Detached cells were then counted by dark field microscopy in a Helber™ bacterial counting chamber (Hawksley, Sussex, UK). Attached bacteria from a total 80 small squares (chosen based on the results of a random number generator) on the counting chamber were enumerated for each of the replicates. The experiment was performed in triplicate for each strain on three independent days. Statistical analysis was performed using SYSTAT 13 (SYSTAT Software Inc., USA) and significance was assessed using an unpaired Students two-tailed *t*-test.

To assess the attachment of Δ*ptlL32* to ECM, the assay outlined above was modified according to the protocol described in Hoke et al. (2008). The ECM preparation used in this study, BD Matrigel™ Basement Membrane Matrix (BD Biosciences, USA), is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, and is composed mainly of laminin, entactin, collagen, and mammalian growth factors (Vukicevic et al., 1992; Hughes et al., 2010). The wells of a 96 well plate were coated with 50 μL of BD Matrigel™ and incubated at 4°C overnight. The wells were then washed three times with PBS to remove excess ECM. The *P. tunicata* bacterial strains (**Table 1**) were grown, washed, and resuspended as described for the polystyrene adhesion experiment above. Fifty microliters of cell suspension for each strain was added to triplicate wells of a plate coated with BD Matrigel™, and incubated for 2 and 6 h at 28°C with gentle shaking. Non-adherent cells were then removed and detached cells enumerated as described for the polystyrene adhesion experiment. Significance was assessed using a One Way analysis of variance (ANOVA).

BACTERIAL ATTACHMENT TO *U. AUSTRALIS*

To assess the adhesion of *P. tunicata* Δ*ptlL32* to a living host surface an attachment assay to *U. australis* was performed as described previously (Dalisay et al., 2006). The *U. australis* samples were collected from Clovelly Bay, Sydney Australia and processed immediately. The algal samples were rinsed four times with 50 ml of autoclaved seawater and thallus sections of approximately 6 mm were excised from the mid thalli using a sterile scalpel. The algal surface was then cleaned to remove the majority of epiphytic bacteria. Briefly individual samples were swabbed with sterile cotton tips, containing 0.012% NaOCl for 5 min, and incubated for 24 h in an antibiotic mixture that consisted of ampicillin (300 μg ml^{-1}), polymyxin (30 μg ml^{-1}), and gentamycin (60 μg ml^{-1}). The alga thalli were then incubated for 1 h in 50 ml of 0.2 μm filtered-seawater to remove residue chemicals.

P. tunicata Δ*ptlL32* was labeled with green florescent protein (GFP) by introducing the broad host range plasmid pCJS10 as described previously for the WT strain (Dalisay et al., 2006) using bi-parental conjugation with an *E. coli* SM10 donor strain (Egan et al., 2002) (**Table 1**). GFP-labeled Δ*ptlL32* ex-conjugants were grown on VNSS agar plates supplemented with chloramphenicol (18 μg ml^{-1}) and confirmed by visual inspection under

the GFP filter cube of an epifluorescence microscope (DM-LB Leica). For the *U. australis* attachment assays, GFP tagged WT (Dalisay et al., 2006) and Δ*ptlL32* (**Table 1**) were grown in VNSS supplemented with chloramphenicol for 24 h at 28°C with shaking. One milliliter of culture was harvested by centrifugation (6000 × g, 2 min), washed three times with NSS, and resuspended at an $Abs_{600\,nm}$ of 0.3. A single thallus section was placed in a well of a 6 well plate (Corning™) containing 1 ml PBS and 1 ml of bacterial culture, and incubated for 6 h with gentle shaking.

After 6 h incubation the algal samples were rinsed three times in 2 ml PBS to remove loosely attached cells and visualized immediately using a Confocal Laser Scanning Microscope (Olympus Fluoview FV1000) under 40× oil magnification and 488 nm excitation. Seven images were taken randomly across each sample and the images were analyzed using ImageJ (Schneider et al., 2012). The number of GFP-fluorescing cells attached to the surface of the algae per mm^2 was quantified using the "analyze particles" function in ImageJ and the results were plotted using GraphPad Prism 6. In addition the number of microcolonies was manually counted (where >10 cells clustered together was classified as a microcolony) and the results plotted using GraphPad Prism 6. The assay was replicated four times in triplicate for each bacterial strain and data analyzed as described for the polystyrene adhesion experiment.

SEQUENCE RETRIEVAL AND PROTEIN ALIGNMENT OF PtlL32 ORTHOLOGS

Bacterial genomes were searched for orthologs to the *P. tunicata* gene PtlL32 using a blastp search tool of both the UniProt database (http://www.uniprot.org/) and the non-redundant database of the National Centre for Biotechnology Information (NCBI) in April 2014 (Altschul et al., 1990). All non-*Leptospira* orthologs with greater than 40% amino acid sequence identity to PtlL32 from *P. tunicata* (**Table 2**) and a further five sequences from representative *Leptospira* species were selected (*L. interrogans*, ADJ95774; *L. kirschneri*, AAF60198; *L. broomii*, EQA47312; *L. borgpetersenii*, ABJ79303; *L. santarosai*, AAS21795). All sequences were aligned with ClustalX using the default parameters (Larkin et al., 2007) and the resulting alignment curated with Gblocks to remove gap positions (Talavera and Castresana, 2007). The resulting alignment of 102 amino acid positions was then subject to maximum-likelihood analysis using PhyML 3.0 with the default LG substitution model and 100 bootstraps (Criscuolo, 2011). Trees were visualized using Dendroscope (Huson and Scornavacca, 2012). Information on the phylogeny and isolation of bacterial strains was obtained from published data and using the genome browser in the Integrated Microbial Genome (IMG) browser (https://img.jgi.doe.gov/cgi-bin/er/main.cgi) in April 2014 (Markowitz et al., 2009).

