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Original article

Pyrosequencing reveals sponge specific bacterial communities in marine sponges of Red Sea, Saudi Arabia

Fehmida Bibi [a,d,*], Sana Akhtar Alvi [a], Abdulmohsin Al-Sofyani [b], Muhammad Imran Naseer [c,d], Muhammad Yasir [a,d], Esam Ibraheem Azhar [a,d]

[a] Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, 21589, Jeddah, Saudi Arabia
[b] Department of Marine Biology, Faculty of Marine Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
[c] Center of Excellence in Genomic Medicine Research (CEGMR), King Abdulaziz University, 21589, Jeddah, Saudi Arabia
[d] Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King AbdulAziz University, 21589, Jeddah, Saudi Arabia

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ABSTRACT

Bacterial communities of marine sponges are believed to be an important partner for host survival but remain poorly studied. Sponges show difference in richness and abundance of microbial population inhabiting them. Three marine sponges belonging to the species of *Pione vastifica*, *Siphonochalina siphonella* and *Suberea mollis* were collected from Red sea in Jeddah and were investigated using high throughput sequencing. Highly diverse communities containing 105 OTUs were identified in *S. mollis* host. Only 61 and 43 OTUs were found in *P. vastifica* and *S. siphonella* respectively. We identified 10 different bacterial phyla and 31 genera using 27,356 sequences. Most of the OTUs belong to phylum *Proteobacteria* (29%–99%) comprising of *Gammaproteobacteria*, *Alphaproteobacteria*, and *Deltaproteobacteria* where later two were only detected in HMA sponge, *S. mollis*. A number of 16S rRNA sequences (25%) were not identified to phylum level and may be novel taxa. Richness of bacterial community and Shannon, Simpson diversity revealed that sponge *S. mollis* harbors high diversity compared to other two LMA sponges. Dominance of *Proteobacteria* in sponges may indicate an ecological significance of this phylum in the Red sea sponges. These differences in bacterial composition may be due to difference in location site or host responses to environmental conditions. To the best of our knowledge, the microbial communities of these sponges have never been studied before and this is first attempt to unravel bacterial diversity using PCR-based 454-pyrosequencing method.

1. Introduction

Microbial communities associated with sponges are diverse and make a mutulistic relationship either as pathogen or symbionts (Wilkinson, 1983; Bavestrello et al., 2000). Associated bacteria may help in nitrogen fixation, removal of waste products and also production of secondary bioactive metabolites (Webster et al., 2001). Sponges have attracted research interest to study associated microbial population and their invaluable metabolites that can be used in pharmaceutical and biotechnological applications. Mutualistic relationship is important ecologically for marine sponges and their associated microbes. As sponge provide place for colonization, shelter from predators and nutrients to microbes and in turn by products of sponges are eliminated by these microbes. Bioactive compounds excreted by these microbes also help their host against different microbial disease (Hentschel et al., 2001).

Culture-dependent and independent techniques used to study sponge-microbe interaction. Cultivation techniques are limited as most of bacteria are not easy to culture and remain untapped. Different culture-independent techniques were used widely to study symbiotic microbiome from various sponge samples (Sharp et al., 2007; Webster et al., 2004). In spite of these various methods pyrosequencing may allow deep and quick analysis of microbial population associated with different samples with much high throughput. This technology enables to determine new and novel taxa and also provide enormous sequences of DNA reads and open

* Corresponding author at: Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, 21589, Jeddah, Saudi Arabia. Tel.: +966-2-64010000 Ext. 73529, Fax: +966-2-6952076.
E-mail address: fnaseer@kau.edu.sa (F. Bibi).
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new avenue for microbial communities of different ecological regions (Huse et al., 2007; Sogin et al., 2006).

Saudi Arabia is largest country bordering and covering around 80% of eastern site of Red sea. Coastal area of Red Sea in Saudi Arabia provide habitats for various communities of mangrove, corals, sea grass and sponges (Price et al., 1998). Sponges from Red Sea have been studied for biologically active compounds as well as for study of microbial communities (Radwan et al., 2010). Previous studies of sponges from Red sea using next generation sequencing have revealed bacterial diversity where new and rare taxa were detected. Using pyrosequencing, they have characterized 2000 different bacterial species belonging to 26 different phyla, 4 novel taxa and 300 archaeal species were recorded from single sponge (Lee et al., 2011).

Three marine sponge species included in this study are well known for their biotechnological importance. *P. vastifica* is biotechnology and ecologically important species capable of producing antimicrobial compounds (Afifi and Khabour, 2017). Triterpenoidal active metabolites were isolated from sponge *S. siphonella*. These compounds showed potent cytotoxic activities against cancer cell lines. *S. mollis* afforded brominated phenolic compounds and purealdin L, aerothionin, and dichloroverongiaquinol compound exhibiting potent antioxidant activity (Abbas et al., 2014). These three sponges didn't show any record of study related to bacterial diversity using culturomics or uncultured methods. Recently we have used only culture based method to study bacterial diversity from *P. vastifica* and *S. siphonella* (Bibi et al., 2018). Therefore, this study provides unique information regarding microbial communities and comparison between bacterial communities among these three marine sponges using Next Generation Sequencing (NGS) approach.

