Population structure and microbial community diversity of two common tetillid sponges in a tropical reef lagoon

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Sponges are predicted to dominate future reef ecosystems influenced by anthropogenic stressors and global climate change. The ecological success of sponges is attributed to their complex physiology, which is in part due to the diversity of their associated microbiome. However, the lack of information on the microbial community of many sponge species makes it difficult to gauge their interactions and functional contributions to the ecosystem. Here, we investigated the population dynamics and microbial community composition of two tetillid sponges identified as *Cinachyrella* sp. and *Paratetilla* sp., which are common on coral bommies in a reef lagoon in Bolinao, northwestern Philippines. The sponges ranged in size from 2.75 ± 2.11 to 6.33 ± 3.98 cm and were found at an average density of 1.57 ± 0.79 to 4.46 ± 3.60 individuals per sq. m. on the bommies. The moon sponge population structure remained stable over the course of four years of monitoring. Microbial communities associated with the sponges were distinct but had overlapping predicted functions. This convergence of functions may reflect enrichment of metabolic processes that are crucial for the survival of the tetillid sponges under prevailing conditions in the reef lagoon. Some differentially enriched functions related to carbon, sulfur, fatty acid, and amino acid metabolism, cellular defense, and stress response, may influence the interactions of moon sponges with other biota on the bommies.

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Abstract

Sponges are predicted to dominate future reef ecosystems influenced by anthropogenic stressors and global climate change. The ecological success of sponges is attributed to their complex physiology, which is in part due to the diversity of their associated microbiome. However, the lack of information on the microbial community of many sponge species makes it difficult to gauge their interactions and functional contributions to the ecosystem. Here, we investigated the population dynamics and microbial community composition of two tetillid sponges identified as *Cinachyrella* sp. and *Paratetilla* sp., which are common on coral bommies in a reef lagoon in Bolinao, northwestern Philippines. The sponges ranged in size from 2.75 ± 2.11 to 6.33 ± 3.98 cm and were found at an average density of 1.57 ± 0.79 to 4.46 ± 3.60 individuals per sq. m. on the bommies. The moon sponge population structure remained stable over the course of four years of monitoring. Microbial communities associated with the sponges were distinct but had overlapping predicted functions. This convergence of functions may reflect enrichment of metabolic processes that are crucial for the survival of the tetillid sponges under prevailing conditions in the reef lagoon. Some differentially enriched functions related to carbon, sulfur, fatty acid, and amino acid metabolism, cellular defense, and stress response, may influence the interactions of moon sponges with other biota on the bommies.

Keywords: 16S rRNA, sponge-associated microbes, moon sponge, coral bommies, *Cinachyrella*, *Paratetilla*

Introduction

Sponges (phylum Porifera) are a diverse group of sessile, filter-feeding invertebrate animals. They are a major component of the benthic ecosystem and are responsible for many ecological processes, such as ecosystem structuring via reef consolidation and bio-erosion (Bell, 2008). Sponges link the whole reef system through the 'sponge loop' whereby dissolved organic matter released by benthic primary producers is made available to higher trophic levels in the form of particulate detritus (de Goeij et al., 2013). Sponges are also consumed as food by some spongivores (e.g. parrotfishes, angelfishes) (Wooster, Marty & Pawlik, 2016) and they offer refuge for juvenile commensal invertebrates (Ribeiro, Omena & Muricy, 2003), reef fish recruits (Cabaitan, Gomez & Yap, 2016), and other macroflora (Carballo et al., 2005; Di Camillo et al., 2017).

Sponges are holobionts that are associated with a diverse array of microorganisms (Pita et al., 2018). These symbionts are essential for nutrition, immunity, defense, and reproduction of the sponge host (Hurst and Werren, 2001; Reiswig, 1975; Scarborough, Ferrari & Godfray, 2005). Sponge-associated microbes are predicted to have the capability for a wide range of metabolic processes, including photosynthesis, nitrogen fixation, ammonium oxidation, sulfate reduction, and sulfur oxidation (Hoffman et al., 2005; Feng & Li, 2019; Pita et al., 2018). The diverse microbial community in sponges may contribute to the ecological adaptability and plasticity of the holobiont, allowing it to thrive even in perturbed environments (Bang et al., 2018; Erpenbeck et al., 2016). However, the stability of sponge microbial communities can vary among host species and under different environmental conditions (Cleary et al., 2013; Morrow, Fiore & Lesser, 2016).

While next-generation sequencing approaches have begun to uncover the diversity of sponge associated microorganisms, the lack of baseline data on the microbial community composition of most sponge species makes it difficult to assess the interactions between microbes and their hosts, as well as the functional contributions of marine sponges at larger ecological scales. This emphasizes the need to better understand the diversity of sponges and sponge-associated microbes and to identify microbially-driven functions in sponges to gain a more comprehensive understanding of the processes within the sponge holobiont that bear implications on ecosystem functions and biogeochemical cycles.

Moon sponges are classified under the family Tetillidae of class Demospongiae and generally possess globular-shaped morphology with crater-like depressions (Rützler and Smith, 1992). The pronounced circular configuration of their megascleres, minimal basal attachment, and almost solid spicule core allow tetillid sponges to inhabit environments that are influenced by frequent disturbances (Byrne, 1987). Tetillids serve as important structural constituents of reef systems where they provide habitats and other functions for many organisms (McDonald, Hooper & McGuinness, 2002; Van Soest and Rutzler, 2002). Moon sponges are a challenge to identify visually in the field, particularly for individuals from closely related taxa or from cryptic sympatric populations (Szitenberg et al., 2013). However, studies have shown that moon sponge species may be differentiated based on their distinct microbial community compositions (Cuvelier et al., 2014; Chambers et al., 2013).

This present work aims to elucidate the distribution and microbial community composition of common moon sponges on coral bommies in a tropical reef lagoon in Bolinao, northwestern

Philippines. This site is influenced by multiple stressors, including rising sea surface temperatures (Dado and Takahashi, 2017; Fang et al., 2006, Peñaflor et al., 2009), increased precipitation and frequent typhoons (Dado and Takahashi, 2017; Fang et al., 2006, Peñaflor et al., 2009). Nutrient loading due to submarine groundwater discharge and nutrient plumes extending from a nearby mariculture zone (Moncada et al., 2019; San Diego-McGlone et al., 2008; Senal et al., 2011; Udarbe-Walker&Magdaong, 2003) is also a persistent condition. The combined effect of these stressors has resulted in several bleaching events that has led to reduced live coral cover (Cabaitan, Gomez & Yap, 2016; Dela Cruz & Harrison, 2017; Gurney et al., 2013), yet sponges like the moon sponge are prevalent in the area.