Table 2 | Characteristics of non-*Leptospira* species that possess orthologs of LipL32.

NCBI accession	Bacterial strain	Taxonomic affiliation	Isolation source
EAR27184	*Pseudoalteromonas tunicata*	C: Gammaproteobacteria O: Alteromondales F: Pseudoalteromonadaceae	Surface of the macroalga *Ulva* spp. (Australia) and the invertebrate *Ciona intestinalis* (Sweden)
ERG54672	*Pseudoalteromonas spongiae*	As above	Surface of the sponge *Mycale adhaerens* in Hong Kong
ERG42655	*Pseudoalteromonas rubra*	As above	Mediterranean seawater of the coast of France
ESP92640	*Pseudoalteromonas luteoviolacea*	As above	Surface of the coral *Montastrea annularis* in reef water off the coast of Florida, USA
ADZ89577	*Marinomonas mediterranea*	C: Gammaproteobacteria O: Oceanospirillaceae F: Oceanospirillales	Mediterranean seawater from the southeastern coast of Spain
EDM65737	*Moritella* sp. PE36	C: Gammaproteobacteria O: Alteromondales F: Moritellaceae	Deep ocean waters of the Pacific Ocean San Diego, USA
ADV49486	*Cellulophaga algicola*	P: Bacteroidetes C: Flavobacteria O: Flavobacteriales F: Flavobacteriaceae	Surface of a sea ice-chain forming pennate diatom, *Melosira* sp. in the Eastern Antarctic coastal zone
ADY29818	*Cellulophaga lytica*	As above	Beach mud in Limon, Costa Rica
CDF79332	*Formosa agariphila*	As above	Surface of the green alga *Acrosiphonia sonder* isolated from the Sea of Japan
EDM44616	*Ulvibacter* sp. SCB49	P: Bacteridetes C, O, F: Unclassified	Surface waters of the Pacific Ocean Southern California Bight, USA
AEE17958	*Treponema brennaborense*	P: Spirochaetes C: Spirochaetia O: Spirochaetales F: Spirochaetaceae	Ulceritive skin lesion on a bovine foot infected with digital dermatitis, Germany

RESULTS AND DISCUSSION

PtlL32 IS NOT REQUIRED FOR ATTACHMENT TO ABIOTIC SURFACES, HOWEVER CONTRIBUTES TO THE ATTACHMENT OF *P. TUNICATA* TO HOST SURFACES

To investigate the role of *ptlL32* in attachment to abiotic surfaces a *ptlL32* knock-out mutant (Δ*ptlL32)* was constructed and was compared to the WT *P. tunicata* strains for its ability to adhere to a polystyrene surface. After 6 h incubation there was no significant difference ($p > 0.8$) between the number of cells attached for the Δ*ptlL32* strain compared to WT (**Figure 1**). These data demonstrate that a mutation in *ptlL32* has no immediate impact on the ability of *P. tunicata* to attach to abiotic surfaces.

Attachment of the *P. tunicata* Δ*ptlL32* strain to ECM structures was also compared to WT and CΔ*ptlL32*, at times that have been characterized as early (2 h) and late (6 h) stages of irreversible attachment in other bacterial species (Hinsa et al., 2003; Palmer et al., 2007; Li et al., 2012). **Figures 2A,B** show the average numbers of cells attached per mm^3 for the three strains after 2 and 6 h, respectively. The mutant strain Δ*ptlL32* exhibited 10-fold reduction ($p < 0.001$) in attachment compared to WT after both 2 and 6 h. There was also a clear increase in attached cells over time for the WT, but not for the mutant strain (Δ*ptlL32)* (**Figure 2**). Complementation of *ptlL32 in trans* (strain CΔ*ptlL32*) restored the WT phenotype, excluding polar effects of the knock-out mutant. Together with the observations that mutations in *ptlL32* had no effect on attachment of *P. tunicata* to abiotic surfaces (**Figure 1**), these data show that Ptlp32 contributes specifically to the ability of *P. tunicata* to adhere to complex biological surfaces.

Bacterial colonization of various marine eukaryotes has been demonstrated in other non-algal systems to be mediated by surface-specific adhesins (Mueller et al., 2007; Bulgheresi et al., 2011; Stauder et al., 2012); and we hypothesize that PtlL32 may facilitate adhesion to ECM-like surfaces in the marine environment, including, but not limited to, its natural host *U. australis.* To further explore this role we assessed the ability of Δ*ptlL32* and the WT *P. tunicata* strains to attach to the surface of *U. australis*. *P. tunicata* Δ*ptlL32* demonstrated a significant reduction in the number of cells attached to the surface of the alga (**Figures 3**, **4A**) compared to WT cells ($p < 0.001$). A reduction in the number of cell aggregates on the surface of *U. australis* in the mutant strain (**Figure 4B**; $p < 0.001$) also indicates that biofilm maturation may be impaired. The reduced attachment of Δ*ptlL32* cells to the surface of the alga compared to WT suggests that PtlL32 may bind to specific host cell wall components. The major cell wall matrix components for *Ulva* species are cellulose and ulvan (Lahaye and Robic, 2007), however the structural details are unknown (Lahaye and Kaeffer, 1997; Robic et al., 2009). Genetic analysis of *U. linza* identified genes involved in the synthesis of hydroxyproline-rich glycoproteins, including collagen (Stanley et al., 2005), which are homologous to components in the ECM preparation used above (Hughes et al., 2010). *Leptospira* LipL32 has also been shown to bind a number of proteins, including different types of collagen (Hauk et al., 2008; Hoke et al., 2008; Chaemchuen et al., 2011). Therefore, the reduced adhesion of Δ*ptlL32* to both ECM and *U. australis* may be the result of interaction of PtlL32 with host proteins, including different forms of collagen.

