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Analysis of Bacterial Diversity in Sponges Collected off Chujado, an Island in Korea, Using Barcoded 454 Pyrosequencing: Analysis of a Distinctive Sponge Group Containing *Chloroflexi*

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The bacterial diversity of 14 sponges belonging to 5 different orders that were collected around Chuja Island, Korea was investigated using barcoded 454 pyrosequencing. The sponges contained many unidentified bacterial groups (e.g. more than half of the taxa at the family level) that were known only in environmental sequences and obtained from culture-independent methods. Five of the sponges were clustered into one notable group (CF group), which was distinguished from the other sponges in accordance with bacterial composition (the other sponges may be separated into more groups but clustering is not clear). The CF group contained high amounts of *Chloroflexi* (25.0–47.7%) and moderate amounts of *Gemmatimonadetes* (2.3–7.0%), AncK6 (0.6–2.2%), PAUC34f (0.8–6.0%), *Acidobacteria* (3.7–9.6%), and SBR1093 (1.8–5.6%) exclusively or almost exclusively to this group. Sponges in the CF group also showed higher diversity (e.g. Shannon index) than the other sponges and contained group-specific taxonomic lineages (e.g. class or family level) from group-specific phyla and even from the *Proteobacteria* and *Actinobacteria,* which were detected in all sponges at the phylum level. The CF group may be one of the most distinctive groups in sponges in terms of bacterial diversity.

Keywords: sponge, bacterial diversity, 454 pyrosequencing, Chujado, *Chloroflexi* group

Introduction

Marine sponges have been an interesting target for microbiologists as they have highly abundant and diverse microbial communities and are a rich source of novel products of biotechnological and pharmaceutical usage such as antibiotic, antiviral, anticancer, and anti-inflammatory agents (Taylor *et al*., 2007). Various kinds of novel natural chemicals have been characterized from sponges and their associated microorganisms (Blunt *et al*., 2013). Microorganisms in some sponges make up as much as 50% of the total biomass (Santavy *et al*., 1990) and have been counted at more than 10^9 cells/ml of sponge tissue (Hoffmann *et al*., 2005). Microorganisms associated with sponges have roles such as food (Pile *et al*., 1996), parasites (Bavestrello *et al*., 2000), or mutualistic symbionts (Wilkinson, 1983).

Microorganisms are important residents of sponges because they can mediate various metabolisms such as photosynthesis, methane oxidation, nitrogen gas fixation, nitrification, sulfate reduction, and reductive dehalogenation that affect nutrient cycles in sponges (Taylor *et al*., 2007). Sponge-associated microbes have been isolated and identified (Wilkinson *et al*., 1981; Webster *et al*., 2011), but many of the residents of sponges have not been isolated or identified through cultivation because it has not been possible to cultivate most of the microorganisms found in this environment (Amann *et al*., 1995). Many unknown microbes and unexpected diversities in sponges have been revealed with culture-independent methods based on 16S rRNA gene PCR (Sfanos *et al*., 2005; Hill *et al*., 2006). Recently, the development of massively parallel 454 pyrosequencing, a next generation sequencing method, has reduced the sequencing cost dramatically (Margulies *et al*., 2005).

The pyrosequencing method has become a promising method in microbial ecology for the investigation of microbial diversity in various ecosystems (Edwards *et al*., 2006; Roesch *et al*., 2007; Kim *et al*., 2008). Tagged or barcoded pyrosequencing was developed to analyze dozens of samples at the same time using sample-specific tags or barcodes composed of oligonucleotides (Binladen *et al*., 2007; Hoffmann *et al*., 2007; Parameswaran *et al*., 2007; Roh *et al*., 2010). The pyrosequencing method has been applied to reveal microbial diversity in marine sponges with highly diverse and species-specific sponge-associated communities identified in sponges from the Red Sea (Lee *et al*., 2011), sponge symbionts in vertical or horizontal modes of transmission identified by pyrosequencing (Webster *et al*., 2010), and descriptions provided of core, variable, and species-specific bacterial members in sponges (Schmitt *et al*., 2012). Also, seasonal variation of bacterial communities was investigated in *Axinella corrugata* sponges using barcoded pyrosequencing (White *et al*., 2012); however, many more sponges remain to be investigated considering that more than 6,000 species of sponges have been described (Hooper and Soest, 2002). In this study, 14 sponges were used to investigate bacterial diversity using

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barcoded 454 pyrosequencing.