Materials and methods

Study site

The study was conducted on five coral bommies (Fig. 1) within the lagoon of the Santiago reef flat in Bolinao, northwestern Philippines (B15: 16°25'50.7"N, 119°55'02.1"E; B16: 16°25'50.6"N, 119°55'07.9"E; B19: 16°25'47.8"N, 119°55'14.1"E; B21: 16°25'48.6"N, 119°55'21.0"E; B22: 16°25'50.2"N, 119°55'24.5"E). The bommies range from 20 to 60 m in diameter and are distributed across a distance of about 500 m. The bommies are located about 200 m north from a populated area on Santiago Island and about 400 m south of the unpopulated side of Silaqui Island. To the west of the bommies is the South China Sea or West Philippine Sea while to the east is the Lingayen Gulf. The bommies are 7-10 km from the mariculture zone in the Guiguiwanen channel to the south of Santiago Island. The organic matter and nutrient-enriched plume from this zone can

be driven by currents around Santiago Island towards the lagoon where the bommies are located (Ferrera et al., 2016; Udarbe-Walker&Magdaong, 2003). In addition, submarine groundwater discharge may be a significant source of nutrients in the reef flat (Senal et al., 2011).

Field surveys

A transect was laid at the base around each bommie about a meter above the sandy substrate. A 1 sq. m. quadrat was placed every 2 m along each transect. All moon sponge individuals found inside each quadrat were counted and photographed. Moon sponge morphology and size were analyzed from the images using Coral Point Count with Excel extensions or CPCe (Kohler and Gill, 2006). Field surveys were conducted in May 2016, August 2017, September 2018, and July 2019. A separate field survey was conducted in September 2019 where small tissue cores were taken from all moon sponges within each quadrat to estimate the abundance of species based on their characteristic internal tissue color.

Measurement of environmental parameters

Environmental parameters were collected at set points around the bommies during each field survey event. A multi-parameter meter (YSI Pro2030) was used to collect information on temperature, dissolved oxygen (DO), and salinity, while a pH meter (SevenGo Mettler Toledo) was used to measure pH. Total suspended solids (TSS) was determined by collecting 500 ml of seawater from the sites, which were filtered through cellulose nitrate membrane filters (0.45 μm pore size, Whatman) that were then oven dried at 70°C. The initial mass of the filter was subtracted from the mass after oven drying to obtain an estimate of the TSS among sites. Sedimentation rates were determined using sediment traps. The traps were deployed at the bommies at a depth of

around 2 m. After 24 hours, the contents of each trap were collected onto combusted, pre-weighed Whatman GF/F filters. Filters were dried at 60 °C to constant weight. Sedimentation rates were computed following the methods of English, Wilkinson & Baker (1997). To determine water turbulence, 8 pre-weighed clod cards were placed at each bommie for 24 hours and the percent difference in the dry weight of the clod cards before and after deployment was computed (Doty, 1971).

Moon sponge characterization

The sponges were characterized in terms of external morphology. Spicule types were determined by bleach digestion followed by microscopic examination (Hooper, 2003). Tissue sections were prepared to examine the sponge skeleton structure. Diagnostic characters were matched to descriptions in the Thesaurus of Sponge Morphology (Boury-Esnault & Ruetzler, 1997), Systema Porifera (Hooper & Van Soest, 2002) and the work of Santodomingo & Becking (2018) to verify sponge identities.

Mitochondrial cytochrome oxidase 1 (CO1) and 28S rRNA gene sequencing was conducted to complement traditional morphological characters and to facilitate species identification. Genomic DNA was extracted using the Hotshot protocol of Montero-Pau, Gómez & Muñoz (2008) followed by PCR amplification of the CO1 and 28S rRNA genes using the degenerate primer pairs LCO1490/HCO2198 (Folmer et al., 2005) and 28S-C2 forward/28S-D2 reverse (Chombard, 1998). The 25 µl PCR mix consisted of 1x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM each of forward and reverse primers, 1 unit Taq DNA polymerase (Invitrogen), and 1 µl of crude DNA extract. PCR amplification was conducted on a

T100 Thermal Cycler (Bio-Rad, Munich, Germany) with an initial denaturation phase of 3 min at 94°C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 20 sec at 45°C, elongation for 60 sec at 72°C, and a final elongation for 5 min at 72°C (Erpenbeck et al., 2016). Purified PCR products were sent to First Base Laboratories (Malaysia) for direct sequencing. Alignment and trimming of sequences and phylogenetic tree rendering using Bayesian inference were done using Phylogeny.fr (Dereeper et al., 2008). Other CO1 and 28S rRNA sequences were downloaded from NCBI GenBank.

Tissue sampling and DNA extraction

Six individuals each of *Cinachyrella* sp. and *Paratetilla* sp. were collected from the easternmost (bommie 22) and westernmost (bommie 15) bommies in December 2016 and April 2017. Sponge sampling was conducted with permission from the Philippines Department of Agriculture Bureau of Fisheries and Aquatic Resources under Gratuitous Permit No. 0125-17 and 0169-19. Sponges were sliced and fragments were washed with sterile seawater to remove any foreign macroscopic debris. To eliminate planktonic or loosely attached microbes and detritus, the cleaned fragments were rinsed with sterile calcium magnesium-free seawater (CMFSW) on a platform shaker at maximum speed for 10 minutes. After washing, fragments were further cut into ~ 0.5 g pieces and total DNA was extracted using PowerSoil DNA Extraction kit (MOBIO) following the manufacturer's protocol. Quality of extracted DNA was checked by agarose gel electrophoresis and concentration was determined using a spectrophotometer (Shimadzu Biospec-Nano) prior to 16S rRNA gene sequencing.

Sequencing and microbial community analysis

Total genomic DNA extracted from 12 tetillid sponge samples (3 biological replicates per species per timepoint) were sent to Macrogen Inc., South Korea, for sequencing on the Illumina MiSeq platform. The V3-V4 region of microbial 16S rRNA gene was amplified using the forward primer Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') and reverse primer Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') (Herlemann et al., 2011). Demultiplexed paired end reads were analyzed using QIIME2 version 2018.11 (https://docs. qiime2.org). Raw data were imported and were renamed according to QIIME2 sample data format Casava 1.8 paired-end demultiplexed fastq. Sequences were denoised by removing chimeric sequences and correcting amplicon errors using the DADA2 package (Callahan et al., 2016). Based on quality plots, reads were trimmed using the following parameters: -p-trim-left-f = 17; -p-trim-left-r = 21; -p-trunc-len-f = 290; and -p-trunc-len-r = 250. For taxonomic assignment, a naïve Bayes classifier was trained on SILVA version 132 (https://arb-silva.de; Quast et al., 2012) with reference sequences trimmed to the V3-V4 region. The trained classifier was applied to the representative sequences to assign taxonomy at 97% sequence identity. Sequence reads classified as chloroplast and mitochondria, as well as singletons, were removed using the commands "qiime taxa filter-table" and "qiime taxa filter-seqs." OTU counts were rarefied to the smallest sample size (20,818 sequences) prior to computation of alpha diversity metrics, such as Observed OTUs, Shannon, and Inverse Simpson. Alpha diversity metrics were computed using phyloseq (McMurdie & Holmes, 2013), Companion to Applied Regression (car) (Fox & Weisberg, 2019) and Ryan miscellaneous (Rmisc) (Hope, 2013) packages. Non-metric multidimensional scaling (NMDS) was used to visualize community distance matrix based on Bray-Curtis using the vegan package (Oksanen et al., 2017). Unrarefied OTU counts were used to calculate nonparametric Permutational Multivariate Analysis of

Variance (PERMANOVA) using the Adonis method and Analysis of Similarity (ANOSIM) using 999 permutations for the comparison of communities. Differentially abundant OTUs were identified using ANOVA-like differential expression (ALDEx2) analysis (Fernandes et al., 2013) with Welch's t test. All packages were implemented in RStudio version 1.2.1335 (RStudio Team, 2018).