2. Materials and methods

2.1. Study site and sample collection

Nine sponge specimens (i.e. three replicates from three sponge samples) located at the depth of 30–40 m and were within few meters distance to each other were collected in single dive in November 2016 at obhur in Red Sea (20°23′8.9664″N and 38°7′21.2124″E) Jeddah, Saudi Arabia. The specimens were inhabiting area that was exposed to sunlight and temperature of the water was 25 °C. For identification of these sponge samples, Dr. Abdulmohsin Al-Sofyani from marine science department King Abdul-Aziz University provided his expertise. These sponges were identified as belong to *Pione vastifica*, *Siphonochalina siphonella* and *Suberea mollis*. After collection, sponges were put inside sterile ziploc plastic bag containing seawater and transferred immediately to the laboratory and were kept at −20 °C until process further.

2.2. Extraction of DNA from sponges

Sponge samples were washed twice with autoclaved distilled water to remove loosely attached bacteria. Sponge specimens were chopped into small pieces and DNA was extracted from three sponge samples using the PowerSoil DNA isolation kit, Mo Bio laboratories (Carlsbad, CA) according to the manufacturer's protocol. Using NanoDrop (ND-1000, Thermo Fisher, USA), quality and quantity of DNA was measured and samples were stored at −20 °C until use.

2.3. Library preparation and emulsion-based PCR (emPCR)

Library was prepared using DNA according to the 454 Seq Sys Amplicon Library Prep Method Manual. Briefly, 20 ng aliquot of each DNA sample was used for a 25ul PCR reaction. The 16S universal primers 27F (5′GAGTTTGATCMTGGCTCAG3′), 518R (5′WTTACCGCGGCTGCTGG3′) were used for amplification of 16S rRNA genes (V1 to V3 regions). FastStart High Fidelity PCR System (Roche, Basel, Switzerland) was used according to the manufacturer's recommendations. Fidelity PCR System (Roche) was used for PCR under the following conditions: 94 °C for 3 min followed by 35 cycles of 94 °C for 15 s; 55 °C for 45 s and 72 °C for 1 min; and a final elongation step at 72 °C for 8 min. Using AMPure beads (Beckman coulter) PCR products were purified. Using the Picogreen assay quantification assay (Life Technologies) libraries were further quantified. To clonally amplify the purified library, emPCR and amplification was carried out as described previously (Udayangani et al., 2017).

2.4. Next generation sequencing using Roche 454 GS-FLX

After PCR amplification, the emulsion was broken chemically and amplified DNA libraries were recovered from the beads after washing by filtration. Using the biotinylated primer A, all positive beads were purified. The magnetic beads were separated from the DNA library beads and single-stranded template DNA bead bound fragments were recovered from double-stranded after melting. The single-stranded DNA was amplified using sequencing primer. Finally, all beads carrying amplified single-stranded DNA were counted using a Particle Counter (Beckman Coulter). Using Genome Sequencer FLX (454 Life Sciences, Branford, CT, USA), sequencing was performed. A 70–75 mm Pico Titer plate (454 Life Sciences) fitted with a 4 lane gasket was loaded with each sample in 2 regions. For precise OTU analysis, data containing sequence error were removed. After this process, clustering was performed. Using CD-HIT-OTU, data removed contains reads that have length shorter than 40% of the library, reads on sequence similarity. OTUs of the remained reads were generated by cluster cutoff value of 97%. The singleton and doubleton created in this process was not used for further analysis.

2.5. Statistical analysis

For OTU analysis and obtaining taxonomy information QIIME (version 1.8) (Caporaso et al., 2010) was used. The major sequence of each OTU is referred to Greengenes and Silva databases. Taxonomic information is obtained with UCLUST taxonomy assigner method. In order to check the diversity and evenness in microbe community, Shannon and Simpson index were calculated. Also alpha diversity was calculated with Rarefaction curve and Chao1 value. Beta diversity (diversity among samples within the group) was calculated based on Weighted UniFrac distance. Genetic relationship among samples was visualized based on PCoA and UPGMA tree. We used R packages heatmap (Kolde, 2012) to generate heatmap figure and Venn diagram programs (Chen, 2012) was used to produce Venn diagrams.

2.6. Nucleotide sequence accession numbers

The pyrosequencing reads were deposited to the European Nucleotide Archive under accession number ERS2923991, ERS2923992 and ERS2923995 for *S. mollis*, *P. vastifica* and *S. siphonella* respectively.

3. Results

3.1. Bacterial diversity and taxonomic composition in sponges

Three sponge samples were collected from different locations (30–40 m in depth) from North Obhur in the Red Sea. Sponges

were identified based on their morphological characteristics by a sponge taxonomist (Table 1). We obtained a total of 55,638 raw sequences from 3 sponge's samples using Roche 454-FLX titanium. A total 27,356 reads were used for diversity and taxonomic analyses. The average reads numbers per sample were ±10,840–28,126 respectively. Bacterial diversity in three sponge species i.e. *P. vastifica*, *S. siphonella* and *S. mollis* were studied at different levels of their taxonomic classification to find bacterial communities. After trimming, denoising and removal of chimera sequences, 27,356 sequences were obtained and further subjected to downstream analysis. Using sequences showing 97% similarities, these sequences were clustered into total of 105, 61 and 43 OTUs respectively for *S. mollis P. vastifica* and *S. siphonella.* Numbers of OTUs were higher in sponge *S. mollis* (105 OTUs) while minimum number (43 OTUs) was observed for sponge *S. siphonella*.

Alpha diversity indicating richness of various taxa and it varies among three sponge species studied. Rarefaction analysis for sequences obtained for three sponge samples showed high sequence coverage values. This is shown by a clear saturated plateau in three sponges (Fig. 1A). It indicates good coverage and sequencing (100%) from these sponge samples. Alpha diversity indices based on OTUs using Chao1 present high richness of bacterial taxa in the sponge *S. mollis* and lowest values were recorded for *P. vastifica* and *S. siphonella* respectively (Fig. 1B). Microbial diversity indices such as Shannon and Simpson diversity indicated that sponge *S. mollis and P. vastifica* were more diverse than sponge *S. siphonella* (Fig. 1C). Bacterial diversity is generally low in two sponges: *P. vastifica* and *S. siphonella* except for sponge *S. mollis*. Shannon's diversity index values of 4.4 and 3.8 were higher for *S. mollis and P. vastifica* respectively as compare to low value of 2.0 for *S. siphonella* indicating minimum bacterial diversity and containing only 43 OTUs. To assess microbial communities across different samples, beta diversity patterns were calculated by using unweighted UniFrac distance matrices in QIIME. This two dimensional PCoA plot have shown differences in three sponge samples as explained by PC1 and PC2 (Fig. 2). This plot clearly distinguishes samples of *P. vastifica, S. siphonella* and *S. mollis*. This difference in sponge phylogeny clearly has shown that bacterial communities have correlation with grouping and taxonomic classification of sponges. This data has shown that sponge studied belong to three different genera and harbor different bacterial communities. Especially variability is noteworthy for the sponge samples of *S. mollis.*

3.2. Taxonomic richness and composition of bacterial community

Using filtered sequence reads of the 16S rRNA gene, we found 10 different bacterial phyla such as *Chloroflexi*, Acidobacteria, Bacteroidetes, Actinobacteria, *Deinococcus-Thermus*, *Proteobacteria*, *Cyanobacteria*, *Firmicutes*, *Nitrospirae*, and the candidate phylum *Poribacteria*) from three sponge species. The dominant groups of each sponge sample are shown in Fig. 3. In three sponge species, *Proteobacteria* (29%–99%) was the dominant phylum consisting of *Alphaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria* where members of *Gammaproteobacteria* show dominance. The 16S rRNA sequence of approximately 4% OTU s belong to *Alphaproteobacteria* and only detected in sponge host *S. mollis* while absent from two other LMA sponges. In sponge *S. mollis*, Alphaproteobacteria comprises of two families: *Rhodobacteraceae* and *Rhodospirillaceae* and most of these sequences remain unknown at genus level and may present a novel species. Members of *Gammaproteobacteria* were dominant and detected in three sponge species. In this study, both LMA sponges showed high proportion (99.9%) of class *Gammaproteobacteria.* Seven different genera: *Pseudoalteromonas*, *Cobetia*, *Halomonas*, *Kushneria*, *Salinicola*, *Psychrobacter* and *Pseudomonas* were observed to this class. Dominance of *Halomonas* and *Psychrobacter* was detected in *P. vastifica* and *S. siphonella* respectively.

While bacterial communities in HMA sponge *S. mollis* were diverse as compare to two other sponge species. *Proteobacteria* (24%) and *Chloroflexi* (20%) were predominant groups following

Table 1
List of sponge species collected and estimation of diversity.