Materials and Methods

Sample collection and DNA extraction

Sponge specimens (n=14) were collected from the shallow seafloor by scuba diving and from the intertidal region in November 2009 near Chujado (Chuja Island), which is about 40 km from Jeju Island (Table 1). Specimens were collected aseptically and transported to the laboratory. Sponge tissues were cut into small pieces (about 1 cm^3) and washed with sterilized seawater. The tissues were frozen at -70°C for 24 h and freeze-dried at -50°C, 0.033 Mbar for 24 h. The lyophilized tissues were homogenized in sterilized mortar. DNA was extracted using G-spin™ Genomic DNA Extraction kit (Intron, Korea).

DNA extraction and barcoded pyrosequencing

The region from V1 to V3 of 16S rRNA gene was amplified using primer sets (V1-9F: 5′-X-AC-GAGTTTGATCMTG GCTCAG-3′ and V3-541R: 5′-X-AC-WTTACCGCGGCT GCTGG-3′), in which X indicates the different oligomers comprised of six nucleotides to tag different samples for barcoded pyrosequencing. Pyrosequencing was performed by a sequencing vendor (Macrogen, Korea) using the system of Genome Sequencer FLX titanium (Roche, Germany) according to the manufacturer's manuals.

Analysis of reads

After sorting according to barcode primer, primer and barcode were removed from the reads. Sequences more than 300 bp in length and with no ambiguous bases, 'N', were selected for further analysis. Potential chimera sequences were checked using the chimer.uchime command in the mothur package (Schloss *et al*., 2009) and on average 0.6% of sequences were discarded as potential chimera sequences. After alignment of the remaining sequences with the silva.gold databases, those showing coverage less than 60% were discarded.

Clustering and taxonomic assignment were carried out using the QIIME package v1.7.0 (Kuczynski *et al*., 2012). Qualified sequences were merged into one file and clustered using the uclust method in the QIIME package based on 97% of similarity. OTUs and representative sequences were formed for each sample. OTUs containing only one read were discarded. Representative sequences were used for taxonomic assignment with the RDP classifier against 97_otus files and 97_otu_taxonomy from the gg_13_05 version of the Greengenes database (McDonald *et al*., 2012). Subsampling and alpha diversity indices calculation such as Chao, Shannon, and Simpson indices was performed with the QIIME package. The Mann-Whitney test was performed to compare the diversity indices of two groups (Mann and Whitney, 1947).

Representative sequences were picked from each OTU group and aligned with the PyNAST program (Caporaso *et al*., 2010). A phylogenetic tree was constructed using the FastTree program (Liu *et al*., 2011). A beta diversity comparison was performed using the Unifrac method with the phylogenetic tree (Lozupone *et al*., 2011). Some reads were found to be related to chloroplasts (0–9.5% of total reads) and mitochondria (0–20.6%) and were removed from the analysis as unwanted sequences should be removed before diversity estimation to prevent any bias in case the number of such sequences is significant. In this analysis, a small change of the Unifrac UPGMA tree topology was observed after removal of the chloroplast-related reads (data not shown).

Results and Discussion

Overall, 14 sponges were collected from 5 different sites in the intertidal region (0 m) and shallow seafloor (14–29 m in depth) around Chuja Island and used to investigate bacterial diversity (Table 1). The sponges belong to 13 different species, 11 different genera, 10 different families, and 5 dif-

