



## Original article

## Analysis of bacterial communities in sponges and coral inhabiting Red Sea, using barcoded 454 pyrosequencing

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## ABSTRACT

Microbial communities are linked with marine sponge are diverse in their structure and function. Our understanding of the sponge-associated microbial diversity is limited especially from Red Sea in Saudi Arabia where few species of sponges have been studied. Here we used pyrosequencing to study two marine sponges and coral species sampled from Obhur region from Red sea in Jeddah. A total of 168 operational taxonomic units (OTUs) were identified from *Haliclona caerulea*, *Stylissa carteri* and *Rhytisma fulvum*. Taxonomic identification of tag sequences of 16S ribosomal RNA revealed 6 different bacterial phyla and 9 different classes. A proportion of unclassified reads were also observed in sponges and coral sample. We found diverse bacterial communities associated with two sponges and a coral sample. Diversity and richness estimates based on OUTs revealed that sponge *H. caerulea* had significantly high bacterial diversity. The identified OTUs showed unique clustering in three sponge samples as revealed by Principal coordinate analysis (PCoA). *Proteobacteria* (88–95%) was dominant phyla along with *Bacteroidetes*, *Planctomycetes*, *Cyanobacteria*, *Firmicutes* and *Nitrospirae*. Seventeen different genera were identified where genus *Pseudoalteromonas* was dominant in all three samples. This is first study to assess bacterial communities of sponge and coral sample that have never been studied before to unravel their microbial communities using 454-pyrosequencing method.

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## 1. Introduction

Microorganisms can inhabit different ecological niches. Especially microbial communities of marine habitat are extremely diverse and play vital role in that environment. In marine ecosystem, microorganisms mostly exist with marine corals and sponges either in mutualistic or symbiotic relationship (Wilkinson, 1983). In low nutrient environment, symbiotic microbial communities provide their host with ambient nutrients like phosphorus, nitrogen and carbon (Bavestrello et al., 2000). Microbial communities of marine sponges are extremely diverse consisting of viruses, bacteria, eukaryotes and archaea (Pita et al., 2018). In marine environ-

ment coral reefs have an immense biodiversity where sponges play an important role in their stability and survival (Bell, 2008). Sponge-associated microbial communities comprises of different archaeal lineages, bacterial, non-representative candidate phyla and contributing for half of the sponge biomass (Taylor et al., 2007). Sponge hosts harbor different taxa forming monophyletic sequence clusters even when they are phylogenetically and geographically different (Chaib De Mares et al., 2017).

Previous studies have highlighted the importance of sponge model to study sponge-microbe association (Hentschel et al., 2012). Recent studies have provided much information regarding sponge-associated microorganisms from different geographic regions of the world, still very limited information available about marine sponges and their associated microbes. In some sponge species, 40–50% of their biomass is representing by associated microorganisms. These sponge-microorganisms mainly include heterotrophic bacteria, cyanobacteria, archaea, algae, diatoms and dinoflagellates (Gaikwad et al., 2016; Hentschel et al., 2002). Sponges used some bacteria as a food source while others involved in symbiotic relationship. These symbiotic bacteria involved in stabilization of host, nutrients acquirement, processing of waste metabolic products and production of bioactive compounds

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(Enticknap et al., 2006). Several previous studies have studied the diversity of microbial communities from marine sponges using cultivation-based and uncultured approaches. It revealed from results that structure of microbial communities can be relatively different using two different approaches (Montalvo et al., 2014). High-throughput DNA sequencing techniques have given understandings into the diversity of the sponge microbiome (Simister et al., 2012). Where next generation sequencing method, the 454 pyrosequencing technology enables to analyze microbial communities of marine sponges from different geographic regions (Gao et al., 2014a; Gaikwad et al., 2016).

Red Sea in Saudi Arabia provide coastal habitats with distinctive and largely underexplored marine ecosystem (Price et al., 1998). Previous studies from Red Sea have revealed biotechnological importance of marine sponges as well as diverse microbial communities (Radwan et al., 2010). In present study we have included two sponge samples i.e *Haliclona caerulea*, *Stylissa carteri* and a soft coral, *Rhytisma fulvum* to study associated microbial communities. Pyrosequencing technique was used to obtain understandings into bacterial communities. Marine sponges and soft coral in this study are already known for their biotechnological importance (Ganapiriya et al., 2012; Tsukamoto et al., 2003). More than 190 secondary metabolites were identified from species of *Haliclona* exhibiting antimicrobial, antimicrobial and cytotoxic activities (Yu et al., 2006; Hoppers et al., 2015). Bioactive compounds exhibiting antibiotic activity were isolated from species of *Stylissa* (Tsukamoto et al., 2003). *Rhytisma fulvum* afforded bioactive compounds, secosterols and nardosinanol (Bishara et al., 2008). These marine sponges and soft coral have shown very little or no work related to study their associated microbial communities. Therefore, present study was undertaken to provide an insight and new information regarding microbial communities from these marine sponges and coral using Next Generation Sequencing (NGS) approach.