Sponge	Sample code	Class	Order	Family	Total reads	Total[a] OTUs	Chao[b]	Shannon[c]	Simpson[d]
P. vastifica	O1	*Demospongiae*	*Hadromerida*	*Clionaidae*	16,672	61	64.3	3.8	0.91
S. siphonella	Sk	*Demospongiae*	*Haplosclerida*	*Callyspongiidae*	10,840	43	48	2.1	0.59
S. mollis	G	*Demospongiae*	*Verongiida*	*Aplysinellidae*	28,126	105	120	4.46	0.91

[a] OTUs: Operational Taxonomic Unit is an operational definition of a species or group of species often used when only DNA sequence data is available.
[b] Chao1: returns the Chao1 richness estimate for an OTU definition.
[c] Shannon: The Shannon index takes into account the number and evenness of species.
[d] Simpson: The Simpson index represents the probability that two randomly selected individuals in the habitat will belong to the same species.

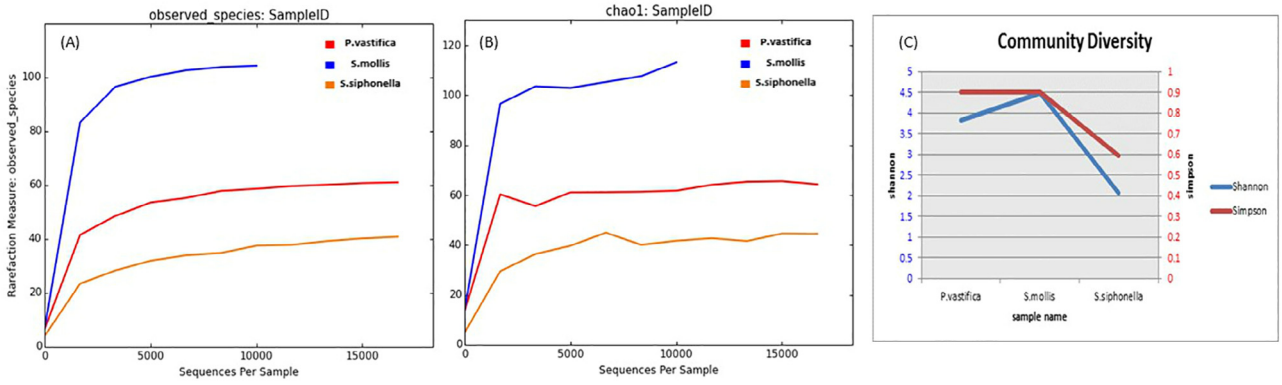

Fig. 1. Alpha diversity indices of microbial communities from 3 sponge samples. Microbial richness was calculated using observed species (A), chao 1 estimator (B) from the sponges *P. vastifica* (red), *S. mollis* (blue) and *S. siphonella* (orange) and using Shannon and Simpson indices (C).

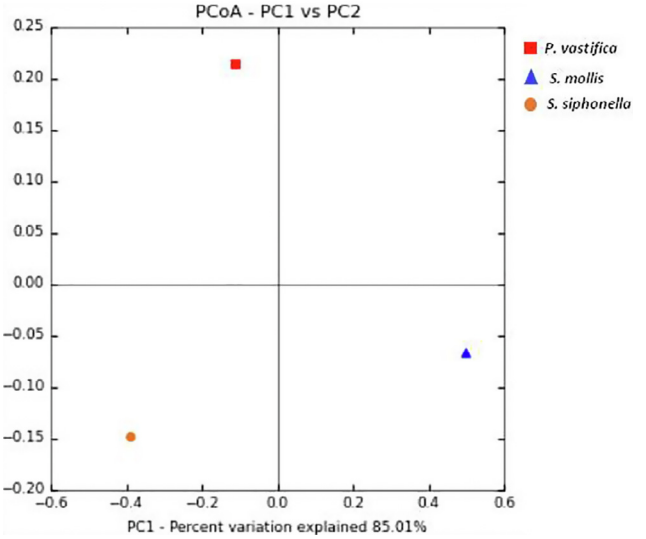

Fig. 2. 2D Principal coordinate analysis (PCoA) plot based on weighted UniFrac distance matrices.

Actinobacteria (8%), *Acidobacteria* (4%), *Bacteroidetes* (0.58%), *Nitrospirae* (0.46%), *Deinococcus-Thermus* (0.31%), candidate phylum *Poribacteria* (0.06%) and *Firmicutes* (0.04%). In addition to *Gammaproteobacteria* and *Alphaproteobacteria*, *Deltaproteobacteria* were only restricted to *S. mollis* host. Members of phylum *Actinobacteria* in host *S. mollis* belong to four different genera *Iamia*, *Streptomyces*, *Psychroflexus* and *Salinimicrobium*. The *Acidobacteria* was another dominant phylum following *Actinobacteria*. This phylum comprises of five subgroups i.e Gp3, Gp6 and Gp9-11. Bacteroidetes was another phylum further comprised of *Psychroflexus*, *Salinimicrobium* and *Salisaeta*. The heat map showed distribution of different taxa reported from three sponge hosts (Fig. 4). At all the taxonomic levels, bacterial communities of host *S. mollis* were more diverse as compare to two other sponge samples in this study. Many unclassified genera belonging to different families were present in all sponge samples, exhibiting complex bacterial community structure. Overall, 203 different genera were detected at genus level taxonomic classification from total sequence reads. Among them 78 OTUs were commonly detected only in sponge sample *S. mollis*.