Table 1. Description of samples of sponges

No	Sample ID	Sponge species	Taxonomy (Class; Order; Family)	Site[a]	Depth (m)
1	Acvu1	*Acanthella vulgata*	*Demospongiae; Halichondrida; Dictyonellidae*	A	18–19
2	Assi1	*Asteropus simplex*	*Demospongiae; Astrophorida; Ancorinidae*	B	14–17
3	Assp1	*Asteropus* sp.	*Demospongiae; Astrophorida; Ancorinidae*	B	18–24
4	Caaw1	*Caminus awashimensis*	*Demospongiae; Astrophorida; Geodiidae*	A	18–19
5	Camo1	*Callyspongia mookriensis*	*Demospongiae; Haplosclerida; Callyspongiidae*	C	0
6	Hapa1	*Halichondria panicea*	*Demospongiae; Halichondrida; Halichondriidae*	C	0
7	Hapa2	*Halichondria panicea*	*Demospongiae; Halichondrida; Halichondriidae*	C	0
8	Hape1	*Haliclona permollis*	*Demospongiae; Haplosclerida; Chalinidae*	C	0
9	Hyfl1	*Hymeniacidon flavia*	*Demospongiae; Halichondrida; Halichondriidae*	C	0
10	Hysi1	*Hymeniacidon sinapium*	*Demospongiae; Halichondrida; Halichondriidae*	C	0
11	Opsp1	*Ophlitaspongia* sp.	*Demospongiae; Poecilosclerida; Microcionidae*	C	0
12	Pein1	*Penares incrustans*	*Demospongiae; Astrophorida; Geodiidae*	D	20
13	Rahi1	*Raspailia hirsuta*	*Demospongiae; Poecilosclerida; Raspailiidae*	D	20
14	Spfa1	Family *Spongiidae*	*Demospongiae; Dictyoceratida; Spongiidae*	E	20

[a] Latitude and longitude of each site are; A, 33.9832733, 126.2959620; B, 33.8670804, 126.3132397; C, 126.3303845, 33.9522348; D, 126.2488228, 33.9876219; E, 126.2888701, 33.96059.

ferent orders. One sponge specimen, Spfa1, was identified up to only the family level and two sponge specimens, Assp1 and Opsp1, up to only the genus level.

Clustering of sponges according to bacterial diversity: emergence of CF group

From the results, a total 64,629 sequences (3,237–7,061 reads per sample) were used for diversity and taxonomic analysis. According to the weighted Unifrac UPGMA tree in Fig. 1A, the bacterial communities of the sponges can be differentiated into one distinct group and the other members. The group (designated CF group because its members contain *Chloroflexi* as the major phylum) was composed of Assp1, Assi1, Caaw1, Pein1, and Spfa1. Other sponges (non-CF group) could be divided into 2–4 groups, but this clustering is not as clear as the clustering of the CF group.

Diversity indices were calculated from the reads subsampled from each sample (3,000 reads per sample) as shown in Table 2. Diversity in the CF group is higher than for the others in this study (Fig. 1B) based on the number of OTUs and estimated OTUs from the Chao method and Shannon indices. Especially, the Shannon index difference is highly significant (P=0.0010) from statistical analysis with the Mann-Whitney test (Fig. 1B, right panel). Also the curve with cumulative relative abundance versus rank showed a different population composition for the CF group compared with the others (Fig. 1C) as in the other samples sharp increases of cumulative abundance in the first few OTUs were observed owing to the high proportion of those OTUs, whereas in the CF group no initial sharp increase was observed because of an absence of a high proportion of any OTUs. In addition, the curves of the CF group were not saturated as did those of the other samples. The Unifrac UPGMA tree, diversity indices, and cumulative relative abundance curves show that the CF group is clearly distinguished from the other sponges in terms of bacterial composition.

Comparison of bacterial composition of sponges between CF and non-CF group

The bacterial composition of the CF group sponges showed several different characteristics from that of the other sponges at the phylum level (Fig. 2). A high proportion (25.0–47.7%) of the bacteria in the CF group sponges were from the phylum *Chloroflexi*, while the proportion of this phylum detected in the other sponges was lower than 0.8%. Also the phylum *Gemmatimonadetes* was contained in a smaller proportion (2.3–7.0%), but this was significantly greater than the proportion detected in the other sponges (less than 0.3%). The phylum AncK6 (0.6–2.2%) was detected only in the CF group. PAUC34f (0.8–6.0%) was detected in all members in the CF group, but not in the other sponges (0.0–0.1%). The phylum *Nitrospirae* (0.9–7.0%) was detected in the CF group as well as in the Acvu1 and Rahi1 samples. The phyla *Acidobacteria* (3.7–9.6%) and SBR1093 (1.8–5.6%) were detected in the CF group and in the Acvu1 sample (less than 0.4% in the other samples). The number of phyla (more than 1% of the total composition) in the CF group (10–12 phyla) is significantly larger than that in the other samples (4–7 phyla).

