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Analysis of Bacterial Diversity in Sponges Collected from Chuuk and Kosrae Islands in Micronesia

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The bacteria resident in sponges collected from Chuuk Lagoon and Kosrae Island of Micronesia were investigated using the 16S rRNA gene PCR-tagged pyrosequencing method. These sponges were clustered into 5 groups based on their bacterial composition. Diversity indexes and cumulative rank abundance curves showed the different compositions of bacterial communities in the various groups of sponges. Reads related to the phylum *Chloroflexi* were observed predominantly (9.7–68.2%) in 9 sponges of 3 groups and unobserved in the other 2 groups. The *Chloroflexi*-containing group had similar bacterial patterns at the phylum and lower taxonomic levels, for example, significant proportions of *Acidobacteria*, *Gemmatimonadetes*, SBR1093, and PAUC34f were observed in most members of this group. The three groups in the *Chloroflexi*-containing group, however, showed some minor differences in the composition and diversity. The other two groups contained high proportions of *Proteobacteria* (>87%) or *Bacteroidetes* (>61%) and different composition and diversity compared to the *Chloroflexi*-containing group and each other. Four pairs of specimens with the same species showed similar bacterial profiles, but, the bacteria in sponges were highly specific at the individual level.

Keywords: bacterial diversity, Chuuk Lagoon, Kosrae Island, Micronesia, sponge, 454 pyrosequencing

Introduction

Marine sponges are a rich natural source of novel chemicals, which are synthesized from the sponges themselves and sponge-associated microorganisms (Webster *et al.*, 2004). These chemicals have been characterized as bioactive compounds with antimicrobial, antitumor, antiangiogenic, antiviral, and anticancer activities (Webster *et al.*, 2004). Sponges are known to contain a very high number (up to 10^9 cells/ml of sponge tissue) of bacterial residents (Hoffmann *et al.*, 2005) and the biomass with bacterial origin makes up to 50% of the biomass of sponges (Santavy *et al.*, 1990). Microorganisms in marine sponges have several roles such as food sources (Pile *et al.*, 1996), pathogens (Bavestrello *et al.*, 2000) or symbionts (Wilkinson, 1983). