BACTERIAL DIVERSITY IN DEMOSPONGES FROM THE CORAL REEFS OF LAKSHADWEEP, INDIA

ANNIE FEBY*, BABY DIVYA*, SHANTA NAIR**

Marine sponges harbor diverse bacterial communities which form about 60% of their biomass and play critical roles in their survival. Due to the ecological and biotechnological importance of sponges, it is important to understand the diversity of bacteria associated with them. In the present study, bacterial diversity in the two demosponges, *Sigmadocia fibulata* and *Dysidea granulosa* inhabiting the coral reefs of Lakshadweep was characterised using PCR-DGGE analysis, during three seasons (premonsoon, monsoon and post-monsoon). The results revealed statistically different banding patterns of bacterial communities in both sponges with *D.granulosa* having greater taxa richness. The common phylogenetic groups associated with both the sponges were Firmicutes, Proteobacteria, Actinobacteria and Acidobacteria. Spirochetes (*Spirochaeta americana*), Thermatogae (*Fervidobacterium pennivorans*) and Chloroflexi (*Dehalogenimonas lykanthroporepellens* and *Caldilinea aerophila*) were exclusively associated with *D. granulosa*, whereas Cyanobacteria (*Procholorococcus marinus*) with *S. fibulata*. Firmicutes was themost abundant bacterial group in all the three seasons. There was no significant temporal variation in bacterial diversity, but abundance of the groups in the two sponges varied over time.

Keywords: Sponge, bacterial diversity, demosponge, DGGE, 16S rRNA, coral reef.

INTRODUCTION

Marine sponges (Phylum Porifera) are the simplest type of metazoans (Li *et al.*, 1998) and represent an ecological niche that hosts a high microbial diversity (Hentschel *et al.*, 2003). Bacteria form about 40–60% (10^8 to 10^{10} bacteria g^{-1}) of their biomass, located both intra and extracellularly, and perform various functions useful for the sponges. The presence of a diverse bacterial community in sponges exposes sponge cells to the chemical products of a wide range of metabolic transformations of carbon, nitrogen and other elements as well as bioactive microbial natural products. Because of this exposure, the sponges benefit from the metabolic diversity of their associated microbes. Demosponges are the most abundant, forming about 85% of the described sponges (Hooper & Van Soest, 2002) and a few studies

have reported their association with large microbial consortia (Hentschel *et al.*, 2003; Imhoff & Stohr, 2003). In addition, Demosponges form a major part of the 486 species of sponges described in the Indian waters (Thomas, 1998; Venkataraman & Wafar, 2005). However, most studies were pertaining to explore microbes for bioactive compounds (Thakur & Anil, 2000; Mohapatra *et al.*, 2003; Selvin *et al.*, 2004; Anand *et al.*, 2006; Thakur *et al.*, 2005; Selvin *et al.*, 2009a; Feby & Nair, 2010). Though studies on the diversity of sponge-associated microbes using cultivation-independent methods have been carried out worldwide (Webster *et al.*, 2001; Hentschel *et al.*, 2003; Noyer *et al.*, 2014; Souza *et al.*, 2017; Bibi *et al.*, 2020) those from Indian waters are very limited (Selvin *et al.*, 2009a, b; Jasmin *et al.*, 2015) and also very little is known about the spatial and temporal variations in diversity.

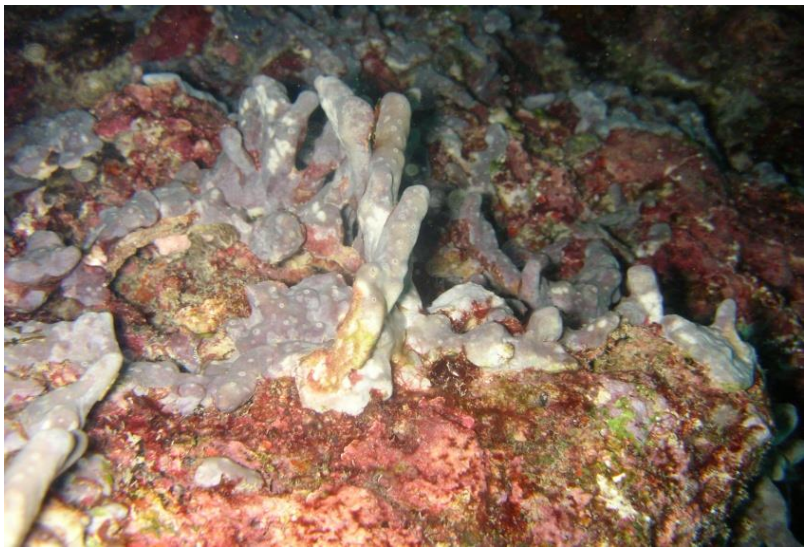
This present study attempts to investigate whether the bacterial community associated with two common and widely distributed tropical Demosponges in Lakshadweep - *Sigmadocia fibulata* and *Dysidea granulosa* vary in space and time.