Prediction of functional genes

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States or PICRUSt2 (Langille et al., 2013) was used to predict functional gene abundance based on OTU taxon affiliations. The software was installed as QIIME2 plugin. The commands "qiime fragment-insertion sepp" and "qiime picrust2 custom-tree-pipeline" setting the --p-max-nsti to 2 were used to generate functional prediction. The relative abundance profiles of predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog (KO) genes were visualized using metaMDS. Linear discriminant analysis (LDA) of effect size or LEfSe was used to identify KOs that distinguish between the two species (Segata et al., 2011). KO terms with an absolute LDA > 2.0 and alpha < 0.05 were considered discriminative features.

Statistics

All data were tested for normality using Shapiro-Wilk test and homogeneity of variances using Levene's test. General Linear Models (GLM) implemented in Statistica v7 were used to examine the differences in mean density of moon sponge among bommies and across sampling periods, and differences in mean density of the two species of moon sponge among bommies, and differences in environmental conditions among bommies. Results from GLM were further tested with Tukey's

HSD post hoc test to see which bommies and sampling periods had significant differences. Kolmogorov-Smirnov tests were conducted to examine the differences in size frequency distributions of moon sponges across sampling periods per bommie. Statistical difference in alpha diversity between the microbial community of the two sponge groups was calculated using Welch's t test. A p-value < 0.05 was considered significant. All the data visualizations were produced using ggplot2 (Wickham, 2016), pheatmap, and Rcolorbrewer in RStudio version 1.2.1335 (RStudio Team, 2018).

Results

Distribution and size frequency of moon sponges on the reef bommies

Moon sponges were observed on all the bommies. The sponges were typically found covered in sediments and overgrown by turf algae, or in close interaction with other types of macroalgae, sponges, and corals (Fig. 2). The average density recorded over four years of monitoring ranged from 1.57 ± 0.79 to 4.46 ± 3.60 individuals per sq. m. per bommie. Sponge density was significantly greater on bommies 21 and 22 than on bommies 15, 16, and 19 (Fig. 3A; Tukey's HSD post hoc tests: $p < 0.05$). There was no change in sponge density over time (GLM: F=2.38, p=0.09) (Fig. 3A). The average size of the moon sponges ranged from 2.75 ± 2.11 to 6.33 ± 3.98 cm, with very few sponges growing larger than 10 cm (Fig. 3B). A significant increase in moon sponge size frequency distribution was noticeable in September 2018 on all the bommies, except for bommie 15 (Fig. 3B; Supplementary Table 6). Environmental parameters measured across the four bommies remained similar over the monitoring period (Supplementary Table 1).

253 **Moon sponge morphology and sequencing**

254 The moon sponges on the reef bommies were identified as *Cinachyrella* sp. and *Paratetilla* sp..
255 *Cinachyrella* sp. exhibited deeper hemi-spherical depressions called porocalices alternatively
256 perforated by a number of small pores, or some oscular tubes, and had yellowish inner tissues
257 (Supplementary Fig. 1; Supplementary Table 2). This sponge possessed spicules characterized as
258 oxea, anatriaene, protriaene, sigmaspires and microoxea (Supplementary Fig. 1; Supplementary
259 Table 2). Sequences of 28S rRNA from these samples grouped with sequences from *C.*
260 *australiensis* and *C. schulzei* (Supplementary Fig. 2).
261
262 *Paratetilla* sp. individuals also had narrow hemi-spherical porocalices that were sometimes closed,
263 and had brown internal tissues (Supplementary Fig. 1; Supplementary Table 2). This sponge
264 possessed oxea, anatriaene, protriaene, sigmaspires, and microoxea spicules, as well as triradiate
265 symmetrical rays (Supplementary Fig. 1; Supplementary Table 2). CO1 sequences from
266 *Paratetilla* sp. samples clustered closely with sequences from *Paratetilla bacca* (Supplementary
267 Fig. 2).
268
269 Although the two species are difficult to distinguish based on their external morphology, a survey
270 that examined internal tissue color of the moon sponges revealed that *Cinachyrella* sp. was
271 distributed on all bommies at almost similar densities and was generally more abundant than
272 *Paratetilla* sp., which was found at greater density only on the easternmost bommie (Fig. 3C).
273
274 **Diversity of moon sponge microbiomes**
275 Sequencing of the 16S rRNA gene V3-V4 region on the Illumina Miseq platform returned a total
276 of 2,068,178 reads. After sequence filtering, a total of 587,405 reads with an average of 48,950 ±

14,495 (mean ± standard deviation) reads per library were obtained from 12 libraries (6 *Cinachyrella* sp. and 6 *Paratetilla* sp. samples). 1,459 operational taxonomic units (OTUs) were identified at 97% sequence similarity and classified into 35 microbial phyla, 78 microbial classes and 176 microbial orders. Rarefaction curves reached a plateau at 20,818 sequences, indicating that the sequencing effort was sufficient to cover most OTUs in each sample (Supplementary Fig. 3). No significant difference was observed in terms of the number of observed OTUs per species (Fig. 4A). However, *Paratetilla* sp. showed greater microbial species richness and diversity compared to *Cinachyrella* sp., as indicated by significantly greater alpha diversity values based on the Shannon and Inverse Simpson indices (Fig. 4A).

The two sponge species possessed distinct microbial communities with only 11% (160 OTUs) of OTUs shared by both tetillids (Supplementary Fig. 4). The difference in microbial community composition between the two species is apparent in the NMDS plot (Fig. 4B) and is statistically supported (PERMANOVA: $R^2 = 0.84118$, $p = 0.001$; ANOSIM: $R = 0.874$, p-value = 0.001) (Supplementary Table 3). In contrast, no statistical difference was observed in the microbial communities of sponge individuals of the same species collected at different times (*Cinachyrella* sp., December16: April 2017 : PERMANOVA: $R^2 = 0.35515$, $p = 0.1$; ANOSIM: $R = 0.4815$, p-value = 0.1; *Paratetilla* sp., December16: April 2017 : PERMANOVA: $R^2 = 0.51471$, $p = 0.1$; ANOSIM: $R = 0.814$, p-value = 0.1) or from different bommies (*Cinachyrella* sp., Bommie 15: Bommie 22 : PERMANOVA: $R^2 = 0.28559$, $p = 0.3$; ANOSIM: $R = 0.2593$, p-value = 0.3; *Paratetilla* sp., Bommie 15: Bommie 22: PERMANOVA: $R^2 = 0.287$, $p = 0.4$; ANOSIM: $R = 0.1852$, p-value = 0.4) (Supplementary Table 3). This suggests that the microbiome associated with each sponge is species-specific.