3.3. Similarity and distribution of bacterial community

We have found differences in bacterial communities detected among three different sponge hosts belong to the same class (*Demospongiae*). Differences between bacterial communities can be clearly evident from clustering of sponge samples based on UPGMA tree in Fig. 5. The UPGMA cluster analysis of three sponges collected from the Red Sea showed similarities between two sponge species i. e *P. vastifica* and *S. siphonella* species and third sponge *S. mollis* grouped separately representing different bacterial communities (Fig. 5). The proportion of OTUs shared among these bacterial communities at genus level for three sponge samples has been demonstrated using a Venn diagram (Fig. 6). The results show that only 5 bacterial OTUs have been shared: for genera *Pseudoalteromonas*, *Cobetia*, *Halomonas*, *Psychrobacter* and *Pseudomonas* were commonly detected in three sponge samples at level of greater than 1% concentration (Fig. 6). While 25 unique OTUs belong to different taxon: Gp10, Gp11, Gp3, Gp6, Gp9, *Iamia*, *Acidimicrobiales*, *Streptomyces*, *Actinobacteria*, *Psychroflexus*, *Salinimicrobium*, *Salisaeta*, *Rhodothermaceae*, *Longilinea*, *Anaerolineaceae*, *Litorilinea*, *Chloroflexi*, *GpIIa*, *Truepera*, *Carnobacterium*, *Nitrospira*, *Poribacteria*, *Rhodobacteraceae*, *Rhodospirillales*, *Alphaproteobacteria*, *Deltapro-*

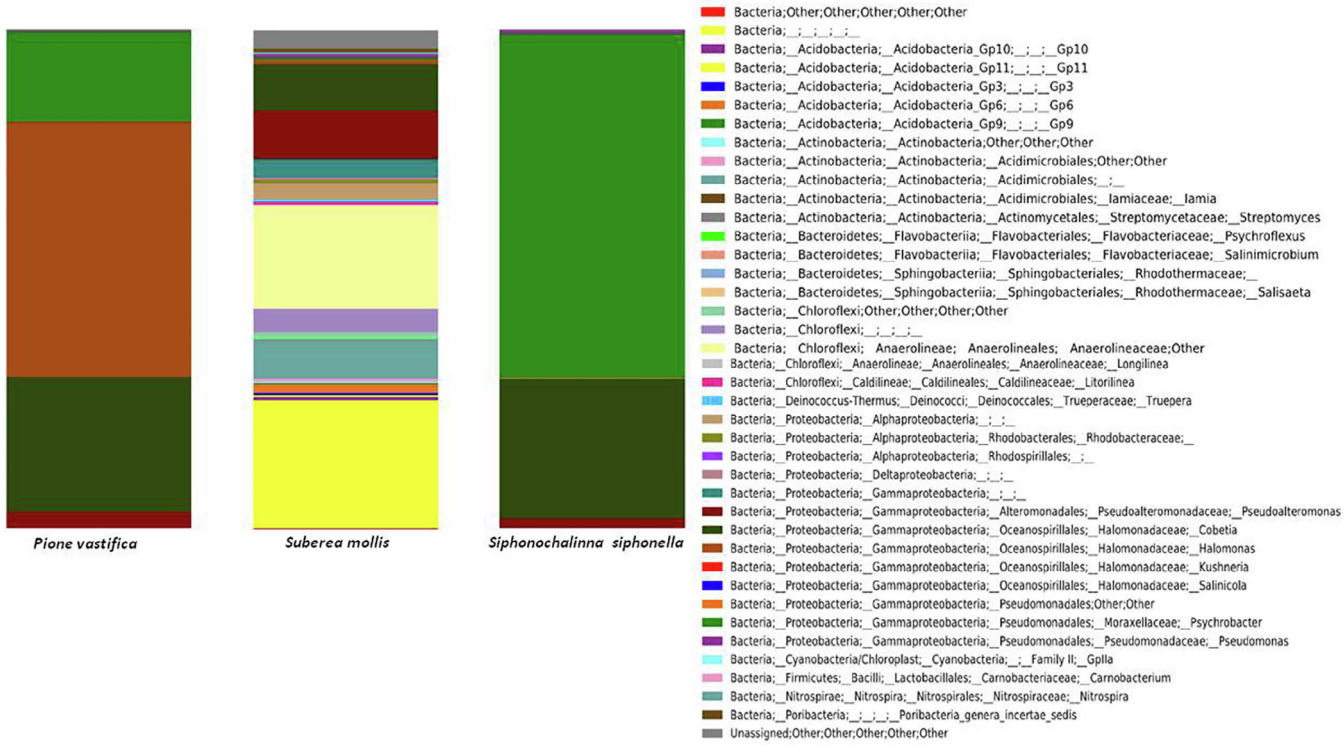

Fig. 3. Bacterial community dynamics based on the taxonomic classification of the 16S pyrosequencing reads at the specie level. (x-axis: sample name; y-axis: OTU s proportions).