MATERIAL AND METHODS

Sponge collection and processing

Sponges *S. fibulata* and *D. granulosa* were collected from inside of the lagoon (KL – 10^0 34' 51" N; 72^0 38' 07" E) and outside the lagoon i.e the oceanic side (KO – 10^0 34' 83" N; 72^0 38' 49" E) respectively at Kavaratti Islandin the Lakshadweep archipelago. The sampling sites KL and KO were 1 km apart and sponges were collected at approximately 4 m and 15 m respectively. The sampling was carried out during three seasons i.e. pre-monsoon (February–May), monsoon (June–September) and post-monsoon (October–January) (Figs. 1–2).

The sponges were collected as aseptically as possible by immediately transferring to Whirlpak sterile sealable sampling bags and sealed underwater to ensure the prevention of contact with air and possible oxidation and contamination. The sponge samples were processed immediately in the field laboratory near to the sampling site. Samples were washed thoroughly with jets of autoclaved filter sterilized (0.22 µm – pore size filter) sea water until the sponges were visibly free of debris and sediments. The pre-washed samples were used for DNA extraction. Sponge samples for DNA extraction were labeled and snap frozen by dipping in Liquid Nitrogen and transferred to the laboratory. The samples were further stored in deep freezer at -80^{o}C until extraction.



(a)

(b)

Fig. 1. *In-situ* photographs of (a) *Dysidea granulosa* and (b) *Sigmadocia fibulata*.

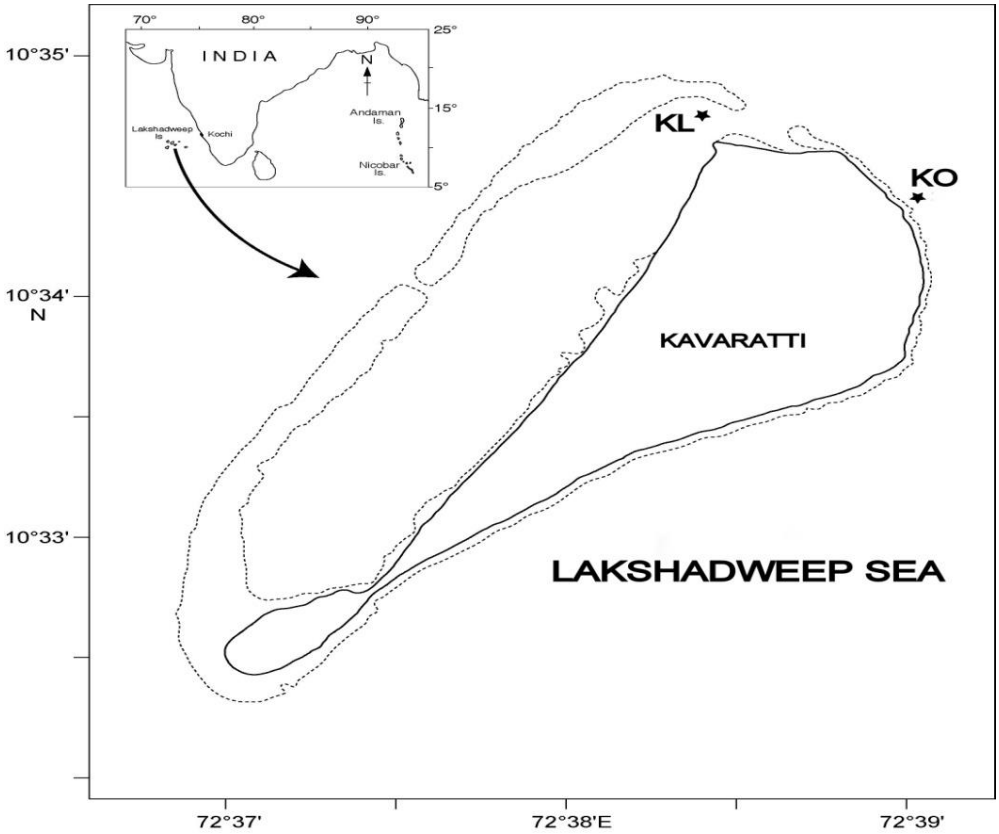


Fig. 2. Sampling sites in lagoon (KL) and oceanic (KO) region of Kavaratti Island.