Some candidate phyla were detected in this study. The PAUC34f group originated from a sequence PAUC34f from the sponge Theonella swinhoei (Hentschel *et al.*, 2002) and the AncK6 group from the sponge Ancorina alata (Kamke *et al.*, 2010). The two groups were related to each other in the phylogenetic tree (Kamke *et al.*, 2010). SBR1093 represents a candidate phylum originally reported from marine basalts (Mason *et al.*, 2009). *Poribacteria* is a candidate phylum first discovered from sponges and is known as a sponge-specific phylum that is abundant and widespread in sponges (Fieseler *et al.*, 2004; Lafi *et al.*, 2009); however, unexpectedly, no *Poribacteria* was detected in this study. The bioinformatics pipeline used in this study could detect the reads of *Poribacteria* in a positive control that contained the sequences related to the *Poribacteria* group. It is noteworthy that some sponges in the orders *Halichondrida*, *Dictyoceratida*, *Haplosclerida*, and *Poecilosclerida* were reported not to contain *Poribacteria* (Lafi *et al.*, 2009), and these four sponge orders are among the five orders found in this study. From the other order found in this study, *Astrophorida*, the species Rhabdastrella globostellata is known to contain *Poribacteria* (Lafi *et al.*, 2009), whereas the sponges from this

Table 2. Diversity estimators calculated using same number of sequences from each sample

Group	Sample	Total reads	Total OTUs	Reads subsampled[a]	OTUs	Chao	Shannon	Simpson
Others	Hyfl1	5280	572	3000	537	674	7.1	0.977
	Hape1	5490	555	3000	456	685	5.95	0.932
	Opsp1	4845	486	3000	407	608	6.71	0.976
	Hapa1	4922	361	3000	308	459	5.53	0.941
	Hysi1	4002	487	3000	455	638	6.66	0.97
	Hapa2	3380	233	3000	225	275	4.47	0.791
	Camo1	3237	232	3000	228	306	5.02	0.917
	Rahi1	4946	298	3000	253	354	5.85	0.959
	Acvu1	3982	537	3000	340	355	6.44	0.964
CF	Spfa1	5535	347	3000	376	546	7.3	0.989
	Caaw1	7062	459	3000	506	750	7.35	0.986
	Pein1	4995	676	3000	446	656	7.62	0.991
	Assi1	4669	533	3000	577	703	8.19	0.994
	Assp1	3926	623	3000	508	637	7.97	0.993

[a] 3000 reads were subsampled from each sample and used to calculate diversity estimators.

order found in this study, *Asteropus simplex*, *Caminus awashimensis*, and *Penares incrustans*, did not contain *Poribacteria*.

The Acvu1 sample showed similarities with the CF group as it contains the phyla *Nitrospirae*, *Acidobacteria*, and SBR1093, but it does not contain the phyla specific for the CF group such as *Chloroflexi*, *Gemmatimonadetes*, and AncK6. It has a similar composition in some phyla with other members of the non-CF group; however, it appears that Acvu1 represents a group separate from the other sponges in both the