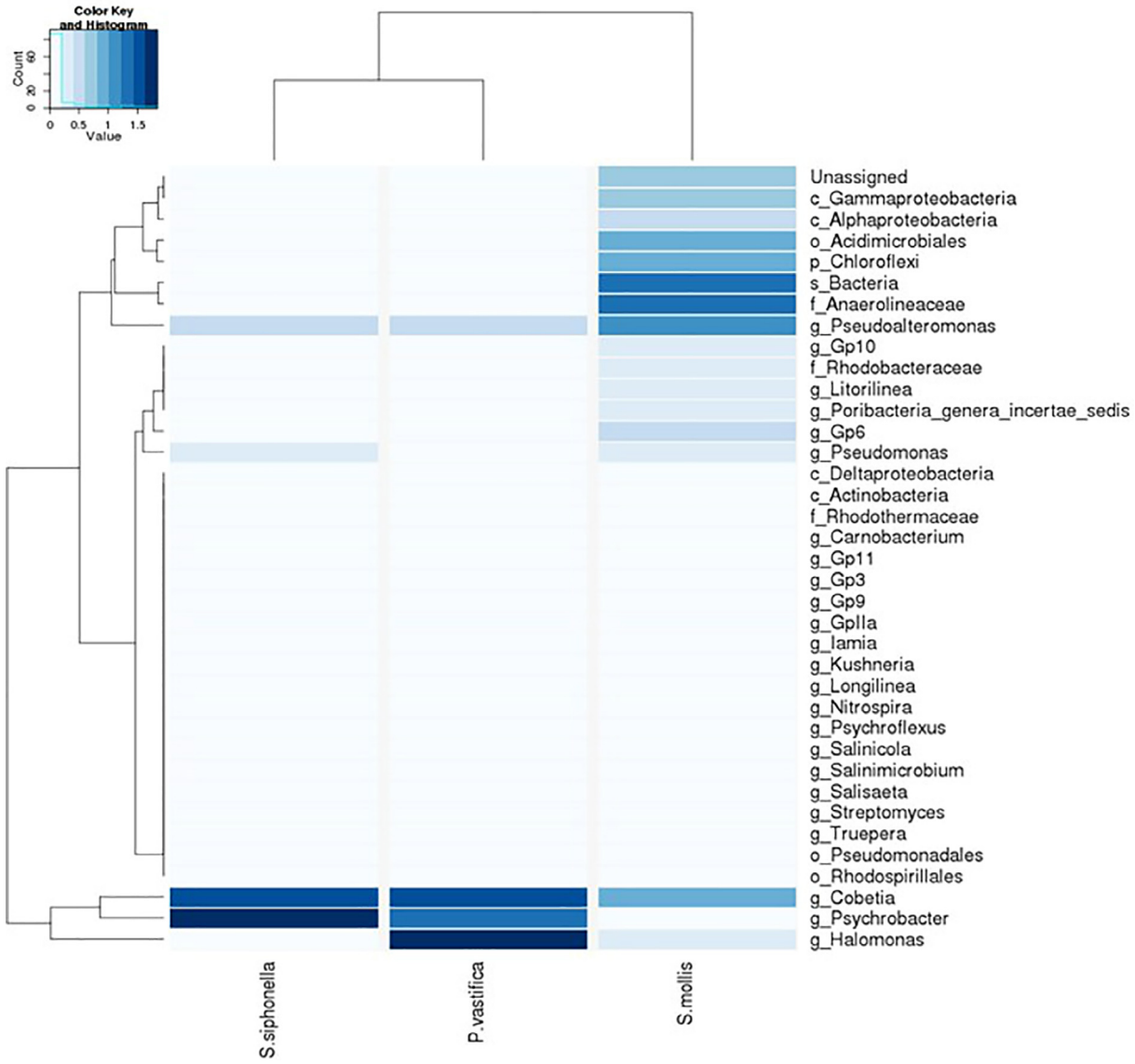

Fig. 4. Heat map showing the richness of 16S rRNA gene sequence reads of bacterial OTUs. All sponge samples are indicated along the x-axis. OTUs are indicated along the y-axis and abundance of each OTU is indicated by colors ranging from white (low abundance or absent) to dark blue (high abundance).

teobacteria, *Pseudoalteromonas*, *Cobetia*, *Halomonas*, *Kushneria*, *Salinicola* and *Psychrobacter*, were identified only in *S. mollis* species.