Total community genomic DNA extraction, PCR amplification and DGGE analysis

Total community genomic DNA from sponges was extracted by standard method (Asahida *et al.*, 1999) an the DNA extracted from the duplicate sponge samples were pooled for downstream processes. Amplification of 16S rRNA gene was done by touchdown PCR using the primers 341F and 907R (Eppendorf, Germany). DGGE as a technique for studying microbial diversity provides an immediate display of the constituents of a population in both a qualitative and semi-quantitative way. The major advantage of DGGE fingerprinting is that it is a rapid and efficient approach for the qualitative comparison of many samples. DGGE was performed in a DCode universal mutation detection system (Bio-Rad Laboratories Inc, USA), by the method described by Muyzer *et al.* (1993). DGGE

gel was extracted by image analysis which resulted in a binary matrix (presence or absence of bands in different samples) and profile consisted of a total of 53 band classes based on the band position.

Twenty six bands were excised with a sterile surgical blade and suspended in 10 µl of deionized water. The tubes were incubated at 4°C for 12 hours followed by a spin for about 15 seconds. 0.5 µl of the supernatant was used as a template for reamplification as above and amplicons were electrophoresed again along with the environmental sample in order to reconfirm the position of the bands. The eluted products were also amplified using PCR primers without GC clamp. The PCR products after the assessment of quality and quantity by agarose gel (1.5%) electrophoresis were cloned using TOPO TA Cloning kit (Invitrogen, USA). Five positive clones were selected at random and their plasmid DNA was extracted using Qiagen Miniprep Kit (Qiagen, USA), following manufacturer's instructions. The PCR products of the plasmid inserts were further sequenced (Bioserve Biotechnologies Pvt. Ltd., Hyderabad). The sequences obtained were edited using the Sequencher and also checked for chimeras using the Pintail Software (Ashelford *et al.*, 2005). The sequences were also compared with those in Ez Taxon Server. The sequences obtained were submitted in the GenBank (Accession Numbers: HQ992829-HQ992941).

Abbreviations:
KL-Kavaratti Lagoon side
KO-Kavaratti Oceanic side
DG PRE – *Dysidea granulosa* Premonsoon
DG MON - *Dysidea granulosa* Monsoon
DG POST- *Dysidea granulosa* Postmonsoon
SF PRE - *Sigmadocia fibulata* Premonsoon
SF MON - *Sigmadocia fibulata* Monsoon
SF POST- *Sigmadocia fibulata* Postmonsoon

RESULTS

DGGE band profile analysis

The DGGE profiles obtained for the sponge samples are shown in Fig. 3.

The 26 prominent bands which were excised and sequenced represented the structure of the most numerically dominant bacterial population and their identity is given in the Table 1.

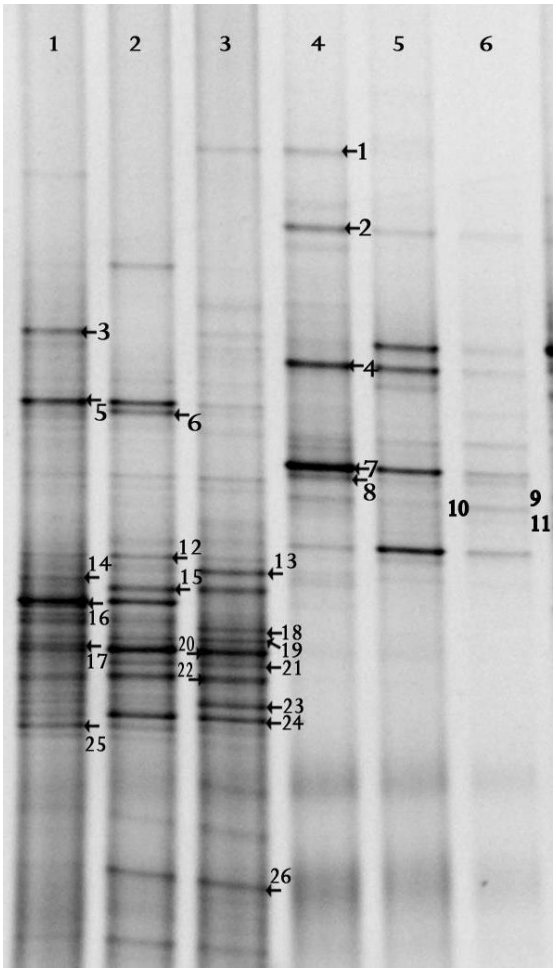


Fig. 3. DGGE profiles 16S rRNA gene fragments from *D. granulosa* (DG) and *S. fibulata* (SF). Lanes 1–3 (DG Postmonsoon, Premonsoon, Monsoon); Lanes 4–6 (SF- Postmonsoon, Premonsoon, Monsoon). Excised bands are numbered in the DGGE gel.

Sequencing analysis showed that sequence similarity of the excised bands ranged from 82–100% to that of the nearest culturable bacteria in the Ez Taxon Server. About 0.7% of the sequenced bands were closely related (>98% similarity) to the sequences deposited in the Ez Taxon Server while the remaining exhibited lower sequence similarity (<97% similarity). The sequences belonged to various bacterial phyla such as Acidobacteria, Actinobacteria, Chloroflexi, Cyanobacteria, Firmicutes, Proteobacteria, Spirochaetes and Thermotogae. In Proteobacteria, Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria were obtained.

Table 1

Identity of excised and sequenced DGGE bands from BLAST search in GenBank.

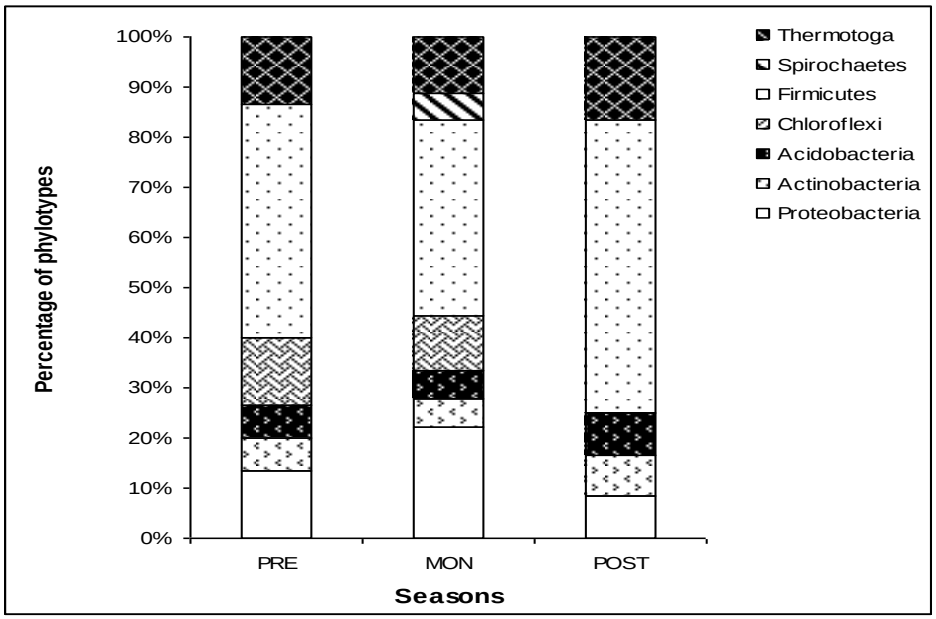
Band Id	Acc. Number	Nearest neighbour	SIM (%)	Affiliation
Band 1	HQ992829	*Rhizobium* sp.	96.8	Alphaproteobacteria
Band 2	HQ992830	*Prochlorococccus marinus*	97.8	Cyanobacteria
Band 3	HQ992831	*Halochromatium salexigens*	90.6	Gammaproteobacteri a
Band 4	HQ992833	*Sporanaerobacter acetigenes*	83.0	Firmicutes
Band 5	HQ992839	*Fervidobacterium pennivorans*	89.1	Thermotogae
Band 6	HQ992845	*Clostridium tepidiprofundi*	83.0	Firmicutes
Band 7	HQ992870	*Rubrobacter xylanophilus*	89.8	Actinobacteria
Band 8	HQ992873	*Micropruina glycogenica*	88.1	Actinobacteria
Band 9	HQ992878	*Desulfovibrio aminophilus*	88.3	Deltaproteobacteria
Band 10	HQ992882	*Spirochaeta americana*	86.0	Spirochaetes
Band 11	HQ992884	*Dehalogenimonas lykanthroporepellens*	90.4	Chloroflexi
Band 12	HQ992889	*Holophaga foetida*	86.9	Acidobacteria
Band 13	HQ992895	*Ruegeria lacuscaerulensis*	100.0	Alphaproteobacteria
Band 14	HQ992899	*Clostridium clariflavum*	87.4	Firmicutes
Band 15	HQ992906	*Euzebya tangerina*	91.2	Actinobacteria
Band 16	HQ992907	*Hippea maritima*	83.1	Deltaproteobacteria
Band 17	HQ992910	*Caminibacter mediatlanticus*	88.9	Epsilonproteobacteria
Band 18	HQ992915	*Dethiosulfatibacter aminovorans*	83.0	Firmicutes
Band 19	HQ992918	*Desulfotomaculum aeronauticum*	88.2	Firmicutes
Band 20	HQ992923	*Caldilinea aerophila*	87.3	Chloroflexi
Band 21	HQ992928	*Sporanaerobacter acetigenes*	83.0	Firmicutes
Band 22	HQ992932	*Fervidobacterium pennivorans*	89.1	Thermotogae
Band 23	HQ992935	*Peptoniphilus gorbachii*	84.4	Firmicutes
Band 24	HQ992941	*Sporanaerobacter acetigenes*	83.7	Firmicutes
Band 25	HQ992935	*Peptoniphilus gorbachii*	84.4	Firmicutes
Band 26	HQ992941	*Sporanaerobacter acetigenes*	83.7	Firmicutes

Temporal variation

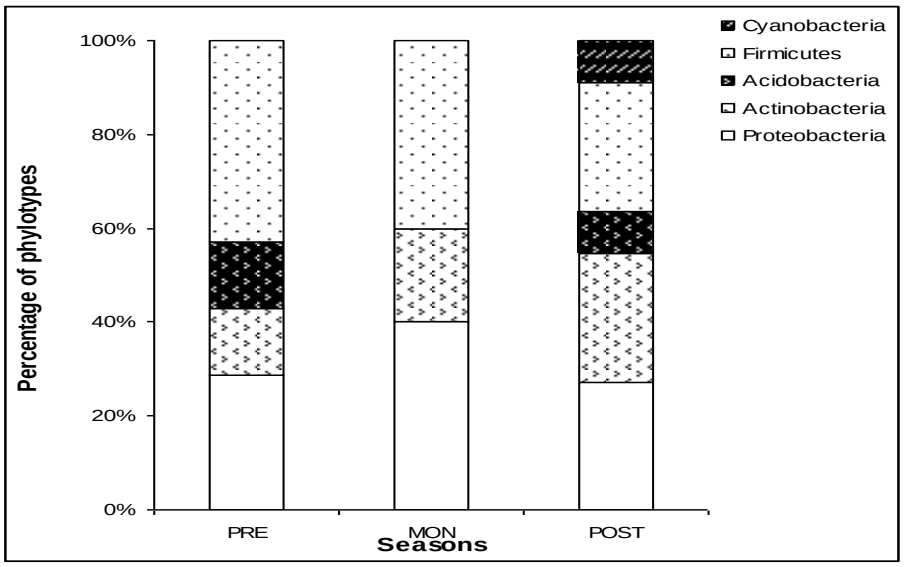
Of the sequenced bands in *D. granulosa*, the maximum number of phylogenetic groups (7 groups) was obtained during the monsoon period. The different groups obtained were Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Firmicutes,

Spirochetes and Thermotogae. This was followed by the premonsoon with six phylogenetic groups and postmonsoon with five phylogenetic groups. In the premonsoon, all the above groups were obtained except Spirochetes. However, in the postmonsoon, Chloroflexi and Thermatogae phyla were absent. Firmicutes was the major group in all the seasons with 58% of the sequenced bands in postmonsoon, 38% in monsoon and 46% in premonsoon. In the monsoon and postmonsoon this was followed by Proteobacteria (22%) and Thermatogae (16%), respectively. Cyanobacteria was completely absent during all the seasons. The percentage of phylogenetic groups during the three seasons in *D. granulosa* is given in Fig. 4 (a).

In *S. fibulata*, the maximum number of phylogenetic groups (five groups) was observed during postmonsoon. The groups were Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes and Cyanobacteria. However, in the premonsoon four phylogenetic groups were obtained namely, Proteobacteria, Actinobacteria, Acidobacteria and Firmicutes. Monsoon had the lowest number of groups (3 groups) which were Proteobacteria, Actinobacteria and Spirochaetes. In the premonsoon, about 42% of the sequenced bands were Firmicutes which formed a majority. However, in monsoon, in addition to Firmicutes, Proteobacteria also formed a major group (40% each). In postmonsoon, three groups were obtained such as Firmicutes, Proteobacteria and Actinobacteria (27% each). Cyanobacteria (9%) were present only in the postmonsoon. Fig. 4 (b) shows the percentage of phylogenetic groups during the three seasons in *S. fibulata.*



a)



b)

Fig. 4. Percentage of phylogenetic groups in three seasons in (a) *D. granulosa* and (b) *S. fibulata*.

A high bacterial diversity in *D. granulosa* and *S. fibulata* was identified (Table 2).

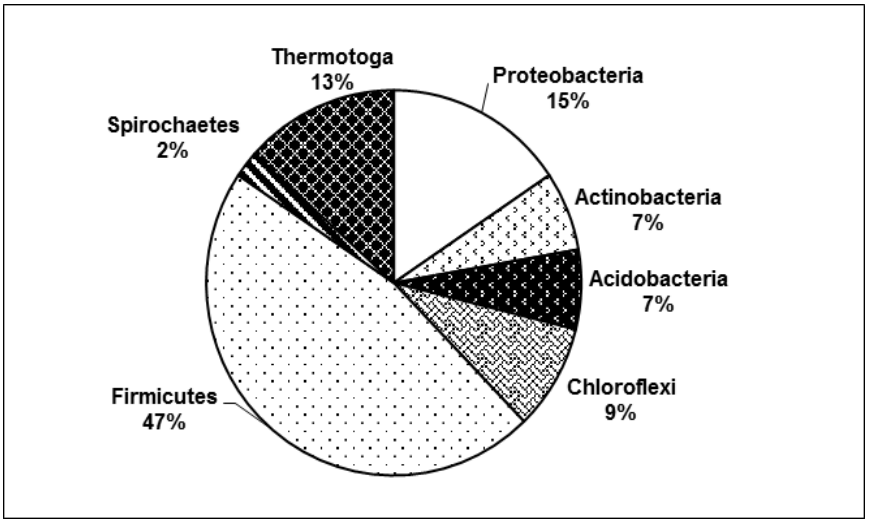
Table 2

Diversity indices of two sponges in varied time

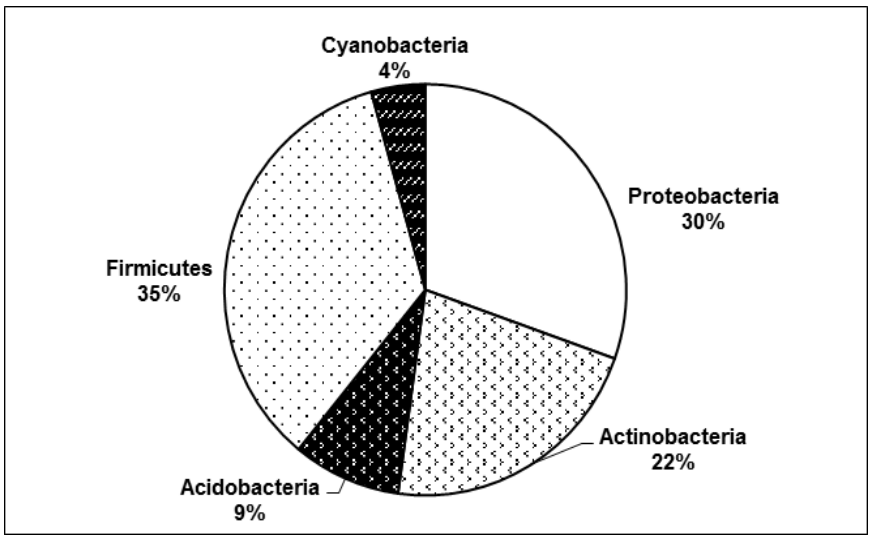
Samples	Abundance	Richness	Evenness	Brillouin	Fisher	Shannon	Simpson
DG PRE	26.0	2971.5	3.1	3.2	3.9	3.2	1.0
DG MON	26.0	3173.2	3.1	3.1	3.9	3.2	1.0
DG POST	26.0	2901.6	3.1	3.2	3.9	3.2	1.0
SF PRE	15.0	1463.8	1.9	2.6	2.3	2.6	0.9
SF MON	16.0	684.5	2.3	2.7	2.9	2.7	0.9
SF POST	17.0	1576.7	2.2	2.7	2.7	2.7	0.9