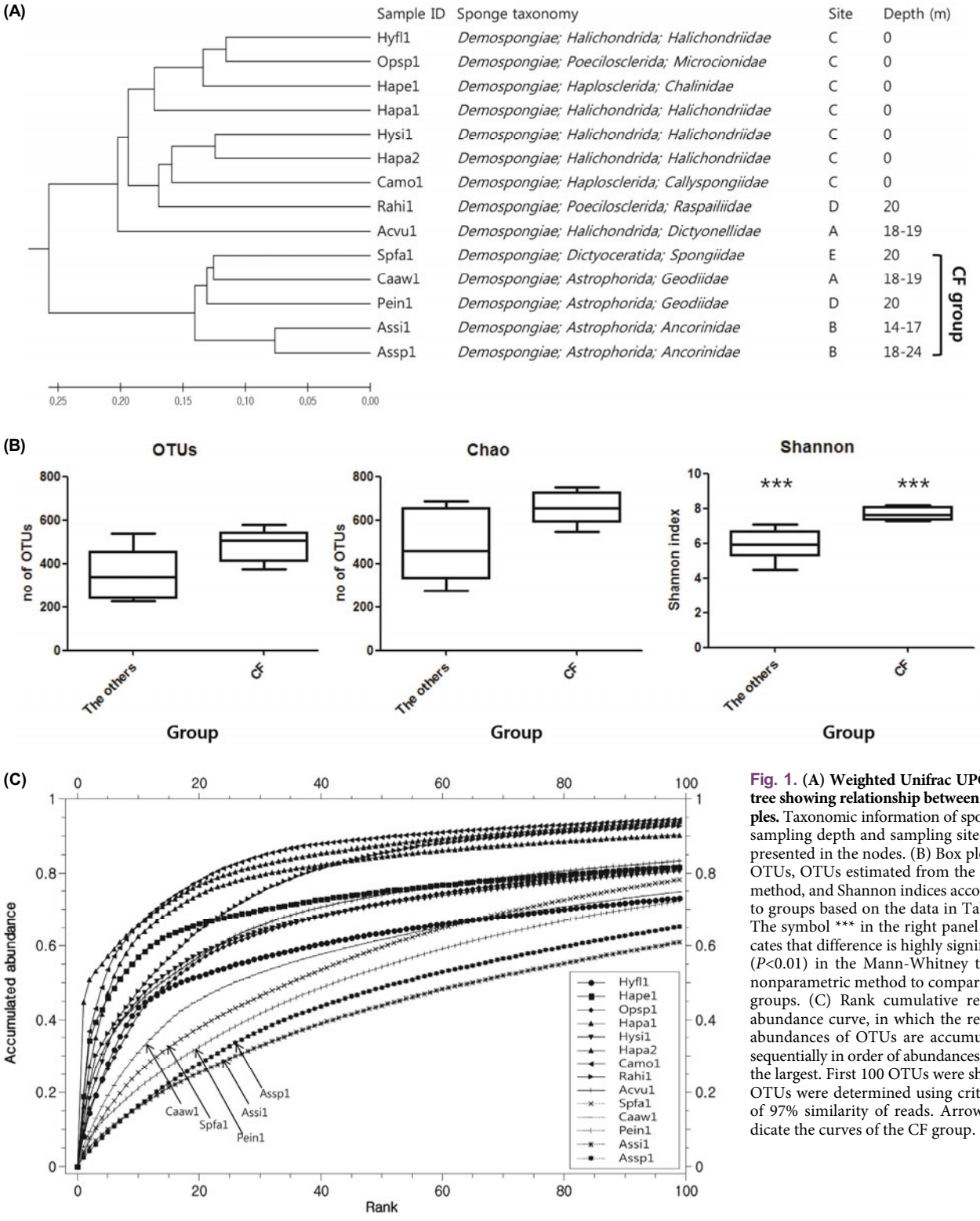

Fig. 1. (A) Weighted Unifrac UPGMA tree showing relationship between samples. Taxonomic information of sponges, sampling depth and sampling site were presented in the nodes. (B) Box plots of OTUs, OTUs estimated from the Chao method, and Shannon indices according to groups based on the data in Table 1. The symbol *** in the right panel indicates that difference is highly significant (P<0.01) in the Mann-Whitney test, a nonparametric method to compare two groups. (C) Rank cumulative relative abundance curve, in which the relative abundances of OTUs are accumulated sequentially in order of abundances from the largest. First 100 OTUs were shown. OTUs were determined using criterion of 97% similarity of reads. Arrows indicate the curves of the CF group.