The observation that PtlL32 mediates colonization only on biotic surfaces stands in contrast to what has been observed for the MSHA-like pili, which mediates adhesion to both biotic and

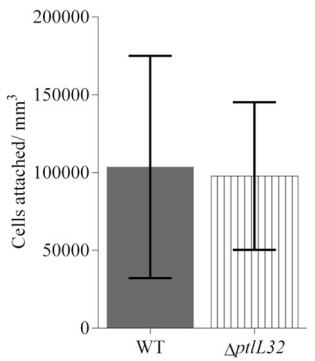

FIGURE 1 | Attachment of *P. tunicata* WT (solid fill) and Δ*ptlL32* (vertical stripes) to a polystyrene well plate after 6 h of incubation. The numbers of cells attached per mm^3 were determined using direct counts of detached bacteria in a Helber bacterial counting chamber. Significance was assessed using the Students unpaired, two-tailed *t*-test ($p > 0.8$). Error bars represent standard deviation.

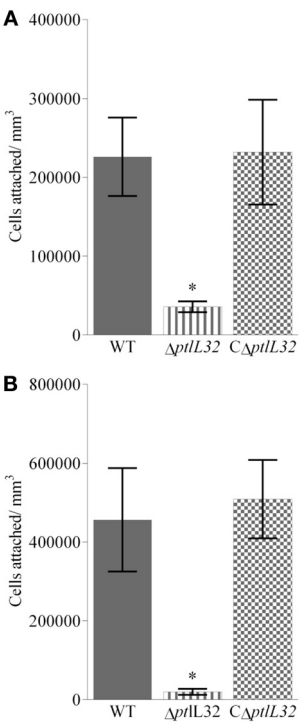

FIGURE 2 | Attachment to Matrigel™ after 2 h (A) and 6 h (B) for Δ*ptlL32* (horizontal stripes) compared to WT (solid fill) and CΔ*ptlL32* (small squares). The number of cells attached per mm^3 for each strain was estimated using direct counts of detached bacteria in a Helber bacterial counting chamber. Averages are shown as columns with $n = 9$. Error bars represent standard deviation. *Indicates significance with a $p < 0.001$ using an ANOVA.

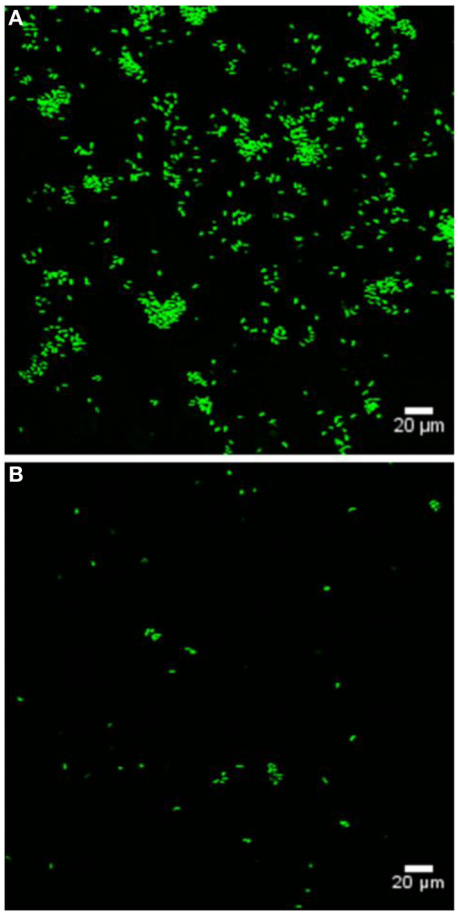

FIGURE 3 | Representative confocal laser scanning microscopy images of GFP-labeled WT (A) and GFP-labeled Δ*ptlL32* (B) attached to the surface of *U. australis*. Green fluorescent cells were enumerated using ImageJ software. Images were captured using a FV1000 confocal laser-scanning microscope. Scale bar represents 20 µm.

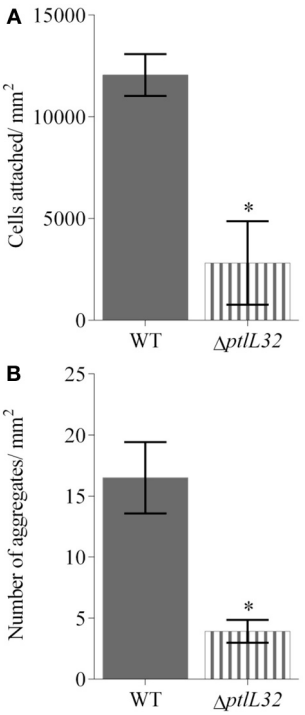

FIGURE 4 | Average number of cells attached to (A), and cell aggregates (defined as a tight cluster of >10 cells) (B) on *U. australis* for GFP-labeled WT (solid fill) and GFP-labeled Δ*ptlL32* (horizontal stripes) after 6 h incubation. The number of green fluorescing cells or aggregates in each field of view was counted using ImageJ and an average expressed for cells per mm^2. Error bars represent standard deviation. Error bars represent standard deviation at 45 replicates and * denotes a significant difference with a $p < 0.001$ using the unpaired Students two-tailed *t*-test.

abiotic surfaces (Dalisay et al., 2006). Changes in the membrane proteome of *P. tunicata* between cells grown on either BSA-coated or ECM-coated surfaces have recently been observed (Hoke et al., 2011) and therefore it is likely that *P. tunicata* utilizes a distinct subset of proteins to adhere to different surfaces. Thus the available data suggests a conserved function for LipL32-like proteins in facilitating interaction with ECM structures (Hauk et al., 2008; Hoke et al., 2008), a novel finding given the absence of an overlapping niche between *P. tunicata* and *Leptospira* species.