## 2. Materials and methods

### 2.1. Sponge sample collection

Six sponges and three coral samples such as three replicates from each sponge sample) located on depth approximately 30–40 m within a few meters distance from each other at North Obhur in the Red Sea Jeddah (20°23'8.9664"N and 38°7'21.2124"E), Saudi Arabia. Sponges were identified using morphological characteristics including size, color shape and oscules. Sterile sample plastic bag containing seawater was used to collect sponge samples. Sponge samples were transferred directly to the laboratory after collection and after identification kept at –80 °C until process further.

### 2.2. DNA extraction from sponges

To clean sponge and coral samples, specimen were washed with sterile water. Sponge specimens were mashed into small pieces, chopped and DNA was extracted using the PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA). Further quantity and quality of DNA was checked using NanoDrop (ND-1000, ThermoFisher, USA).

### 2.3. Preparation of library and emulsion-based PCR amplification

16S rRNA gene library was generated using DNA according to the 454 Seq Sys Amplicon Library Prep Manual. We used primers as described previously (Bibi et al., 2020) to amplify the hyper-variable regions of the 16S rRNA gene (V1-V3) to generate the 454 libraries. The FastStart High Fidelity PCR System (Roche, Basel,

Switzerland) was applied using conditions such as: 94 °C for 5 min for 35 cycles of 94 °C for 20 s; 55 °C for 50 s and 72 °C for 60 s; and elongation step at 72 °C for 10 min. PCR products were purified further using AMPure beads (Beckman coulter). Libraries were quantified using the Picogreen assay quantification assay (Life Technologies). Clonal amplification of the DNA sequenced library, emPCR and subsequent amplification was conducted according to the manufacturer's recommendations and as described previously (Udayangani et al., 2017).

### 2.4. Next generation sequencing and clustering of operational taxonomic units (OTUs)

DNA libraries generated after PCR amplification were recovered from the beads and counted as described previously (Bibi et al., 2020). Further sequencing was performed using Genome Sequencer FLX (454 Life Sciences, Branford, CT, USA). Data containing sequence error or mismatches, length smaller than 40% of the library were removed for further OTU analysis. Sequences that have been passed through the quality check using CD-HIT-OTU were merged and clustering was performed.

### 2.5. Statistical analysis

QIIME (version 1.8) was used for operational taxonomic units (OUTs) and obtaining taxonomy information (Caporaso et al., 2010). Greengenes in conjunction with Silva databases were used to assigned taxonomic designations of major sequence of each OTU. UCLUST based taxonomy assigner was used to obtain taxonomic information. Microbial diversity and evenness were calculated using Shannon and Simpson indices. Alpha diversity metrics were calculated with Chao1 value and Rarefaction curve. Weighted UniFrac distance metric was used to calculate phylogenetic beta diversity. Genetic dissimilarity and relationship among samples was determined by Principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) dendrogram. Heatmap figure was generated to produce Venn diagrams R packages heatmap (Kolde, 2013) and Venn diagram programs (Chen, 2012) were used.

### 2.6. Nucleotide sequence accession numbers

The result of pyrosequencing reads were deposited to the European Nucleotide Archive under accession number ERS2923990, ERS2923993 and ERS2923994 for *H. caerulea*, *S. carteri* and *R. fulvum* respectively.

## 3. Results

### 3.1. Diversity of bacteria and taxonomic composition in sponges and coral

We have assessed two sponge and a coral associated bacterial communities collected from Red Sea in Saudi Arabia. A total of 41,216 raw sequences were obtained from the sponges and coral samples from pyrosequencing-based analysis of 16S rRNA gene sequences. The number of reads detected per sample ranged from 12,021 to 16,212 with an average of 13,738. For diversity and taxonomic analyses of total 23,170 reads were used. The Good's coverage indices were 99%, 99% and 99% for *Haliclona caerulea*, *Stylissa carteri* and *Rhytisma fulvum* respectively. The observed bacterial diversity and richness significantly differed between three samples. Sequences showing 97% similarities were used for downstream analysis and clustered into 61, 55 and 52 operational taxonomic units (OTUs) for *H. caerulea*, *R. fulvum* and *S. carteri*.