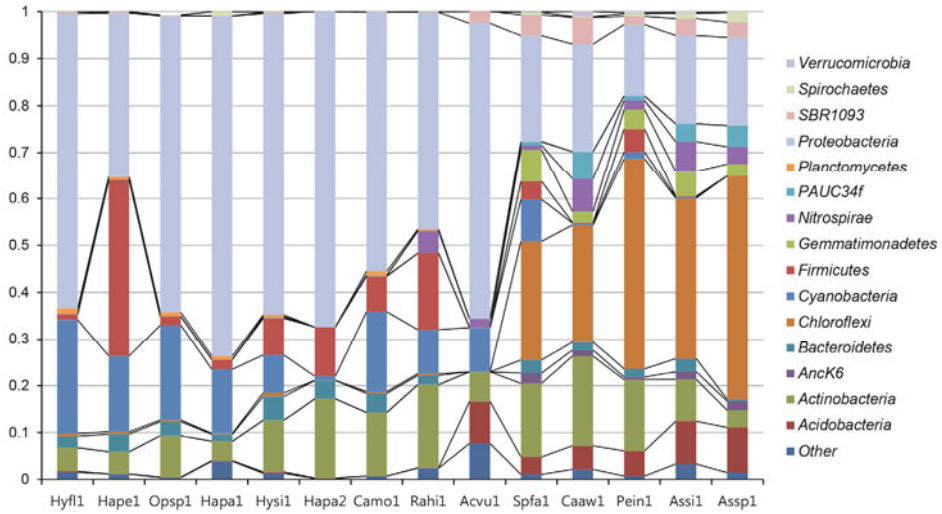

Fig. 2. Taxonomic classification of pyrosequencing reads in the phylum level. The order of samples was the same with that of the weighted Unifrac UPGMA tree shown in Fig. 1. Only phyla which have abundance more than 1% in at least one sample were shown in the figure. Summation of reads in phyla more than 1% was 96.8–99.9% among total reads.

CF and non-CF groups because it has the least common number of OTUs in comparison with other sponges in the level of OTUs (Fig. 4) and is distinguished from all the other sponges in the clustering tree (Fig. 1A).

The members in the non-CF group showed similar bacterial community patterns at the phylum level, that is, they contained high proportions of the phyla *Proteobacteria* (35.0–72.6%, average 58.9%) and *Cyanobacteria* (0.9–24.4%, average 13.8%) and no or a small proportion of the phyla, *Chloroflexi*, *Gemmatinomoadetes*, AncK6, and PAUC34f, which were observed in the CF group. It is interesting that the seven samples in the non-CF group were obtained from the surface area of the intertidal region, while members in the CF group were obtained from a deeper depth (14–29 m).

Nitrospirae, the phylum represented by the genus *Nitrospira*, a nitrite-oxidizing group, was detected from seven sponges, and it is interesting that all of the samples were collected from the subsurface (14–29 m), not from the surface water. The nitrifying ability of *Nitrospira* might be correlated with the depth of its habitat and further studies should be performed to determine whether or not this is an actual correlation.

A small proportion of *Spirochaetes* (0.2–1.9%) was observed in the CF group, while the other sponges also contained a small proportion (0.1–0.9%) or no sequences. Sequences from *Spirochaetes* have been detected frequently from sponges (Taylor *et al.*, 2007; Isaacs *et al.*, 2009) and many of them are known to be specific to sponges (Neulinger *et al.*, 2010; Simister *et al.*, 2012). It was reported that different genotypic and morphotypic spirochaetes could exist in the same sponges (Neulinger *et al.*, 2010), but little is known about the roles of the spirochaetes associated with sponges.

Differences in abundance at the family level for the samples are shown as heatmaps in Fig. 3. The columns of the left heatmap show normalized relative abundance and provide a comparison of the differences of abundance between different families in the same sample. This shows that high amounts from a few families occupied most of the reads in each sample. For most samples, the most predominant family was different, especially in the non-CF group, and could represent a taxon specific to each sponge. The rows of the right heatmap show normalized relative abundance and provide a comparison of the differences of abundance between different samples in the same family. As predicted from phylum level diversity, several families in CF group-specific phyla were observed only in the CF group. In addition, some families such as *Syntrophobacteraceae*, *Ectothiorhodospiraceae*, *Piscirickettsiaceae*, *'Entotheonellaceae'*, and the HTCC2089-related family in *Proteobacteria* were observed only in the CF group although *Proteobacteria* was observed ubiquitously in all sponges at the phylum level in Fig. 3. This observation shows that detailed classification to lower levels of taxonomic rank could reveal unidentified differences in microbial communities.