PtlL32 ORTHOLOGS HAVE A PATCHY DISTRIBUTION ACROSS SPECIFIC GROUPS OF ENVIRONMENTAL BACTERIA

Given that PtlL32 is important for mediating host colonization in *P. tunicata* we sought to determine the distribution and relationship of orthologous proteins across other non-*Leptospira* organisms. *In silico* analysis of publically available bacterial genomes revealed sequences similar to PtlL32 in eleven other species, of which five were isolated from eukaryotic hosts, ten are marine bacteria and only the spirochete *Treponema brennaborense* has been associated with disease (**Table 2**). This distribution is interesting considering that until relatively recently LipL32-like proteins were thought to be unique to pathogenic *Leptospira* (Hoke et al., 2008; Murray, 2013). Given the untapped bacterial diversity in environmental ecosystems these data further suggest that the distribution of LipL32 orthologs may be greater still.

Multiple alignment of the protein sequences revealed a total of 29 amino acids positions that are fully conserved across all sequences; with increased conservation of amino acids at positions 85–107 (Supplementary Material). The regions of sequence conservation also include components of the protein that are postulated to mediate binding of host structures in *Leptospira*, including the polypeptide binding function located in the C-terminal (Supplementary Material, amino acids 188–272) (Hauk et al., 2008; Hoke et al., 2008) and an acidic loop at amino acids 164–178 (AKPVQKLDDDDDGDD) (Hauk et al., 2009; Vivian et al., 2009). These regions of amino acid conservation were previously highlighted in the crystal structures of three non-*Leptospira* LipL32 proteins for their potential role in conferring similar binding properties to the orthologous proteins (Hauk et al., 2009; Vivian et al., 2009).

Analysis of the phylogenetic relationship between the PtlL32 orthologs provides new insight into its evolutionary origins. Most striking is the observation that the *P. tunicata* sequence clusters with the *Leptospira* and not with those from other

Pseudoalteromonas species or Gammaproteobacteria (**Figure 5**). Likewise, a PtlL32 ortholog of *T. brennaborense* (phylum Spirochaetes) does not cluster with the other members of the Spirochaetes (i.e., *Leptospira* spp.) (**Figure 5**, **Table 2**). This clustering of LipL32 sequences outside of their taxonomic relatives is in support of an acquisition via horizontal gene transfer (HGT) and/ or maintenance only in the pathogenic *Leptospira* and a selected group of environmental organism. In support of this theory, an examination of the GC content of the *lipL32* genes revealed a deviation from the genomic GC content in examples of *Leptospira lipL32* genes, but not in the non-*Leptospira* orthologs. Given the diversity of the ecosystems in which many of the LipL32 containing strains are found (**Table 2**) it possible that the *Leptospira* LipL32 has evolved from environmental strains, where it facilitates commensal interactions, to a role in pathogenic interaction with animals. This proposed "dual function" for LipL32 is in line with observations of other environmentally acquired pathogens, where certain colonization traits mediate pathogenesis in a host-associated context, whilst facilitating microbial survival and persistence in the environment (Casadevall et al., 2003; Vezzulli et al., 2008). Indeed an environmental origin of pathogenic *Leptospira* has also been recently suggested and is supported by observations that the closest orthologs to many hypothetical *Leptospira* genes are from environmental rather than pathogenic bacteria (Murray, 2013).

CONCLUSION

Understanding of the mechanisms that facilitate bacterial interactions with marine surfaces is poorly understood compared to that of medically relevant systems. Here we have begun to fill this knowledge gap by investigating the mechanisms that facilitate interactions between the marine bacterium *P. tunciata* with its macroalgal host. We found that a *P. tunicata* Δ*ptlL32* strain attached with a greatly reduced capacity to the biotic surfaces (**Figures 2–4**), a finding that is in line with previous reports indicating that the colonization of *U. australis* by *P. tunicata* involves multiple adhesins (Dalisay et al., 2006; Thomas et al., 2008). Having multiple mechanisms for adhesion may represent a means by which the bacterium can mediate host specificity and/or modify its interaction with the host in response to environmental variability. A degree of functional overlap has also been reported for adhesive structures in a diverse range of bacteria where the apparent redundancy may reflect the ability of these organisms to interact with multiple eukaryotic hosts or tissues (Ramey et al., 2004; Clarke and Foster, 2006; Rodriguez-Navarro et al., 2007; Pruzzo et al., 2008). For example, collagen is also a component of the ECM of the tunicate *C. intestinalis* (Vizzini et al., 2002), since *P. tunicata* has also been isolated from *C. intestinalis* it is possible that PtlL32 also has a function in mediating colonization to both macroalgae and marine invertebrates.

To the best of our knowledge this is the first study to investigate the function for a LipL32 ortholog in an organism outside the *Leptospira* species and the results speak to a conserved function for this protein in mediating association with host cell matrix components. Phylogenetic analysis suggests the *Leptospira* LipL32 was acquired from environmental bacteria and that LipL32 proteins act as "dual function" traits (Casadevall et al., 2003; Casadevall, 2006; Hoke et al., 2008; Murray, 2013) facilitating both pathogenic interactions by *Leptospira* spp. and host colonization by the environmental bacterium *P. tunicata.* This study therefore adds weight to the hypothesis presented by Egan et al. (2013a), that such dual function traits may facilitate interactions between bacteria and macroalgae; a relationship that has important implications for macroalgal health, as well as nutrient cycling, and homeostasis in the temperate ocean environment (Wahl et al., 2012; Egan et al., 2013b).