Higher numbers of bacterial taxonomic diversity within the microbiome were observed in *H. caerulea* (61 OTUs) while lower number (55 OTUs) was reported for soft coral *R. fulvum* (52 OTUs) and sponge *S. carteri* (Table 1).

Richness of various taxa (alpha-diversity) varies among three samples studied. Alpha-diversity estimates based on OTUs using Chao1 display richness and abundance of bacterial communities in the sponge *H. caerulea* while low values were noted for *R. fulvum* and *S. carteri* respectively (Fig. 1A and B). Rarefaction analysis revealed good sequence coverage values for three samples as shown by a clear saturated plateau (Fig. 2). Shannon and Simpson diversity indices provide more inference about the composition of bacterial community. These indices indicated that soft coral, *R. fulvum* and sponge *S. carteri* showed more evenness within their microbial communities than in *H. caerulea* (Fig. 2A). Higher values of Shannon's diversity i.e. 3.5 and 3.4 were for recorded for *R. fulvum* and *S. carteri* correspondingly as compare to low value of 2.9 for *H. caerulea* indicating minimum bacterial diversity. Beta diversity was calculated to determine microbial communities through different samples. Two dimensional PCoA plot represents differences in three sponge samples as shown by PC1 and PC2 (Fig. 2B). This plot clearly separates three sponge samples and indicated their unique clustering. These analyses showed clearly that three sponge species studied belong to three different classes, harboring different bacterial communities.

### 3.2. Taxonomic richness and diversity of microbial community

Sequence reads of the 16S rRNA gene from three samples were clustered into six different phyla such as *Bacteroidetes*, *Planctomycetes*, *Proteobacteria*, *Cyanobacteria*, *Firmicutes* and *Nitrospirae*. Results indicated the dominant groups of each sponge sample are presented in Fig. 4. Nine different classes were detected where *Gammaproteobacteria* and *Alphaproteobacteria* were predominant groups following *Deltaproteobacteria*, *Flavobacteria*, *Phycisphaerae*, *Cynobacteria*, *Chloroplast*, *Bacilli* and *Nitrospira*. Overall, all three sponge species were dominated by *Proteobacteria* (88–95%) where the dominant class comprising members of class *Gammaproteobacteria*. Approximately 68–92% OTUs belong to *Gammaproteobacteria* and detected in three sponge species. Overall seventeen different genera *Photobacterium*, *Vibrio*, *Agarivorans*, *Colwellia*, *Ferrimonas*, *Idiomarina*, *Pseudoalteromonas*, *Shewanella*, *Alcanivorax*, *Endozoicomonas*, *Cobetia*, *Halomonas*, *Kushneria*, *Salinicola*, *Psychrobacter*, *Pseudomonas* and *Methylophaga* were observed to this class. Where members of genus *Pseudoalteromonas* were dominant in all three sponge species ranging from 6% to 29%. Second dominant class was *Alphaproteobacteria* in all three sponge species. This class mostly comprises of unclassified bacteria within genus *Aurantimonas* and *Thalassospira*. *Proteobacteria* in *R. fulvum* were dominated by *Alphaproteobacteria* (20%) after *Gammaproteobacteria*. *Deltaproteobacteria* were only restricted to *R. fulvum* and *S. carteri* while absent from *H. caerulea*. Similarly members of class *Phycisphaerae* were only detected in sponge *H. caerulea* while absent from two others. Bacterial communities of *R. fulvum* and *S. carteri*

are more diverse. *Bacteroidetes* was comprising of only two genera i.e. *Psychroflexus* and *Salinimicrobium* and was second dominant class following *Proteobacteria* in *R. fulvum*. At phylum level, members of class *Planctomycetes* and *Firmicutes* were only detected in sponge *H. caerulea*. While members of phylum *Cyanobacteria* were distributed among all samples studied. The phylum *Nitrospirae* was only detected in sponge *S. carteri*. As shown in Fig. 4, many sequence reads from *R. fulvum* and *S. carteri* were associated with unclassified groups at genus level (Fig. 3). Bacterial communities of sponge *H. caerulea* were more diverse at all taxonomic level as compare to other two samples in this study. Differences in richness and abundance at the genus level for the sponge samples shown in heatmaps Fig. 4.