As shown in Fig. 3, many reads from sponges were related to unclassified groups that are known only by environmental sequences and not by cultivated strains and are unclassified in the various taxonomic positions such as class, order, and family. Taxonomic names such as c__, o__, f__, c__TK17, and c__Gemm-4 in the figure mean that they are not classified properly in each taxonomic rank and have no name or temporary names like TK17 or Gemm-4 (McDonald *et al.*, 2012). Forty one among 72 families represented in Fig. 3 were identified as unclassified families. To understand their roles in sponges, more research should be conducted to isolate the strains in unclassified taxonomic ranks. Recently, genomic sequences of uncultured strains of *Poribacteria* were obtained without cultivation and their functions were predicted with *in silico* analysis through culture-independent methods such as metagenomics (Fieseler *et al.*, 2006) and single cell genomics (Siegl *et al.*, 2011).

Relationship of CF group with other parameters

It is interesting that all 4 sponges in the order *Astrophorida* belonged to the CF group and not to the other sponges group. The other sponge in the CF group belongs to the order *Dictyoceratida*. Sponges in the order *Dictyoceratida* are known to show similarities to those in the order *Astrophorida* with regard to bacterial composition from comparison at the phylum level (Schmitt *et al.*, 2012). It was reported that similarities within the same order were not necessarily greater than similarities between different orders and that bacterial