4. Discussion

High-throughput 454 pyrosequencing of bacterial 16S rRNA (V1 to V3 regions) was carried out in our study to examine bacterial composition in three sponges belonging to same class (Table 1). Class *Demospongiae* is more diverse class in phylum *Porifera* and comprising of more than 5000 species. In marine sponges dominance of different phyla of bacteria vary depending upon taxonomy of sponge and their geographical location. High microbial abundance (HMA) sponges generally are rich in bacterial diversity while low microbial abundance (LMA) sponges usually harbor low bacterial taxa (Giles et al., 2013; Hentschel et al., 2003). Previous studies have demonstrated lower diversity of bacterial communities in LMA sponges in comparison with HMA sponges with high diversity (Weisz et al., 2007; Kamke et al., 2010). In our study bacterial richness varied from 43 to 105 OTUs which is consistent with the range reported in previous studies (Giles et al., 2013; Hentschel et al., 2006). Recent survey of marine sponges has shown that they significantly contribute to the microbial population of the Sea where microbial richness ranges from 50 to 3820 distinct OTUs per host (Thomas et al., 2016). Two sponge species: *P. vastifica* and *S. siphonella* harbor low OTUs, low bacterial richness and *Proteobacteria* as the most prominent phylum thus affiliated with LMA sponges. While one sponge, *S. mollis* contains high abundance and diverse bacterial communities that is typical of HMA sponges. Similarity of sponge species to HMA and LMA was based exclusively on the diversity and richness of microbial communities as no histological examination was performed for the confirmation of microbial abundance. Vertical transmission of bacteria is common feature of HMA sponges (Schmitt et al., 2008). While in LMA sponges bacteria were taken up by sponge through selective mechanism hence dominated by the same group of *Proteobacteria* (Weisz et al., 2007).

Low bacterial abundance is observed for *S. siphonella* and *P. vastifica* sponge species in this study. In a previous study, sponge of same genus *Callyspongia vaginalis* showed low diversity mainly

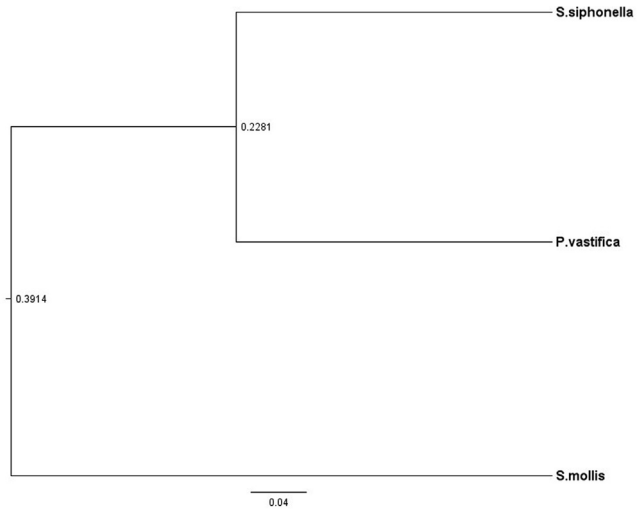

Fig. 5. UPGMA tree plotted using UniFrac distance matrices. The tree showing similarities of OTUs (%) of 16S rRNA gene sequences obtained from different bacterial taxa associated with sponge samples.

consisting of species of *Proteobacteria* (Hentschel et al., 2003). No record is available for bacterial diversity related studies for species of sponge genus *Pione*. This is a first study to report bacterial communities from *P. vastifica*. High diversity in the sponge *S. mollis* was recorded in present study that is concordant with previous study where 206 OTUs were recorded in sponge from genus *Suberea* (Turon et al., 2018). In all sponge species, *Proteobacteria* (29%–99%) was the dominant phylum. High proportion of *Proteobacteria* was also reported in previous studies of marine sponges. This high proportion of *Proteobacteria* in LMA sponges is consistent with many previous studies where LMA sponges showed high proportion of *Proteobacteria* as compare to HMA sponges (Jeong et al., 2013). *Proteobacteria* perform different functions in host including nitrogen fixation and involve in host defense mechanism (Webster et al., 2013; Li et al., 2006). Class *Gammaproteobacteria* was dominant in three sponge species where high percentage was recovered from LMA sponges in this study. Two genera, *Halomonas* and *Psychrobacter* were dominant in *P. vastifica* and *S. siphonella* respectively. Results of this uncultured study are concordant with our recent study where strains exhibiting antimicrobial activity belong to these two genera were isolated from *P. vastifica* and *S. siphonella* (Bibi et al., 2018). Different species of the genus *Halomonas* and *Psychrobacter* from sponges are already known to exhibit antimicrobial activities (Matobole et al., 2017; Bibi et al., 2018) thus playing their role in host defense mechanism.