Differentially abundant microbes in *Cinachyrella* sp. and *Paratetilla* sp.

The *Cinachyrella* sp. microbiome was dominated by members of phylum Proteobacteria (90.28%), followed by Bacteroidetes (2.30%) and Nitrospirae (1.86%) (Supplementary Fig. 5A). These included members of class Gammaproteobacteria (52.49%), Alphaproteobacteria (37.02%), Bacteroidia (2.29%) and Nitrospira (1.86%) (Supplementary Fig. 5B). Amongst OTUs classifiable to the order level, the greatest proportion were affiliated with Rhodobacterales (18.68%), Nitrosoccocales (16.09%) Betaproteobacteriales (12.19%), Parvibaculales (10.54%), KI89A clade (3.67%) and Nitrospirales (1.86%) (Fig. 4C).

On the other hand, the *Paratetilla* sp. microbiome was dominated by phylum Proteobacteria (59.52%), followed by Chloroflexi (17.95%), Dadabacteria (6.68%), Verrucomicrobia (5.32%), Actinobacteria (3.73%), Nitrospirae (2.20%), Patescibacteria (1.69%) and Bacteroidetes (1.39%) (Supplementary Fig. 5A). This included members of class Gammaproteobacteria (29.53%), Alphaproteobacteria (18.26%), Dehalococcoidia (17.84%), Deltaproteobcateria (10.97%), Dadabacteriia (6.68%), Verrucomicrobiae (5.32%), Acidimicrobiia (3.68%), Nitrospira (2.20%), Parcubacteria (1.64%) and Bacteroidia (1.37%) (Supplementary Fig. 5B). Amongst OTUs classifiable to the order level, the greatest proportion were affiliated with the SAR202 clade (17.84%), NB1-j (9.29%), Betaproteobacteriales (7.46%), Dadabacteriales (6.68%), JTB23 (6.11%), Pedosphareales (5.27%), Nitrosococcales (3.83%), Microtrichales (3.66%), and Nitrospirales (2.20%) (Fig. 4C).

Forty eight OTUs differed significantly in relative abundance between the two sponge species (Fig. 4D) based on ALDEx2 analysis with Welch's test (p-value < 0.05). Twenty OTUs affiliated

with class Gammaproteobacteria (10 OTUs), Alphaproteobacteria (6 OTUs), Dadabacteriia (1 OTU), Nitrospira (1 OTU), class Nitrososphaeria under Thaumarchaeota (1 OTU), and one unclassified bacterial OTU were found at relatively greater abundance in *Cinachyrella* sp.. On the other hand, 28 OTUs belonging to class Gammaproteobacteria (8 OTUs), Alphaproteobacteria (7 OTUs), Nitrospira (3 OTUs), Deltaproteobacteria (2 OTUs), Acidimicrobiia (2 OTUs), Parcubacteria (2 OTUs), Dehalococcoidia (1 OTU), Dadabacteriia (1 OTU), one unclassified Proteobacteria OTU, and one unclassified bacterial OTU were found at higher relative abundance in *Paratetilla* sp.. Different OTUs of Nitrospiraceae, Betaproteobacterales EC94, Dadabacteriales, and Gammaproteobacteria KI89A clade were enriched in each sponge species.

Predicted functional genes in tetillid-associated microbes

Functional prediction was conducted using PICRUSt2, a software tool that predicts the functional profile of a microbial community based on 16S rRNA sequences (Langille et al., 2013). To improve accuracy of metagenome prediction, the weighted Nearest Sequenced Taxon Index (NSTI) value for the analysis was set to < 2.0 (Langille et al., 2013). However, it is important to note that this method is predictive and does not completely substitute for whole metagenome profiling (Langille et al., 2013; Weigel and Erwin, 2017). Nevertheless, it provides a starting point for understanding functions potentially represented within a microbial community.

PICRUSt2 predicted a total of 6892 KEGG ortholog (KO) genes from the microbial communities associated with the two sponge species. Of these, 6234 KOs (90.5%) were present in both microbial communities, while 405 (5.9%) were present only in the *Cinachyrella* sp. microbiome and 253 (3.7%) were found only in the *Paratetilla* sp. microbiome. The predicted KO profiles of

346 the microbial community of each sponge could be differentiated by NMDS (Fig. 5A) and these
347 differences were statistically supported (*Cinachyrella* sp. : *Paratetilla* sp.*,* PERMANOVA: R^2 =
348 0.79417, p = 0.003; ANOSIM: R = 1, p-value = 0.002) (Supplementary Table 4). LEfSe analysis
349 revealed an enrichment of KOs associated with ABC transporters, biosynthesis of secondary
350 metabolites, fatty acid metabolism, glutathione metabolism, microbial metabolism in diverse
351 environments, quorum sensing, sulfur metabolism, and terpenoid backbone biosynthesis in
352 *Cinachyrella* sp.. KOs involved in bacterial chemotaxis, biosynthesis of amino acids and
353 antibiotics, carbon fixation, citrate cycle, galactose metabolism, glycolysis/gluconeogenesis,
354 methane metabolism, and pentose phosphate pathway were enriched in *Paratetilla* sp. (Fig. 5B).
355
356
357 **Discussion**
358
359 **Moon sponge population dynamics**
360 Moon sponges were abundant on the coral bommies and populations were stable over the course
361 of 4 years of monitoring. The easternmost bommies (bommie 21 and 22) had the highest density
362 of sponges compared to the others. These bommies face the Lingayen Gulf and are likely to be
363 less exposed to strong wave action during typhoons. Most sponges exhibited an average diameter
364 of about 3 to 6 cm, with very few growing to larger size. This further suggests that the sponges
365 may be affected by various physical disturbances, such as grazing or predation, strong wave action,
366 and sedimentation, all of which can limit growth or cause tissue loss or mortality. A change in size
367 frequency distribution was observed in 2018 on all bommies, except on bommie 15, although the
368 cause remains unknown. The dynamics of the moon sponge population on these bommies are in