Seven phylogenetic groups were obtained in *D. granulosa*, while only five groups were obtained in *S. fibulata*. The major group obtained in the two sponges was Firmicutes (46% and 34% respectively, for *D. granulosa* and *S. fibulata*). The 16S rRNA gene based DGGE fingerprinting provided a general insight into the bacterial community of the sponges temporally.

nMDS plotting of DGGE band profiles of the sponge *D. granulosa* in premonsoon and postmonsoon (DG PRE AND DG POST) clustered at 64.5% of similarity. Monsoon sample (DG MON) of *D. granulosa* showed similarity to non-monsoon and clustered at 46.8% (Fig. 5).



(a)



(b)

Fig. 5. Percentage of phylogenetic groups in (a) *D. granulosa* and (b) *S. fibulata* irrespective of seasons.

SF PRE and SF POST clustered at 58%. SF MON has 34.9% similarity with non-monsoon cluster of *S. fibulata.*

DISCUSSION

Bandprofile analysis

The abundance of bacteria in the sponge samples were evaluated by number of DGGE bands. The average number of bands obtained in *D. granulosa* was 26 while that of *S. fibulata* was 16. *D. granulosa* had a higher number of phylotypes than *S. fibulata*. A definite difference in the banding pattern itself was obtained for the two sponge species as can be observed from the gel image, though temporally this difference was not very obvious. This suggests the presence of sponge-specific bacterial strains. The number of bands obtained in this study was consistent with the other studies carried out. One such study is by where five different sponge genera were collected from San Juan Island (Lee *et al.*, 2009). The number of bands obtained from the sponge samples ranged from 5 -15 which is much lower than that of what has been obtained in this study. Studies conducted by Wichels *et al.* (2006) in *Halichondraia panacea* on the diversity associated with the aquiferous system and tissue showed that banding patterns differed in the two. Distinct banding patterns were also observed for three sponges (*Ircinia felix, Aplysina cauliformis, Niphates erecta*) collected from two different sites in Key Largo, one a inshore patch reef and the other in outer reef tract (Weisz *et al.*, 2007).

There was no variation in the number of bands during the three seasons for *D. granulosa*, but the positions and intensities were different. This indicates that there may not be much variation in composition, but the abundance and dominant groups may vary. In *S. fibulata*, temporal variation in the number of bands was observed during the three seasons. Such variation in bacterial community structures has been documented in the DGGE profile analysis of two sponges, *Hyrtios erectus* and *Amphimedon* sp. from the Red Sea and showed that the bacterial communities associated with the two sponges had different banding patterns from each other (Radwan *et al.*, 2010).

Taxonomic diversity

In this study, diverse bacterial communities of the sponges were observed in the two sponges of *D. granulosa* and *S. fibulata*. The common bacterial groups encountered in both the sponges were Proteobacteria, Firmicutes, Acidobacteria and Actinobacteria. Earlier surveys of sponge-associated bacteria from the Mediterranean Sea, the Red Sea, the Sea of Japan, and the Pacific reported 14 different monophyletic sponge-specific bacterial clusters belonging to seven bacterial divisions (Hentschel *et al.*, 2003). A few of the sponge-associated bacteria reported have been Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria,