### 3.3. Shared bacterial community associated with sponges and coral

Differences have been found in bacterial communities of two sponges belong to same class of *Demospongiae* and a soft coral. The percentage of OTUs shared between microbial communities (genus level) for three samples verified using a Venn diagram (Fig. 5). In comparing all three samples of marine sponges and coral, three families (*Rhodobacteraceae*, *Rhodobiaceae*, *Rhodospirillaceae*) were exclusive to *H. caerulea*. Members of Class *Proteobacteria* were common in three samples where *Gammaproteobacteria* were abundant and most commonly shared bacteria in all three samples. These shared OTUs of bacteria belong to 12 different genera including *Cobetia*, *Candidatus Pelagibacter*, *Gp11a*, *Halomonas*, *Methylophaga*, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*, *Psychroflexus*, *Salinimicrobium*, *Thalassospira* and *Vibrio*. Members of 5 different genera (*Alcanivorax*, *Aurantimonas*, *Colwellia*, *Idiomarina*, *Salinicola*) were exclusive to *R. fulvum* and *S. carteri*. Members of three genera, *Bacillariophyta*, *Ferrimonas* and *Nitrospira* are exclusive to *S. carteri*.

## 4. Discussion

Recently marine sponges gain much interest due to their diverse symbionts, bioactive secondary metabolites and biotechnological importance (Alex and Antunes, 2015; Perdicas et al., 2013; Bibi et al., 2020). Sponge and coral-associated bacterial communities vary with respect to different environmental parameters including species diversity, health status, site and season of collection (Taylor et al., 2007; Zhang et al., 2015). However, our knowledge of sponge-associated microbial symbionts and their diversity is limited. Several previous studies have shown that 454 tag pyrosequencing explored more diverse bacterial communities and was more efficient than other methods such as Sanger sequencing and DNA library construction (Moitinho-Silva et al., 2014a; Gao et al., 2014b).

Class *Demospongiae* that comprises of nearly 8800 species is largest and diverse class of sponges. In marine sponges, abundance and dominance of bacterial communities vary depending upon taxonomy and geographical location of sponge. Using pyrosequencing technology we studied microbial symbionts of two sponges

**Table 1**  
Sponge and coral sample used in this study and calculations of diversity.

Sponge	Class	Order	Total Reads	Total <sup>a</sup> OTUs	Chao <sup>b</sup>	Shannon <sup>c</sup>	Simpson <sup>d</sup>
<i>S. carteri</i>	<i>Demospongiae</i>	<i>Scopaliniida</i>	12,021	52	62.5	3.4	0.84
<i>H. caerulea</i>	<i>Demospongiae</i>	<i>Haplosclerida</i>	12,983	61	64	2.9	0.69
<i>R. fulvum</i>	<i>Anthozoa</i>	<i>Alcyonacea</i>	16,212	55	58.3	3.5	0.87

<sup>a</sup> OTUs: Operational Taxonomic Unit.

<sup>b</sup> Chao1: richness estimation for an OUT.

<sup>c</sup> Shannon: index showing the number and evenness of microbial species.

<sup>d</sup> Simpson: represents the probability values of microbial species.

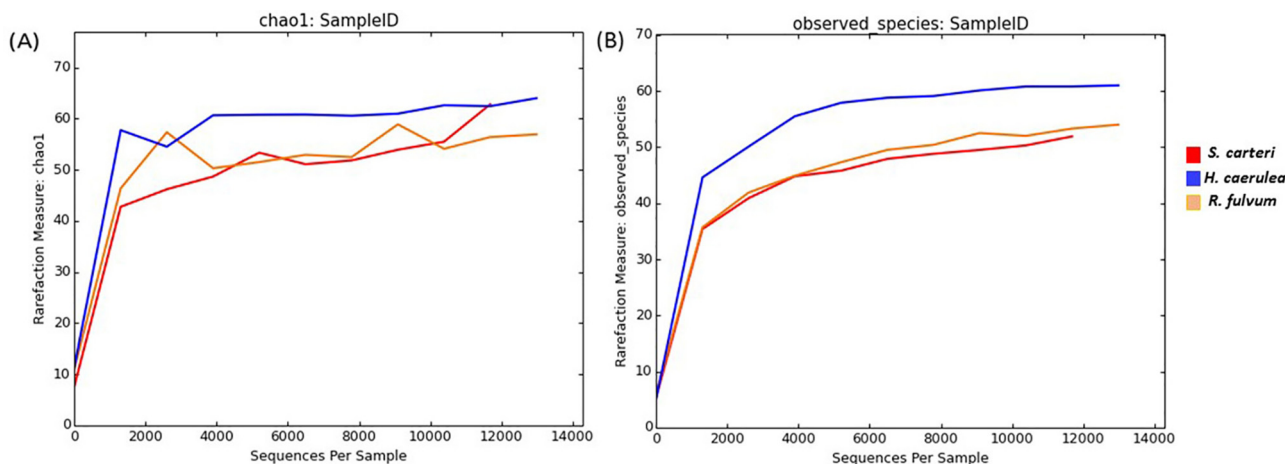


Fig. 1. Diversity indices (alpha) of microbial communities from sponges and coral sample. Calculation of microbial richness using observed species (A), chao 1 indices from sponges *S. carteri* (red), *H. caerulea* (blue) and *R. fulvum* (orange) (B).