369 contrast to that of *Xestospongia muta* in Florida, which showed an increase in abundance over the
370 course of 6 years due to increased recruitment owing to suitable environmental conditions
371 (McMurray, Henkel & Pawlik, 2010).
372
373 The stable population of the moon sponges suggests that individuals are long-lived and slow-
374 growing. In fact, another tetillid sponge, *C. cavernosa,* has been found to increase in mean diameter
375 by just 0.1–0.2 cm per year, with specific growth rates decreasing as sponge size increases (Singh
376 & Thakur, 2015). Similarly, growth rate of settled buds of *Tethya citrina* decreased with sponge
377 age (Cardone, Gaino & Corriero, 2010). Growth rates were affected by temperature, silicate
378 concentration, dissolved oxygen, and the presence of competitors (Singh & Thakur, 2016). On the
379 bommies, moon sponges were typically found interacting with or in close proximity to algae,
380 corals, and other sponges (Fig. 2). These organisms are known to produce allelochemicals and may
381 inhibit sponge growth, similar to the reported growth-limiting effect of zoanthids on *C. cavernosa*
382 (Singh & Thakur, 2016). Further studies to test the impact of other benthic reef organisms on the
383 growth of moon sponges remain to be conducted.
384
385 **Species specificity of moon sponge microbial communities**
386 The sponge-associated microbiota is host-specific and compositionally distinct from surrounding
387 seawater and sediments (Reveillaud et al., 2014; Thomas et al., 2016). The microbial community
388 of *Cinachyrella* sp. can be distinguished from that of *Paratetilla* sp., although these two sponges
389 co-exist in the same biogeographic location and experience similar environmental conditions. The
390 *Paratetilla* sp. microbial community composition reported here is similar to what has been
391 reported for related species in other biogeographic regions, with the dominance of Proteobacteria,

392 Chloroflexi and Actinobacterial OTUs (De Voogd et al., 2018). The *Cinachyrella* sp. microbiome
393 from this study also showed some common OTUs with other *Cinachyrella* microbiomes, which
394 are dominated by Proteobacteria, Bacteroidetes, Cyanobacteria and Actinobacteria (Cleary et al.,
395 2013; Cleary, Polonia & De Voogd, 2018). In addition, differentially abundant microbial taxa
396 found in both moon sponges have previously been reported as symbionts of other sponge species.
397 For example, Nitrospiraceae was dominant in the *Rhabdastrella globostellata* microbiome
398 (Steinert et al., 2016), Betaproteobacteria EC94 was abundant in *Callyspongia* sp. (Steinert et al.,
399 2016), and Gammaproteobacteria K189A was abundant in *Petrosia ficiformis* (Burgsdorf et al.,
400 2014).
401
402 The microbial community in each sponge species remained similar in samples taken during
403 different times and from different bommies. This mirrors findings from other studies in marine
404 sponges that suggest that microbial communities are shaped by host identity (Chambers et al.,
405 2013; Naim et al 2014; Souza et al. 2016; Steinert et al. 2016). The sponge microbiome has been
406 shown to be stable even in individuals taken from different sampling locations or depths (Pita et
407 al., 2013; Reveillaud et al 2014) and can even withstand moderate pollution stress (Baquiran &
408 Conaco, 2018; Gantt, Lopex-Legentil & Erwin, 2017).
409
410 Specificity of the sponge microbial community may be attributed to sponge host-related factors.
411 Demosponges, such as *Haliclona tubifera* and *H. amboinensis*, have been shown to possess
412 different complements of scavenger receptor cysteine-rich (SRCR) domain-containing genes
413 (Guzman & Conaco, 2016). The repertoire of SRCR-like domains is expanded in low microbial
414 abundance (LMA) sponges, like *Stylissa carteri*, but is reduced in high microbial abundance

(HMA) sponges, such as *Xestospongia testudinaria* (Ryu et al., 2016). The expression of SRCR receptors is influenced by exposure to microbe-derived molecules (Pita et al., 2013). It is hypothesized that the presence of this diverse class of receptors allows the sponge to be more selective in the types of microbes that interacts with.

Bacteria-bacteria interaction may also play a role in structuring the sponge microbial community. Some sponge-associated microbes can inhibit the growth of other members of the community through the production of various compounds and regulatory signals (Esteves, Cullen & Thomas, 2017; Fuerst, 2014; Gutierrez-Barranquero et al., 2017). For instance, *Bdellovibrio,* which is enriched in *Paratetilla* sp., is an active predator of other microorganisms and produces compounds that attack the cell walls of other bacteria (Beck et al., 2004). Other sponge-associated microorganisms are attracted to sponge host-derived compounds, indicating an active role of the microbes in initiating the species-specific partnerships (Tout et al., 2017; Lurgi et al., 2019). This is supported by the predicted abundance of genes related to bacterial chemotaxis in both moon sponge species. On the other hand, microbes with reduced genomes may exist in the community as ectosymbionts or parasites, relying on the biosynthetic capabilities of the host associated microbiome (Nelson & Stegen, 2015). An example of this is Parcubacteria, which was detected in *Paratetilla* sp..

Predicted functions of tetillid-associated microbes

Sponge-associated microbes fulfill functions that provide important benefits to the host. They can also influence ecosystem health and function through a number of roles, including primary production, nutrient cycling (sponge loop, inorganic nitrogen and phosphorus cycling), and

438 regulation of the benthic food web (chemical defense, predation and competition) (Bell, 2008;
439 Taylor et al., 2007; Thomas et al., 2010). In the present study, genes critical for metabolism,
440 defense, and stress response were predicted to be present in the microbiomes associated with
441 *Cinachyrella* sp. and *Paratetilla* sp..
442
443 Various differentially abundant OTUs in the moon sponges were affiliated with taxa known to be
444 involved in the nitrogen cycle. Nitrifying microbes, such as Nitrosopumilaceae (Feng et al., 2016;
445 Li et al., 2014), the AqS1 group of Nitrosococcaceae (Rua et al., 2015; Feng & Li, 2019), and the
446 SAR202 clade of phylum Chloroflexi (Mincer et al. 2007; Morris et al., 2004), transform ammonia
447 to nitrite. Members of *Nitrospira,* some of which are enriched in *Paratetilla* sp., may contribute to
448 the conversion of nitrite from ammonia oxidation into nitrate (Daims & Wagner, 2018; Hentschel
449 et al., 2002). Members of Proteobacteria and Bacteroidetes, which were also identified in the moon
450 sponges, may play a role in denitrification (Feng & Li, 2019). Denitrification removes excess
451 nitrate from the sponge tissues and takes place when oxygen becomes deficient (Hoffman et al.,
452 2009). This condition may occur when sediment levels in the water column are high, causing moon
453 sponges to close their oscules and cease pumping in order to prevent clogging of the aquiferous
454 system (Strehlow al., 2017). The potential co-existence of nitrification and denitrification
455 functions in the moon sponge microbiota suggests that affiliated microbes can adapt to shifts from
456 aerobic to anaerobic conditions inside the sponge (Schlappy et al., 2010). In addition, to supplying
457 the nitrogen requirements of the holobiont, nitrogen metabolism by sponge-associated microbes
458 may also benefit other biota, such as macroalgae and other organisms, in the surrounding area
459 (Davy et al., 2002).
460