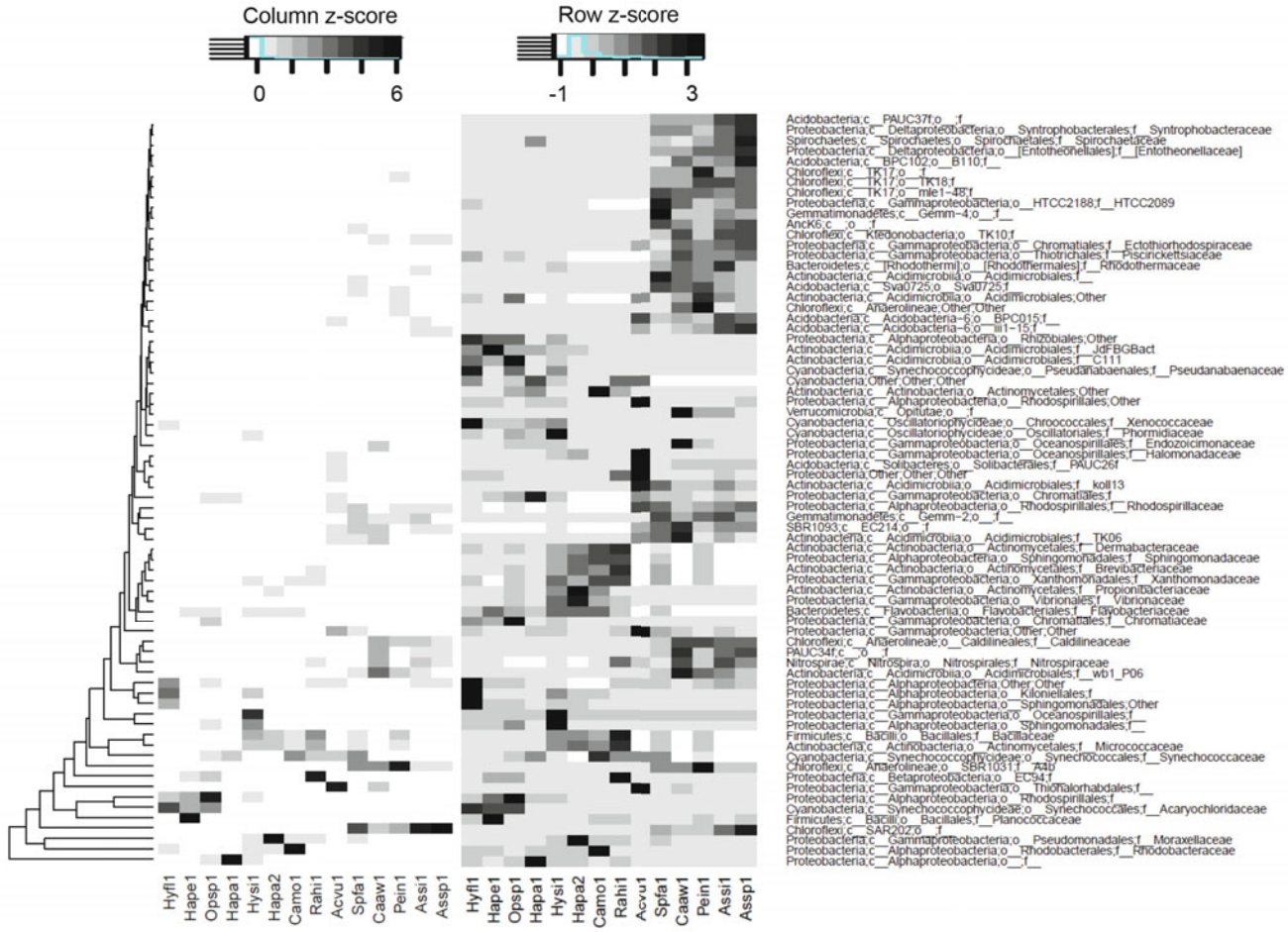

Fig. 3. Heatmaps in the family level. The left heatmap shows normalized relative abundance using the equation z = (value in each spot - average of values in each column)/(standard deviation of values in each column). The right heatmap shows normalized relative abundance using the equation z = (value in each spot - average of values in each row)/(standard deviation of values in each row). Only families which have abundance more than 0.1% in at least one sample were shown.

communities have no correlation with host taxonomy (Schmitt *et al*., 2012). The other sponges in the non-CF group also have no tendency to cluster in accordance with their order and the members from *Halichondrida*, *Haplosclerida*, and *Poecilosclerida* were not clustered into an exclusive group based on each order. The factor that all 5 sponges in the CF group were collected at the depth of 14–20 m may also give a partial clue to the differences in the bacterial profiles of the sponges (Fig. 1). Seven sponges among the other sponges were collected from an intertidal region close to the seawater surface (sampling depth is 0 m).

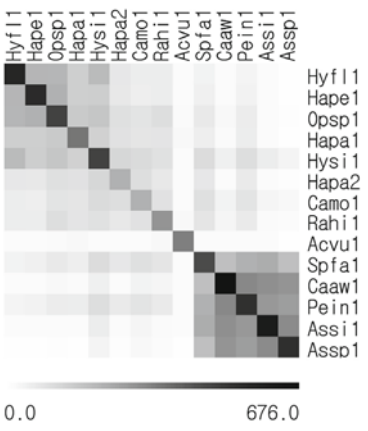

Fig. 4. Heatmap from pairwise matrix of shared OTU number. The number of OTUs shared by two sponges was indicated at each spot. Highest number of OTUs is 676 of the Pein1 sponge. OTUs were determined using criterion of 97% similarity of reads.

Interestingly, a relatively high amount of *Cyanobacteria* were observed in the group collected from the intertidal region, but this was not the case in the CF group except for Spfa1 (Fig. 2). Considering that *Cyanobacteria* need sun light for photosynthesis, the amount of *Cyanobacteria* is dependent on the depth and intensity of light. As all members of the CF group were collected from a depth of 14–20 m, there seems to be a correlation between the sample depth and clustering as the CF group; however, this explanation should be treated with caution because the number of samples and sample sites was insufficient. More samples, environmental parameters, or controlled experiments are needed to reveal any correlation between taxonomy and environmental parameters for bacterial communities.

Considering that more than 6,000 species of sponges have been identified so far (Hooper and Soest, 2002) and that many

sponges are known to contain unique microbial members (Schmitt *et al.*, 2012), the microbial diversity of sponges is potentially enormous and has not been revealed sufficiently yet. In this study, many unclassified bacteria were identified from 14 sponges with massive sequencing technology. In addition, a distinctive group of sponges, the CF group, was identified based on bacterial community diversity and differences in the bacterial composition of the sponges collected were discussed.

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