High microbial abundance was observed in *S. mollis* in contrast to *P. vastifica* and *S. siphonella*. Approximately 4% OTUs were unassigned in *S. mollis* host at phylum level. Previous studies also reported presence of unassigned OTUs ranging from 34 to 36% and could not be assigned to any bacterial phylum (White et al., 2012; Cleary et al., 2013). A large number of (25%) 16 S rRNA sequences from *S. mollis* were not matched to bacteria at genus level so may present some novel taxa. Therefore, these sponge species may be a reservoir of novel bacteria that have not been identified so far. Candidate phylum *Poribacteria*, *Chloroflexi*, *Actinobacteria and Acidobacteria* were only detected in sponge *S. mollis. Proteobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, Firmicutes, Acidobacteria* from marine sponges are already known to produce biologically active compounds (Brinkmann et al., 2017). *S. mollis* also harbor candidate phylum *Poribacteria* that was first discover from sponge tissues and widely spread among members of class *Demospongiae* (Lafi et al., 2009). *Poribacteria*, *Acidobacteria* and *Chloroflexi* are commonly detected in HMA sponges belonging to different phyla (Kamke et al., 2014). To compare bacterial communities UniFrac analysis was performed. No correlation was observed according to distribution of bacterial communities in sponge samples i.e. they were collected from same geographical

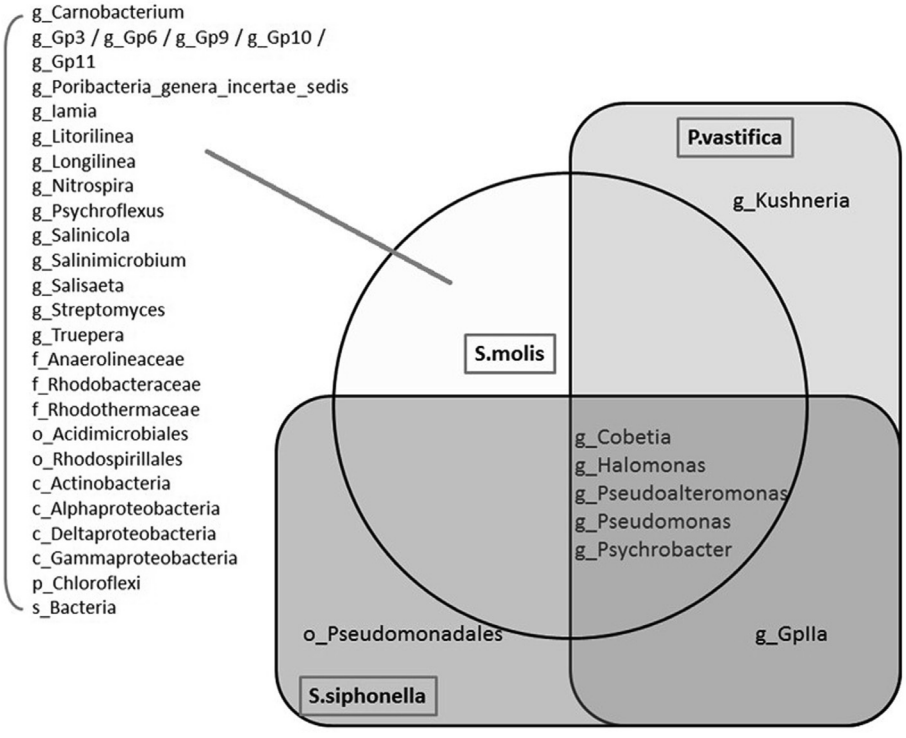

Fig. 6. Venn diagram at the genus level that exhibit the relationship between OTUs detected in three sponge samples.

location so they could harbor similar bacterial communities. Another study reported composition of complex microbial communities in HMA sponges is influenced by both environmental factors and host phylogeny (Erwin et al., 2012). This reveals that environmental factors might play a role in defining difference in pattern and structure of microbial taxa in marine sponges.

5. Conclusions

Our investigation reveals pattern and distribution of bacterial taxa in LMA and HMA sponge species. These sponges were not studied before and our data showed that three sponge samples belong to two different groups i.e. LMA and HMA on the basis of low and high microbial abundance. The community structure of each sponge showed dominance of phylum *Proteobacteria* where *Gammaproteobacteria* was dominant as a class. Richness and abundance of bacterial phyla typical of HMA sponges are absent in LMA sponges. This difference might indicate that different factors related to host and environmental may define composition of bacteria in LMA and HMA sponges. This work increases our knowledge about bacterial communities of marine sponge species that are of medical significance from previous literature.

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Conflict of interest

There is no conflict of interest.

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