461 Genes related to sulfur metabolism, including sulfur oxidation (e.g. K17218) and sulfate reduction
462 (e.g. K00381), were predicted to be present in the moon sponge microbiomes (Supplementary
463 Table 11). These two processes may be coupled, as has been demonstrated in the cold water sponge
464 *Geodia barretti* (Jensen et al., 2017). Sulfur oxidation is a potential mechanism for the removal of
465 toxic metabolic end-products, such as hydrogen sulfide, that are produced by the sponge host
466 (Jensen et al., 2017). The existence of an anoxic micro-ecosystem in the moon sponges is further
467 supported by the presence of sulfate-reducing bacteria (SRB), such as members of
468 Deltaproteobacteria and Dadabacteria (Hug et al., 2015; Wasmund, Mußmann & Loy, 2017). SRB
469 that thrive under anoxic conditions but that can also tolerate oxic conditions have previously been
470 reported in tetractinellid demosponges (Minz et al., 1999; Schumann-Kindel et al., 1997;
471 Sigalevich et al., 2000).
472
473 Genes in key biosynthetic pathways were predicted to be present in the moon sponge microbiomes.
474 ATP-binding cassette (ABC) transporters, which are essential for nutrient and metabolite
475 acquisition by the symbionts (Hentschel et al., 2012; Pita et al., 2018), as well as amino acid
476 biosynthesis-related genes, were abundant in both species. The moon sponges also harbored
477 members of Proteobacteria, Bacteroidetes and Acidobacteria that may play a role in CO_2 fixation,
478 as previously described in the deep-sea sponge *Neamphius huxleyi* (Li et al., 2014). The
479 microbiome of *Paratetilla* sp.*,* in particular, was enriched for genes in the carbon fixation pathway,
480 pentose phosphate pathway, galactose metabolism, glycolysis, and citrate cycle. Translocation of
481 fixed carbon to the sponge would provide a valuable source of alternative nutrition for the host,
482 analogous to photosynthates from autotrophic microbes (e.g. Cyanobacteria) (Kandler et al.,
483 2018).

The *Cinachyrella* sp. microbiome was enriched for fatty acid metabolism genes. This suggests that this species may have an abundance of lipids in its tissues (Luskow et al., 2019), as well as a diverse array of fatty acids (Rod'kina, 2005), which may serve as a potential energy store or as building blocks for bioactive compounds. The enrichment of the terpenoid biosynthesis pathway in the *Cinachyrella* sp. microbiome further suggests an active involvement in secondary metabolite production, as has been reported for other species of sponges (Cleary, Polonia & De Voogd, 2018; Steinert et al., 2019). Secretion of secondary metabolites, including terpenoids, may have allelopathic effects on other organisms and may contribute to the differential distribution of the two sponge species. However, a detailed assessment of the secondary metabolites produced by each moon sponge remains to be conducted.

Genes related to antibiotic production and glutathione synthesis were also predicted to be present in the *Cinachyrella* sp. and *Paratetilla* sp. microbial consortia. *Endozoicomonas*, which is abundant in *Paratetilla* sp., can produce quorum sensing metabolites and demonstrates antimicrobial properties against potentially harmful microbes (Esteves, Cullen & Thomas, 2017; Mohamed et al., 2008; Morrow et al., 2015; Rua et al., 2014). Genes related to production of the antioxidant glutathione were predicted to be enriched in *Cinachyrella* sp.. The presence of these genes may protect the symbionts from damage caused by environmental stressors (Cleary, Polonia & De Voogd, 2018). We hypothesize that the abundance of protective genes in the moon sponge-associated symbionts may be an adaptation to stressful conditions, such as high temperatures, high sedimentation rates, and eutrophic waters, that are frequently encountered in the reef lagoon.

Although the microbial composition of the two tetillids were distinct from each other, it is interesting to note that the predicted functions represented in the microbiomes were similar. These observations support the concept of functional redundancy, which states that similar functions may be present across multiple taxa through the combined effect of environmental selection and horizontal gene transfer events (Louca et al., 2018). The functional equivalence of the communities in the moon sponges indicates the presence of core functions that may be critical for the health and survival of the tetillids in the area (Fan et al., 2012), and may partly explain the stability of the sponge populations on the bommies. Differentially enriched functions, on the other hand, may indicate species-specific adaptations influenced by host metabolism or chemistry (Cleary et al., 2015; Steinert et al., 2019).

Conclusion

In this study we identified two tetillid species, *Cinachyrella* sp. and *Paratetilla* sp., that are found in abundance on coral bommies within a reef lagoon. The density and size frequency of the sponge populations remained relatively stable over the course of the monitoring period (~4 years), although *Cinachyrella* sp. was dominant on more bommies. The sponges host distinct microbial communities, supporting the idea of species-specificity of the sponge microbiome. However, predicted functions represented within the microbiota of the two species present a large overlap, indicating functional equivalence of the communities driven by prevailing environmental conditions at the site. Nevertheless, certain functions could be distinguished as differentially enriched between species, particularly pathways related to carbon, sulfur, fatty acid, and amino

529 acid metabolism, cellular defense, and stress response. These likely indicate microbiome-specific
530 adaptations to host metabolism and may influence the interactions of the sponges with other biota
531 on the bommies. Further validation of the functional profiles of the moon sponge-associated
532 microbiota using metagenome or metatranscriptome approaches are warranted in order to verify
533 the genes that are present and expressed, as well as the microbial players contributing to functions
534 of interest.
535
536
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541
542
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Figure 1

Study site.

Moon sponge surveys were conducted on five coral bommies (bommie 15, 16, 19, 21, and 22) in the Santiago reef flat in Bolinao, Pangasinan, northwestern Philippines (inset).