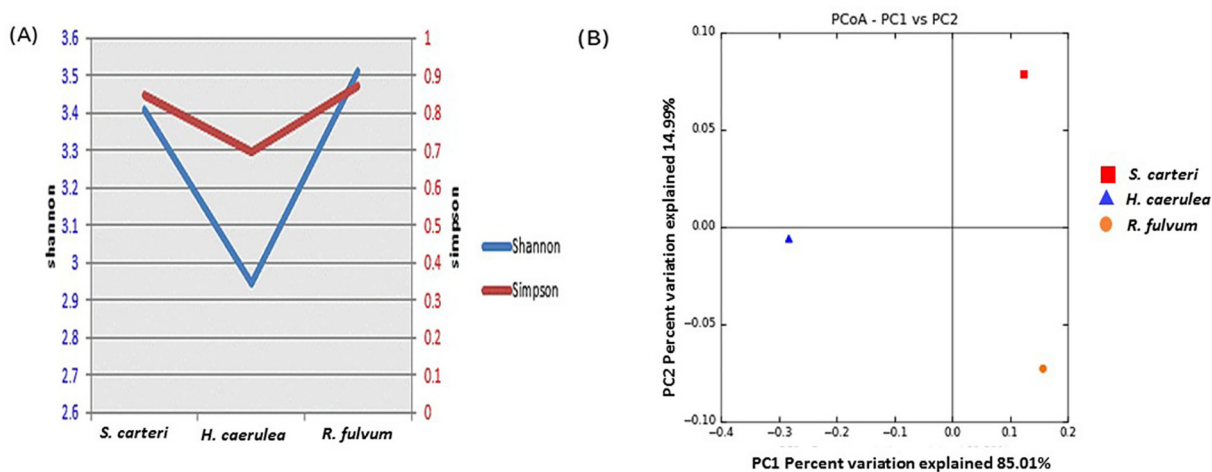


Fig. 2. Analysis using Shannon and Simpson (A) and (B) Principal coordinate analysis (PCoA) plot.