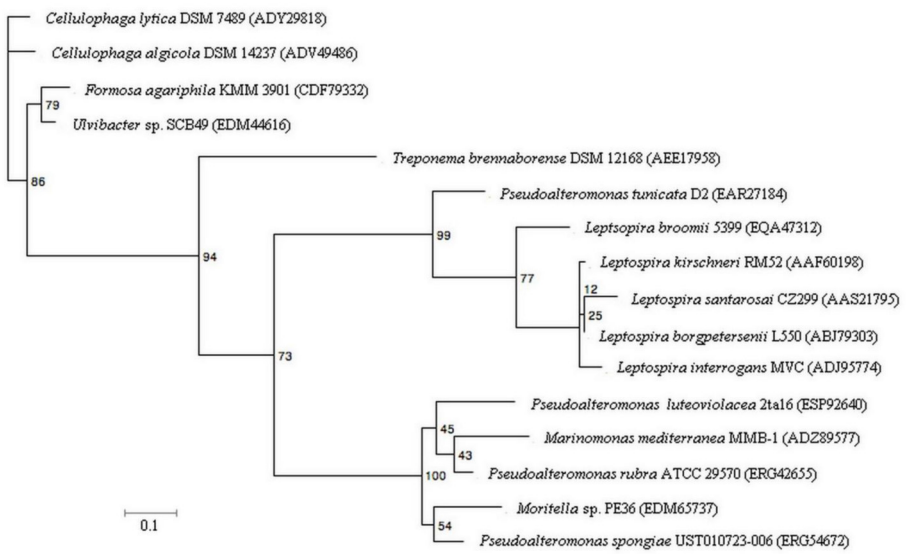

FIGURE 5 | Maximum-likelihood tree of PtlL32 and orthologous protein sequences in non-*Leptospira* species. Five representative *Leptospira* LipL32 sequences were included for comparison with the non-*Leptospira* sequence. NCBI GenBank accession numbers for each protein sequence is provided in brackets. Bootstrap values for 100 replicates are shown for each node. The scale bar represents 10% sequence divergence.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00323/abstract

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Think laterally: horizontal gene transfer from symbiotic microbes may extend the phenotype of marine sessile hosts

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Since the origin of the animal kingdom, marine animals have lived in association with viruses, prokaryotes and unicellular eukaryotes, often as symbionts. This long and continuous interaction has provided ample opportunity not only for the evolution of intimate interactions such as sharing of metabolic pathways, but also for horizontal gene transfer (HGT) of non-metazoan genes into metazoan genomes. The number of demonstrated cases of inter-kingdom HGT is currently small, such that it is not yet widely appreciated as a significant player in animal evolution. Sessile marine invertebrates that vertically inherit bacterial symbionts, that have no dedicated germ line, or that bud or excise pluripotent somatic cells during their life history may be particularly receptive to HGT from their symbionts. Closer scrutiny of the growing number of genomes being accrued for these animals may thus reveal HGT as a regular source of novel variation that can function to extend the host phenotype metabolically, morphologically, or even behaviorally. Taxonomic identification of symbionts will help to address the intriguing question of whether past HGT events may constrain contemporary symbioses.

Keywords: lateral gene transfer, marine invertebrate, symbiotic bacteria, extended phenotype, host–symbiont interactions

ANIMAL–MICROBIAL SYMBIOSES PROVIDE AMPLE OPPORTUNITY FOR HGT

It is increasingly apparent that all sessile marine animals live in intimate association with a diversity of viruses, prokaryotes and unicellular eukaryotes, many of which may function as beneficial symbionts at some or all stages of the animal life cycle (Douglas, 2010; McFall-Ngai et al., 2013). This is not surprising given that animal–microbial interactions have been shaped by more than 600 million years of evolutionary history, traceable back at least to a common ancestor of all modern animals that lived in an ocean of microbes. This long and constant association has provided ample opportunity for all manner of interactions to evolve between kingdoms. The growing number of documented cases of astonishingly intimate symbioses between animal hosts and bacterial symbionts in particular (reviewed in McFall-Ngai et al., 2013), facilitated frequently by vertical transmission, almost certainly represents just the tip of the iceberg. The field of aquatic microbiology is well-poised to benefit from this growing appreciation of animal–bacterial interactions (e.g., Dubilier et al., 2008; Hentschel et al., 2012) by drawing upon the huge diversity of sessile marine animals and their equally diverse symbionts, coupled with lateral thinking about the nature of the interactions that might occur between them. In doing so, we have the opportunity to apply knowledge of animal–microbe interactions to fundamental concepts in biology, and to marine biotechnological problems and applications. There is hope even that investigations in this area "may inform our understanding of diseases like cancer" (Robinson et al., 2013).

Querying the nature and extent of animal–microbe interactions can increasingly be done at the level of the genome. Genomes clearly reflect the deep, shared ancestry between animals and microbes; all three domains of life share approximately one third of their genes, notably including many that encode central metabolic pathways (Domazet-Loso and Tautz, 2008). Beyond this, however, we are becoming increasingly aware of diverse ways in which the genomes and genome products of animal hosts and their microbial symbionts can interact. Host–symbiont interactions revealed to date include critical roles for bacteria in marine invertebrate host nutrition, defense, reproduction, and development (McFall-Ngai et al., 2013). The sharing of gene products impacts directly on the ecological versatility of the host, because the co-opting of a more diverse genetic repertoire present in its bacterial partners (Lapierre and Gogarten, 2009) allows a host to rapidly expand its metabolic potential. Intracellular bacterial symbionts, for example, often encode proteins for metabolic capabilities that are lacking in the animal host, such as synthesis of essential amino acids, photosynthesis, or chemosynthesis (Unson and Faulkner, 1993; Dubilier et al., 2008; Venn et al., 2008; Wilson et al., 2014). Some marine invertebrates that feed on algae are even able to maintain symbioses with photosynthetically active algal plastids and use their photosynthate directly as a food source (Rumpho et al., 2011).