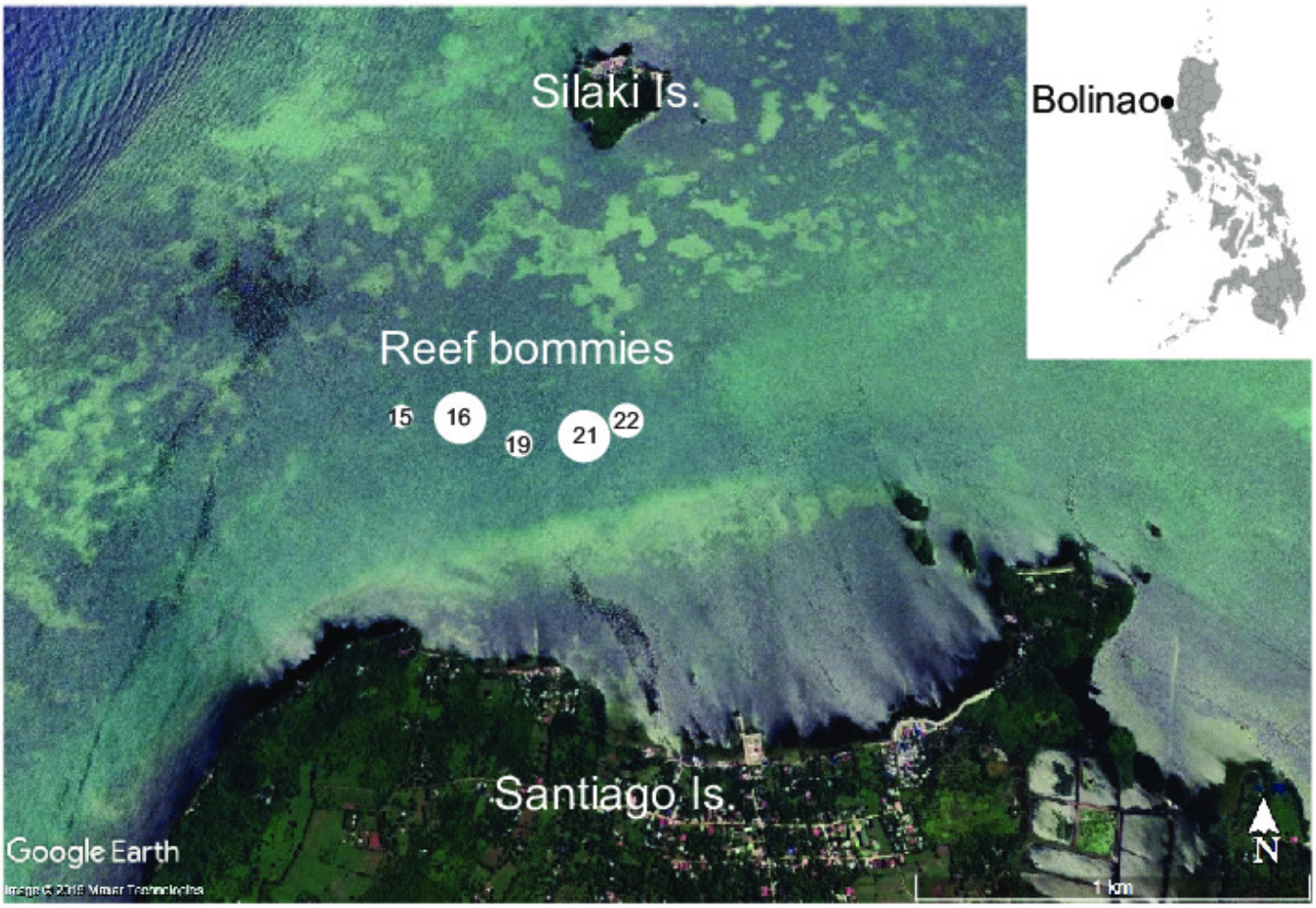

Figure 2

Moon sponges are typically covered by sediments (A) or turf algae (B), and can be found in close interaction with other types of algae (C), sponges (D-E), and corals (F).

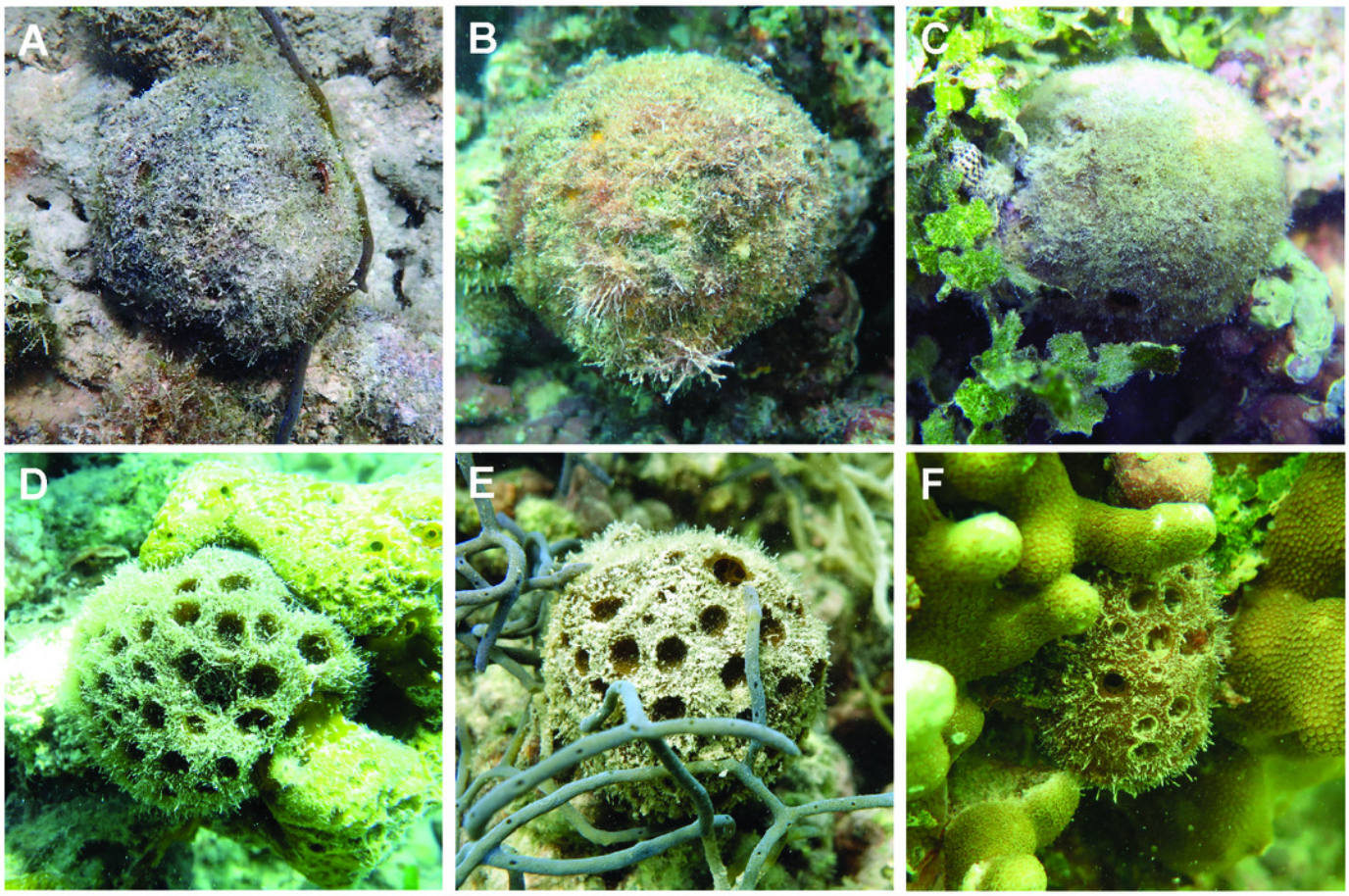

Figure 3

Sponge population dynamics.

(A) Moon sponge population density on the coral bommies from 2016 to 2019. (B) Size frequency distribution of moon sponges on the bommies. Bommie 16 was not included in the May 2016 survey. (C) Distribution of *Cinachyrella sp.* and *Paratetilla sp.* on the bommies based on a survey conducted in September 2019.