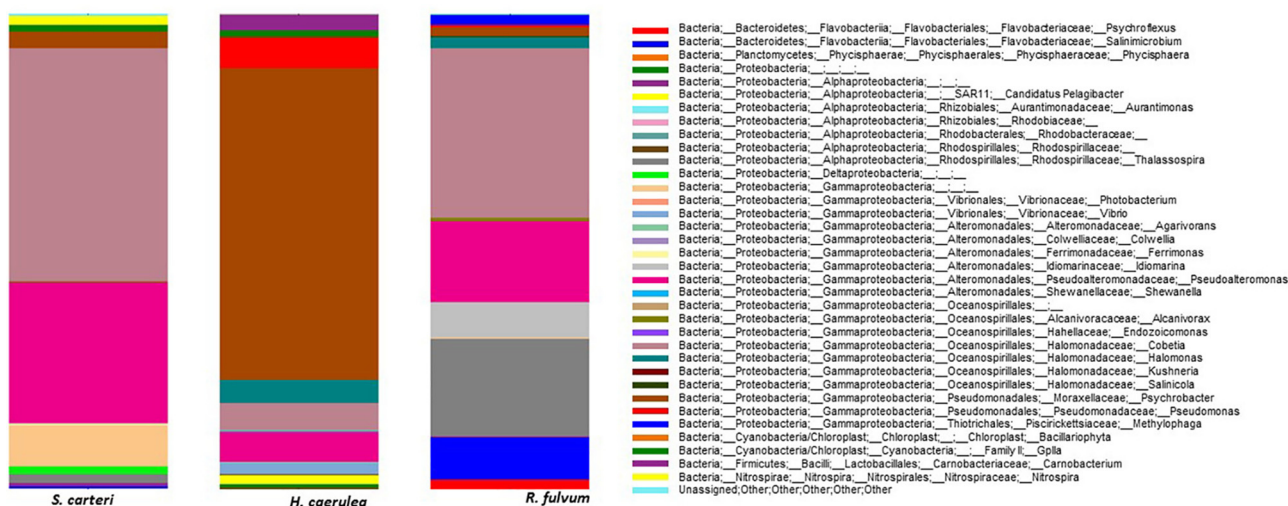
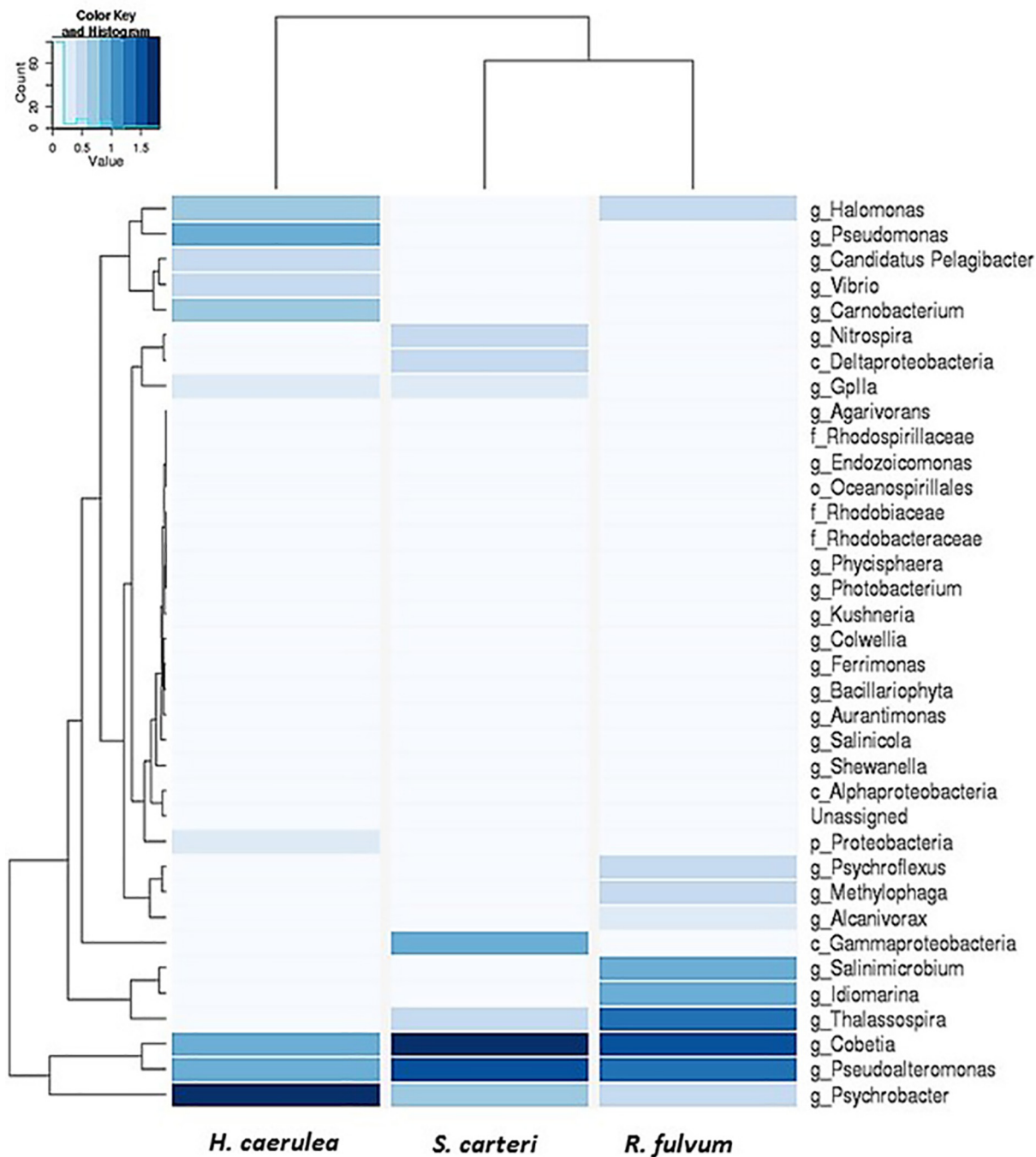


Fig. 3. 16S pyrosequencing reads (genus level) based microbial community analysis. (x-axis: sample name; y-axis: OTUs).