A less broadly appreciated outcome of host–symbiont interactions is the potential through deep evolutionary time for novel functions to be conferred via inter-kingdom horizontal (lateral) gene transfer (HGT), broadly defined as "non-genealogical transmission of genetic material from one organism to another" (Goldenfeld and Woese, 2007). Although most animal genes that can be assigned as homologs of microbial genes are unambiguously derived by descent (Domazet-Loso and Tautz, 2008), there are an increasing number of cases that instead seem to have

originated via HGT (Hughes and Sperandio, 2008; Keeling and Palmer, 2008; Boto, 2014; Schönknecht et al., 2014). The full extent to which HGT has occurred across the animal kingdom is currently unknown and the concept of HGT is not yet a mainstream paradigm in the fields of animal evolution and ecology. Recent technical advances, however, now mean that genomic (and proteomic, metabolomic) resources are rapidly becoming available for an increasing number and diversity of animal hosts and their microbial symbionts. For the first time, there is the promise of comprehensively exploring the way in which microbial genomes, past and present, can shape those of their animal hosts via both shared gene products and HGT. Better understanding of these kinds of host–symbiont crosstalk will also facilitate the proper orchestration of these symbiotic relationships in culture.

Horizontal gene transfer could be another way for symbionts to significantly affect host morphology, physiology, or even behavior, in manner akin to the 'extended phenotype' proposed by Dawkins (1982) to describe the effects of parasites on their animal hosts. The aim of this perspective is to provide a framework for investigating the role of HGT of protein-coding genes in the evolution and ecology of marine sessile invertebrates. Many marine invertebrates transmit their symbionts via the egg to the early embryo (Douglas, 2010; Schmitt et al., 2011), which could increase the potential for HGT into germ cells that are yet to be differentiated. This potential is increased even further in animals that present no germline barrier to HGT because they do not establish a dedicated germline early in development (Zhaxybayeva and Doolittle, 2011); this applies especially to sponges (Phylum Porifera), whose cells remain pluripotent throughout adult life (Ereskovsky, 2010). In addition, the life cycle of many marine invertebrates includes budding or excision of pluripotent somatic cells, which provides another opportunity for genetic exchange and subsequent vertical transmission of acquired sequences (Boto, 2014).

In recent years, the number of confirmed cases of HGT into animal genomes has been steadily growing as interest is rekindled and growing databases of genomic resources make evidence more robust (reviewed by Hotopp, 2011; Schönknecht et al., 2014). In a recent review on the subject (Boto, 2014), more than half of the reported cases involved marine sessile invertebrates, albeit still in small numbers from just three phyla (Porifera, Cnidaria, and Chordata).

HOW CAN HGT EVENTS BE IDENTIFIED WITH CONFIDENCE?

The difficulty of unambiguously identifying HGT events is well-known. Early reports of acquisition of bacterial genes by the human genome, for example, were received with excitement but later shown to be erroneous (Salzberg et al., 2001). Importantly, these early falters served to demonstrate that "surrogate" methods to detect HGT (Ragan et al., 2006), based primarily on sequence similarity, are hopelessly insufficient unless followed up at least by phylogenetic analysis. Phylogenetic incongruence has sometimes been accepted as a gold standard for demonstrating HGT, but has two major limitations. First, deep ancestry can mask evidence of a bacterial heritage if genes continue to evolve in their new genomic context, meaning that only recent HGT events likely retain sufficient phylogenetic signal to be detected (Keeling and Palmer, 2008). Second, and even more critical in this age of genomics, phylogenetics alone cannot discriminate between foreign genes present in an animal genome due to contamination (from symbionts or other sources) and those present due to HGT. Both scenarios result in a phylogenetic distribution of the gene of interest that does not reflect the known evolutionary relationships of the species in which it is found.

Compelling identification of an HGT-acquired gene thus requires at a minimum: (i) phylogenetic evidence that the candidate gene is more closely related to foreign than to animal genes; (ii) genome data showing the candidate gene assembles into a contiguous stretch of DNA with neighboring genes unambiguously of animal origin; and (iii) gene sequence revealing metazoan-like compositional traits, including presence of introns, GC content and codon usage. The second of these requires, of course, the availability of a sequenced and assembled animal genome; the more complete the assembly, the more confident the HGT identification. Where possible, gene expression data showing active transcription of candidate genes in animal cell nuclei can enormously strengthen a case, and also addresses the issue of whether or not the HGT-acquired gene is active in its new genomic context, as discussed below.

WHAT ARE THE IMPORTANT QUESTIONS ONCE HGT-ACQUIRED GENES HAVE BEEN IDENTIFIED?

Only HGT-acquired genes that are functionally integrated into the animal host genome have the potential to affect host phenotype. Functional integration does not always happen, and should not be assumed based solely on presence of the non-animal gene in the animal genome. Some of the best-studied animal germline symbionts – the *Wolbachia* parasites of arthropods and nematodes – have produced several cases of strong evidence for HGT, but interestingly only some of which have resulted in functional genes in the recipient animal genome (Blaxter, 2007; Ioannidis et al., 2013). Evolutionary theory predicts that successful integration of a transferred gene requires both that it is active in the recipient genome and that it is maintained in that genome over evolutionary time (Blaxter, 2007). To fully explore HGT thus requires answering multiple questions.