Acidobacteria, Actinobacteria, Nitrospira, Bacteroidetes, Cytophaga/Flavobacterium, Chloroflexi, Chlorobi and Planctomycetes (Webster *et al.*, 2001; Hooper & Van Soest, 2002; Souza *et al.*, 2017; Li *et al.*, 2006a; Cuvelier *et al.*, 2014; Glasl *et al.*, 2018). The novel sponge - specific group, Poribacteria, which has a large degree of homology (75%) with other phylogenetic groups of bacteria (Fieseler *et al.*, 2004) was not detected in the present study. Interestingly, members of phylum Cyanobacteria, Spirochaetes, Thermatogae and Chloroflexi were associated with either one of the sponges. DGGE-based community fingerprinting studies showed the existence of highly stable bacterial communities in the demosponges, *Callyspongia* sp., *Cymbastela concentrica* and *Stylinos* sp. and minimal timescale variability was detected for *C. concentric* (Taylor *et al.*, 2004). Also, studies on the variation in bacterial diversity of distantly related sponges such as *Theonella swinhoei* and *Aplysina aerophoba* from western Pacific and Mediterranean respectively, suggested a high degree of uniformity among sponge-associated microbial communities (Hentschel *et al.*, 2003). The result of the present work was also consistent with the findings from other sponge species (Li *et al.*, 1998; Li & Liu, 2006b; Thiel *et al.*, 2007; Meyer & Kuever, 2008; Turque *et al.*, 2008; Noyer *et al.*, 2014; Bibi *et al.*, 2020). Taylor *et al.* (2007) reported 16 bacterial phyla and showed that sponge associated microbial community contains a mixture of generalist and specialist microorganisms and that these microbial communities were generally stable in both space and time.

Proteobacteria and all classes of the phylum such as Alpha, Gamma, Delta and Epsilon were present in this study as elsewhere (Friedrich *et al.*, 2001; Webster *et al.*, 2001, 2004; Jasmin *et al.*, 2015). Firmicutes formed the dominant group in both the sponges under investigation, *D. granulosa* and *S. fibulata*. The presence of this group has been reported in sponges such as *Craniella australiensis*and other sponges (He *et al.*, 2006; Jeong *et al.*, 2015). Acidobacteria was present in the two sponges. The presence of this group has been reported elsewhere (Larkum *et al.*, 1988; Weisz *et al.*, 2007; Noyer *et al.*, 2014). However, the significance of this group in the sponge-bacterial association is yet to be investigated.

Temporal variation

DGGE analysis indicated that the sponge associated bacterial community appeared to be relatively similar with respect to several bacterial groupsin *D. granulosa* and *S. fibulata* temporally. Bacterial groups such as Firmicutes, Actinobacteria, Proteobacteria were the robust ones observed in all the seasons in the two demosponges. Acidobacteria was almost present throughout the seasons in the two sponges but was completely not detected during monsoon in *S. fibulata*. The only exceptions were with bacterial groups Thermatoga, Spirochetes and Cyanobacteria which showed some kind of specificity to the organisms and the period of occurrence. Earlier, temporal variability in marine sponges had been examined for field based (Taylor *et al.*, 2004) and aquarium based (Friedrich *et al.*, 2001) populations of

sponges. It has been reported that in natural populations of three sponges from southeastern Australia, temporal variability was minor (Taylor *et al.*, 2004). Hentschel *et al.* (2003) affirmed that sponge-associated microbial communities are stable within individuals through time and it can be conservatively stated that specific subsets of the overall community occurred consistently within the same sponge species from different locations.

Sponge-specific groups

Micropruina belonging to Actinobacteria was found to be only associated with *S. fibulata* and not detected in *D. granulosa*. This association was exclusively temporal as it was detected during the monsoon. Similarly, the Cyanobacteria, *Prochlorococcus* sp. has been encountered only in the sponge *S. fibulata* and has also has been reported to be associated with sponges collected from various locations as Caribbean, Mediterranean and Indian Ocean (Steindler *et al.*, 2005; Erwin & Thacker, 2008). However, Chloroflexi was associated with *D. granulosa* and was not found in *S. fibulata*. Chloroflexi which are anoxygenic photosynthetic bacteria were present in the *D. granulosa*. Such association of Chloroflexi has been reported (Schmitt *et al.*, 2011). Spirochaetes also was associated with *D. granulosa*. Spirochaetes are regularly been identified with the sponges, however no information is available on the specificity of this association (Hill *et al.*, 2006; Taylor *et al.*, 2007; Isaacs *et al.*, 2009). Also, Thermotoga which are chemoorganotrophs, was also found to be specific to one of the sponges *D. granulosa*. There are no reports of the association of this group with the sponges and should be further investigated.

CONCLUSIONS

The present study showed that the demosponges of Lakshadweep harbor highbacterial diversity belonging to eight phyla. A significant finding was the presence of certain stable bacterial groups widely associated in the two sponges across various seasons and thought to play an important role in the metabolic activities of sponges. A few sponge specific groups such as, Thermatoga, Spirochetes, Chloroflexi, and Cyanobacteria, were also obtained. However, further investigations at the cellular and molecular levels are necessary to get a thorough understanding of the relationship between sponges and bacteria.

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