(*H. caerulea*, *A. carteri*) belong to Class *Demospongiae* and one soft coral (*R. fulvum*) from Red Sea in Saudi Arabia. The present study

revealed abundance and diversity of microbial communities in bacterial symbionts of three samples. In addition, similarities





**Fig. 4.** Heat map of microbial OTUs showing bacterial richness. (x-axis: sample name; y-axis: OTUs where abundance is indicated by low (white) to high (blue). abundance indicated by colors ranging from white (low abundance or absent) to dark blue (high abundance).

found in the sponge and coral associated microbial communities where some were sponge specific. Using 454-pyrosequencing and bioinformatics approaches products of hypervariable regions were identified as different taxonomic groups of bacterial symbionts. The bacterial richness found in our samples varied from 52 to 61 OTUs that is low and might be related to selection of primers (V1–V3), environmental or geographical differences. To the best of our knowledge, bacterial symbionts from these samples were not studied before as there is no scientific literature available except pharmacological survey (Carneiro et al., 2013; Trifman et al., 2016). Since no previous literature on bacterial symbionts

of *H. caerulea*, *A. carteri* and *R. fulvum* are available for comparison; hence we could not assign any OTU as species-specific.

Previous studies have demonstrated that bacterial diversity was low in sponges belong to species of *Haliclona* and *Stylissa* and are low in microbial abundance (Jasmin; Coelho).

In our study, low bacterial richness of 61 and 52 for *H. caerulea*, *S. carteri* and dominance of *Proteobacteria* affiliated these sponges to low microbial abundance sponges. Number of OTUs and diversity index was higher in *H. caerulea* as compare to other two samples. A study from Gulf of Mannar reported *Proteobacteria* as prominent group from the microbial communities associated with

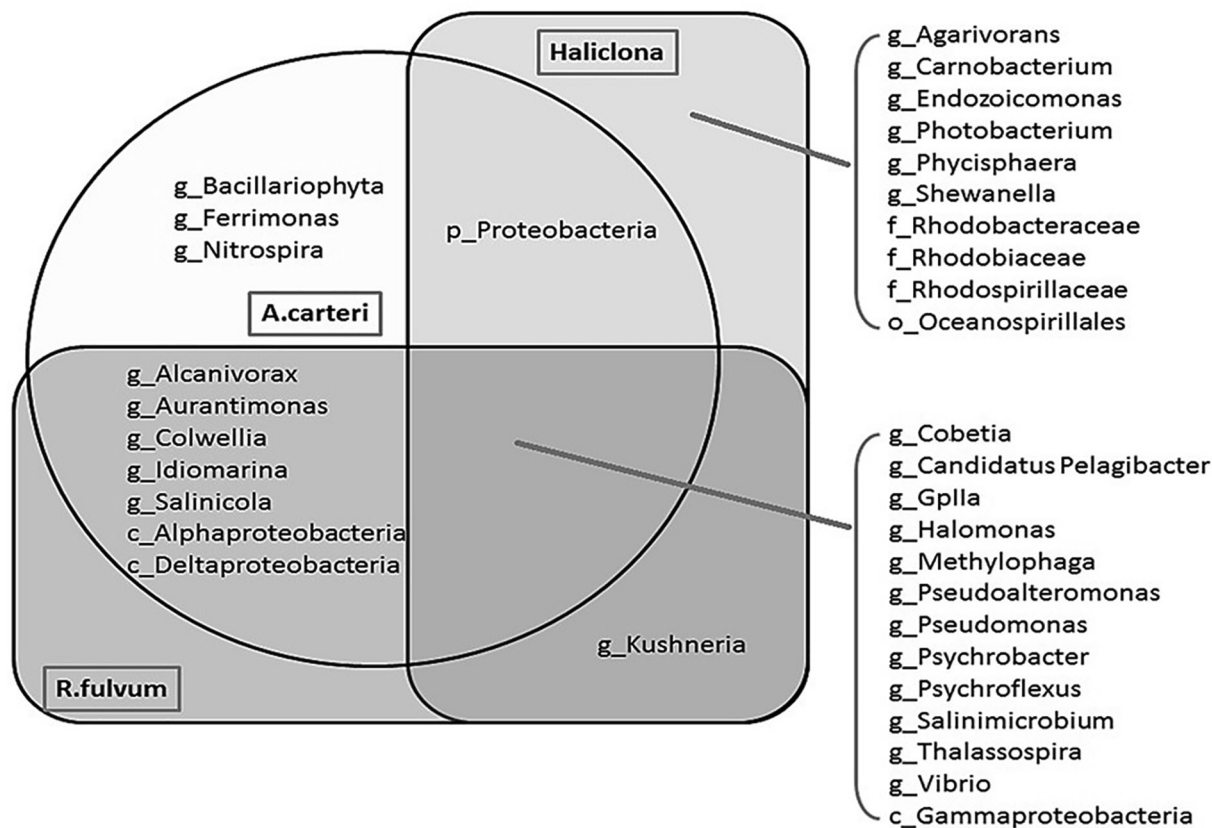


Fig. 5. Identification of core and unique OTUs at genus level using Venn diagram analysis.