WHAT EVIDENCE EXISTS THE HGT-ACQUIRED GENE IS FUNCTIONAL?

Interplay between genome-encoded metabolic pathways of hosts and symbionts is likely to reflect contemporary ecological demands and must necessarily involve only functional gene products (e.g., Dubilier et al., 2008; Wilson et al., 2014). The same is not true for HGTs, which may reflect deep evolutionary consequences of past ecologies and thus may not presently be functional, even if they have been at some time in the past (Hotopp, 2011). Alternatively, HGT-acquired material that is currently non-functional could potentially be co-opted into novel functions in the future. Further complicating this issue, a transferred sequence may only be partial, or may start accruing mutations immediately upon incorporation into a host genome, rendering it non-functional or tending towards pseudogenization from the start.

Demonstrating active transcription of an HGT-acquired gene, using transcriptomics or gene-specific reverse transcriptase-PCR, both now quite routine methodologies, can provide the first line of evidence relatively simply and cheaply. Transcriptomic

approaches in particular are accessible even in species lacking extensive genomic resources (Hofmann and Place, 2007). Evidence that a gene is transcribed and available as an mRNA for translation also demonstrates that it has the necessary *cis*-regulatory module/s to be integrated into an existing animal gene regulatory network. Perhaps less routine, but ultimately more compelling, is the demonstration a gene product beyond an mRNA, which is best achieved by proteomics approaches (Hartmann et al., 2014). Substantial evidence can be drawn bioinformatically by showing that the coding sequence contains all elements necessary for a functional protein (e.g., Gladyshev et al., 2008). Careful examination of coding sequence should also reveal evidence of pseudogenization, which is a demonstrated fate of some HGT-acquired genes (e.g., Kondrashov et al., 2006; Gladyshev et al., 2008; Campbell et al., 2012; Strese et al., 2014). Where possible, spatial expression data of gene transcripts or gene products can determine in which tissues or cells an HGT-acquired gene is active, which can go part way toward predicting possible function of that gene.

One caveat of transcriptomic and proteomic approaches is that mRNA or protein detected as present could feasibly be derived from symbiotic or contaminating bacteria that were present in the original biological material. Specific methodologies that target only polyadenylated transcripts largely protect against the detection of bacterial-derived mRNAs in transcriptomic approaches, but proteomics approaches require a clear isolation of animal from bacterial cells. Where there is doubt about the presence of confounding bacterial cells, spatial expression data showing transcripts confined to animal cells provides strong evidence that the transcript originates from the animal, rather than the bacterial, genome (e.g., Gladyshev et al., 2008). A second caveat is a gene may not be active at all stages of an animal life cycle, or in all tissues, so that biological sampling should encompass multiple life history stages and multiple tissue types to optimize one's chances of detecting active transcription or translation.

DO HGT-ACQUIRED GENES TEND TO HAVE COMMON FUNCTIONS?

Might there be particular characteristics of genes that make them more likely to be integrated into the recipient genome post-transfer? Here I exclude consideration of transposable elements, which are often horizontally transferred (see Chapman et al., 2010 for a marine invertebrate example) but which are outside of the scope of this perspective. Genes that encode for a function that is of benefit to the host may be subject to positive selection, thus increasing their chance of stable integration. In particular, interactions between intracellular bacterial symbionts and their animal hosts include sharing of metabolic capabilities (Dubilier et al., 2008; Venn et al., 2008; Rumpho et al., 2011), so we might reasonably ask whether HGT-acquired genes might also tend to be of a metabolic nature. It is unclear whether gene products derived from extracellular symbionts as metabolic add-ins (and used by the animal for defense, for example) can actually be incorporated into animal cells, or even whether this is required for genuine metabolic interplay. HGT, on the other hand, provide a means for gene products to be generated directly inside the animal cells, and thus always accessible to those cells. An interesting extension of this consideration is to ask whether genes involved in host–symbiont metabolic interplay in ecological time might be predisposed to acquisition via HGT through evolutionary time.

In bacterial–bacterial systems, HGTs that functionally integrate into the recipient genome do indeed very often involve genes encoding metabolic enzymes (Ochman et al., 2000). However, the more critical factor appears to be that transferred genes are more likely to be integrated if they encode proteins with low network connectivity, regardless of their function (Jain et al., 1999; Cohen et al., 2011). The small number of currently reported cases of HGT between bacteria and aquatic sessile invertebrates do not yet strongly support one scenario over the other, but the fact that operational genes such as metabolic genes tend to have less connectivity than informational genes (Jain et al., 1999; Cohen et al., 2011) points to their increased likelihood of horizontal transfer.

Instances of metabolic gene transfers include bacterial genes acquired by the genome of (i) the sea anemone *Nematostella vectensis*, specifically genes involved in glyoxylate (Kondrashov et al., 2006) and shikimic (Starcevic et al., 2008) pathways; (ii) the ascidian *Ciona intestinalis*, specifically a cellulose synthase gene (Nakashima et al., 2004); and (iii) the freshwater *Hydra magnipapillata*, specifically genes encoding sugar-modifying enzymes (Chapman et al., 2010). A similar number of cases exist, however, of transfers involving non-metabolic genes. These include transfer into (i) the octocoral mitochondrial genome of a gene encoding a MutS protein with putative mismatch repair activity (Bilewitch and Degnan, 2011); (ii) the sponge genome of a gene encoding a biomineralization-related protein apparently involved in biocalcification (Jackson et al., 2011); and (iii) the genome of cnidarians *Nematostella* and *Hydra* of bacterial pore-forming toxin genes of the aerolysin family (Moran et al., 2012).