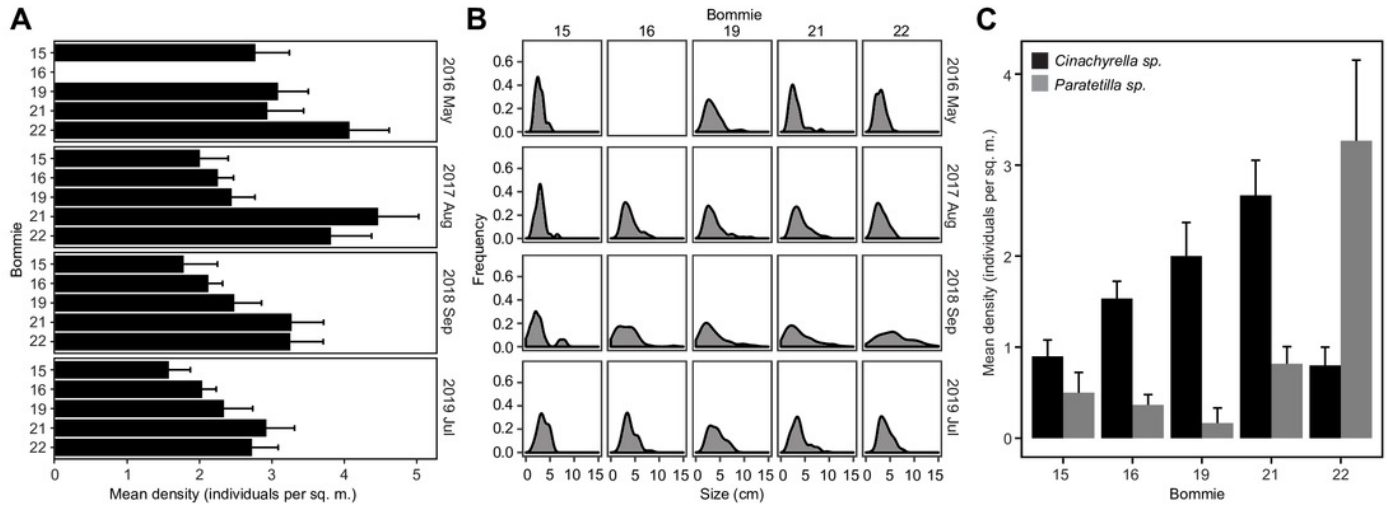

Figure 4

Microbial community characteristics of the two tetillid sponges.

(A) Comparison of alpha diversity indices between sponge microbial communities in the two moon sponge species. (B) Non-metric multidimensional scaling (NMDS) illustrating dissimilarity of microbial communities in *Cinachyrella* sp. (square) and *Paratetilla* sp. (circle) individuals collected at different times (white, Dec 2016; black, Apr 2017). (C) Taxonomic assignments at order level showing the relative abundance of the 16S rRNA gene sequences of microbes associated with the two sponge species collected at different times from different bommies. Orders representing less than 0.4% of the total community are represented as “other microbial orders.” Colored bars represent the relative abundance of microbial taxa in each replicate sample. (D) Scaled heatmap of 48 differentially abundant microbial OTUs based on ALDEx2 analysis (p-value < 0.05). Colors represent row z-scores of each microbial taxon (red, high; blue, low).

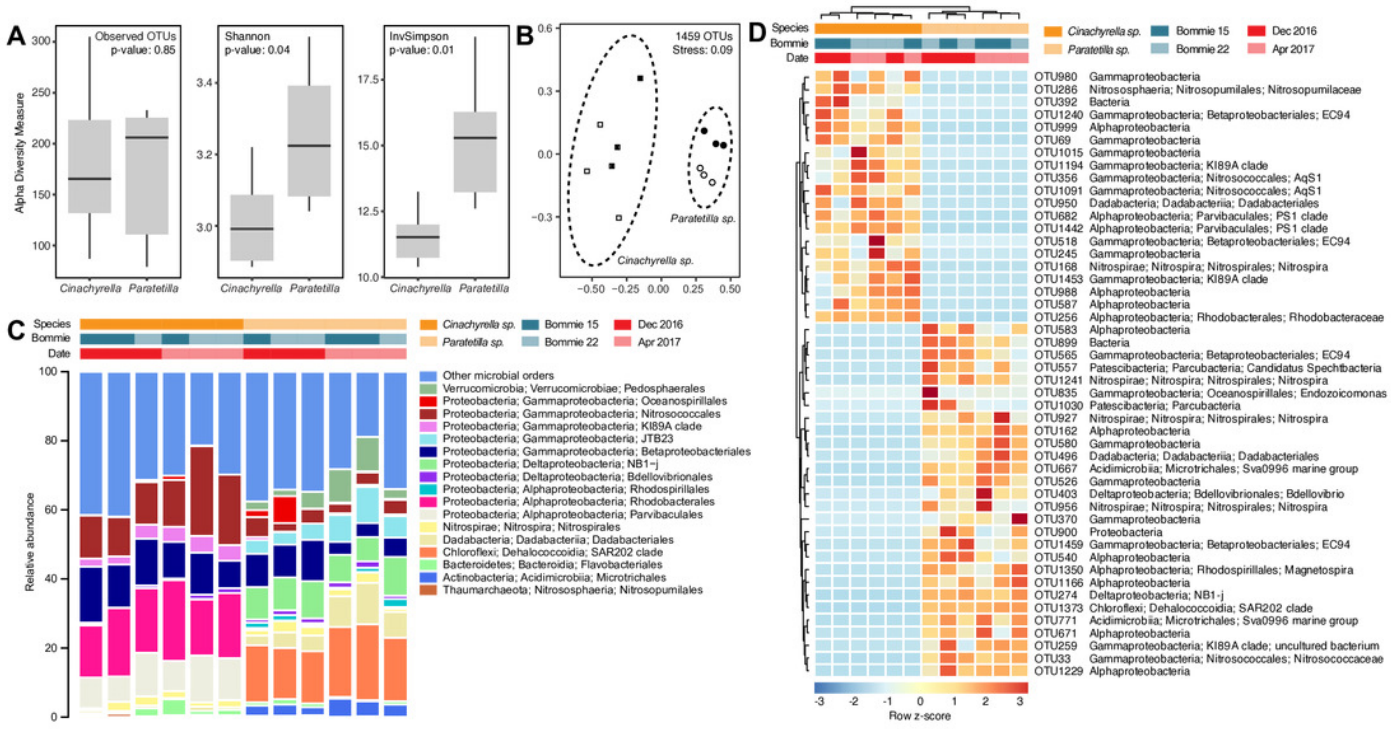

Figure 5

Functional gene predictions.

(A) Non-metric multidimensional scaling (NMDS) plot illustrating dissimilarity of the relative abundance profiles of PICRUSt2-predicted KEGG ortholog (KO) genes in the microbial communities associated with *Cinachyrella* sp. (square) and *Paratetilla* sp. (circle) individuals collected at different times (white, Dec 2016; black, Apr 2017). (B) Average sums of the relative abundance of KOs in selected pathways. Only differentially enriched KOs in either species with LEfSe LDA > 2.0 and p-value < 0.05 were included.