sponge *Haliclona* sp (Jasmin et al., 2015). Relative abundance of OTUs assigned to the phylum *Proteobacteria* were higher in sponge species and coral sample. This phylum is commonly dominant and abundant in marine environment whether as planktonic or as symbiotic (Stabili et al., 2014). Previous studies have reported the dominance of *Proteobacteria* in different species of sponges from different geographical locations (Jasmin et al., 2015). They contribute to biogeochemical cycles by producing extracellular enzymes. They also perform different function in sponges especially nitrogen fixation, help in manipulating reproduction, secondary metabolite production and chemical defense of host (Li et al., 2006). From GenBank database, half of 16S rRNA gene sequences of sponge-associated bacteria comprised of *Proteobacteria* and rest half make up other phyla (Webster and Taylor, 2012). Our sponge samples also showed that *Proteobacteria* is dominant phyla. Class *Gammaproteobacteria* showed dominance followed by *Alphaproteobacteria* and *Deltaproteobacteria* in three samples studied. This bacterial group always remain dominant in marine sponges from different habitat (Preston et al., 1996). Sponge *H. caerulea* contained many phyla and this high proportion is similar to previous studies in low microbial abundance sponges (Giles et al., 2013). Dominant OTUs from three samples were identified as belonging to the *Gammaproteobacteria* were similar to studies where in both culture and uncultured approaches in marine sponges from different geographic locations reported dominance of this class of bacteria (Sfanos et al., 2005).

Our study reported diverse microbial communities in sponges and coral from Red Sea and supports the idea that in low abundance sponges are composed of diverse communities of organisms (Hughes et al., 2001). Furthermore, our study revealed a small proportion of unclassified reads in sponges that may represent novel species never been reported. Small proportion (<1%) of Cyanobacte-

ria were also present in sponge as well as in coral sample. A previous study of functional expressed prokaryotic genes from Red Sea in sponge *S. carteri* revealed the association of functional genes expression with microbial communities to unravel functional roles of microorganisms in sponges (Moitinho-Silva et al., 2014b). The phylum *Cyanobacteria* was second dominant phyla with respect to bacterial expressed gene functions implying their role in photosynthetic carbon fixation. Members of the phylum *Nitrospirae* are usually responsible to convert ammonia into nitrate in marine sponges (Han et al., 2013). In the present study, phylum *Nitrospirae* (2%) was identified only in sponge *S. carteri*. Our study showed similar results as recorded in other pyrosequencing studies where *Nitrospirae* was detected in a small proportion (<3%) among several sponge samples studied (Webster et al., 2010; Gaikwad et al., 2016). Both sponges (*H. caerulea*, *S. carteri*) and coral sample (*R. fulvum*) showed presence of OTUs related to *Bacteroidetes* where high percentage was evident in *R. fulvum*. A culture independent study has revealed strong association of *Bacteroidetes* in disease free colonies of corals while their absence in diseased colonies of corals (Godwin et al., 2012). Presence of *Bacteroidetes* might be strongly associated with healthy coral tissues are their role in defending the coral against infection. Approximately 4% of OTUs showed similarity with phylum *Firmicutes* in *H. caerulea*. In spite of the essential role of *Firmicutes* they are typically absent in other two samples. Members of this phyla have been reported in marine sponges from different geographical locations (Giles et al., 2013; Bayer et al., 2014). The representative OTUs sequences in our study related mainly to sequences from marine environments. UniFrac analysis showed no correlation according to distribution of different bacterial phyla in marine sponges as they were collected from same geographical locations so could harbor same bacterial groups. Although differences were found in richness and diversity of different bacte-

rial groups shows that although there is a difference in sponge-associated microbial communities, due to influence of the host but certain degree of influence of environmental factors in microbial communities among different habitats. This improve our knowledge that how environmental factors play a role for the defining the structure of microbial signature in marine sponges.

## 5. Conclusions

Our results indicate that Red Sea sponges and coral harbor diverse microbial communities and reveals pattern and distribution of bacterial communities. These sponges and coral were studied first time and our data indicates that community structure of marine sponges and coral exhibited dominance of phylum *Proteobacteria*. OTU-based analysis revealed bacterial communities belong to six different bacterial phyla. Bacterial symbionts in these sponges and coral from Red Sea may suggest their role in nitrogen and carbon cycle and dominance of *Proteobacteria* in chemical defense of the studied sponges. More research work is needed to understand the sponge-associated microbial communities from Red Sea in Saudi Arabia. Especially functional studies of these sponge-associated microbial communities to unravel their functions and role in marine environment.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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