These latter two cases are of particular interest in the context of commonality of function of HGTs, because both cases reflect similar events in multiple eukaryote taxa. With respect to the sponge biomineralization-related gene, Ettensohn (2014) has recently proposed multiple, independent HGT origins for Msp130 proteins, also implicated in biomineralization, into the genomes of several invertebrate deuterostome and one protostome (molluscs) clade. In each recipient animal lineage, the transferred msp130 genes appear to have undergone independent, parallel duplications (Ettensohn, 2014), suggestive of positive selection. Similarly, aerolysin toxin genes appear to have been independently acquired via HGT not only by cnidarians, but also by other animals such as insects, and even other eukaryotes such as plants and fungi, where they likely function in predation and defense (Moran et al., 2012). Whether these multiple, independent acquisitions reflect strong positive selection on the acquired gene, possibly coupled with their occurrence as genes with low network connectivity, remains to be determined.

A very different, and especially fascinating, instance of non-metabolic HGTs involves eukaryote-like proteins (specifically ankyrin-repeat proteins) putatively transferred *from* a sponge host *to* the bacterial symbionts; these appear to facilitate manipulation by the symbiont of host cell behavior to the benefit of the bacterial symbiont (Nguyen et al., 2014). This, of course, is completely consistent with the role of HGT between bacterial symbionts and their hosts resulting in an extended phenotype, and future outcomes of

this research will be exciting to watch. This example also is relevant to the next question posed below.

DO HGT-ACQUIRED GENES TEND TO HAVE A TAXONOMIC ORIGIN THAT REFLECTS CONTEMPORARY SYMBIONTS?

Our ability to address this question depends upon (i) the comprehensiveness of bacterial genome databases that permit assignment of HGTs to a specific taxonomic source, and (ii) knowledge of the taxonomy of contemporary symbionts of the recipient animal host. Data in both of these areas is growing rapidly, so that any patterns that do exist should emerge within the next few years. At present, for many of the demonstrated cases of HGT described above, the taxonomic source of the transfer is currently unknown. One reason for this, as exemplified by the biomineralization gene transferred into sponges (Jackson et al., 2011), might be that the event happened so long ago that phylogenetic signal of the source has been eroded. In other cases (e.g., *Hydra*; Chapman et al., 2010), the transfers appear attributable to diverse bacterial phyla, with no enrichment for bacterial relatives of present-day symbionts. In contrast, the shikimate pathway genes identified in *Nematostella* as HGTs from bacteria can be traced phylogenetically to a *Tenacibaculum*-like (Flavobacteria) origin; *Nematostella* appears to host a bacterial symbiont of the same taxonomy, which is present even in early life history stages (Starcevic et al., 2008).

This question is important for two reasons. First, it speaks to the temporal and spatial stability of host–symbiont relationships, in that HGT events reflecting current relationships suggest those particular taxonomic associations have persisted over evolutionary time. Where possible, adding a temporal component to the evolutionary source of the transfer can indicate how long ago the gene may have been transferred (what last common ancestor) and thus how long it has persisted. This returns to the question of longevity as demonstration of genuine integration into the recipient genome, and is best served by HGTs for which evidence in multiple animal lineages permits tracing back to a last common ancestor. Second, it addresses whether animals may be choosy in their source of HGT, even if only as a consequence of accessibility to bacterial DNA. If the phylogenetic source of HGT-acquired genes reflects taxonomy of contemporary symbionts, might this suggest constraint on associations? Could HGT events resulting from symbioses deep in the evolutionary past constrain the taxonomic associations possible in the ecological present? Symbioses between arthropod and nematode hosts and their alpha-proteobacterial *Wolbachia*, which have resulted in multiple HGT events (Blaxter, 2007; Le et al., 2014), will no doubt lead the way in how to investigate these interesting considerations.

CONCLUDING REMARKS

Given the temporally very long, and spatially very close, associations between marine invertebrate animals and bacteria, it is entirely feasible that HGT from bacterial to marine animal genomes is much more prevalent than currently appreciated. That marine animal–bacterial symbioses are both ubiquitous and simultaneously exclusive strongly suggests that host and symbionts carefully choose their partners, rather than relying on random associations (Douglas, 2010; McFall-Ngai et al., 2013). Might both of the same things be true for HGT? The exact nature of the sophisticated conversation that must be required for animal and bacterial partners to assess, respond to and regulate each other currently is unknown, but a number or players are emerging. Among these are innate immune gene pathways (e.g., Franzenburg et al., 2012; Degnan, 2014), eukaryote-like domains (e.g., Nguyen et al., 2014), quorum-sensing (e.g., Zan et al., 2012), and nitric oxide (e.g., Perez and Weis, 2006). Could HGT be another possibility? Could HGT provide a means for bacteria to gain some control over animal function in a manner that ultimately serves both the symbionts and thus also the host? Might HGT in this context be a means for constraining future symbionts to bacteria of particular taxa?

In insects and vertebrates in particular, evidence is mounting for anatomical, cellular, and molecular determinants that act during early development to prepare progeny for their lifetime of interactions with microbes (reviewed in Pradeu, 2011; Ezenwa et al., 2012). Simultaneously, there is a growing understanding of the diverse mechanisms by which microbes act directly as agents of post-embryonic development (reviewed in McFall-Ngai et al., 2013). The integration of genes horizontally transferred from bacterial symbionts into animal host genomes is a potentially potent mechanism by which bacteria can further influence their host environment to ensure their own persistence through evolutionary time. Increasing availability of genomes for marine invertebrate hosts and their bacterial symbionts now allows us to comprehensively address this question for the first time.

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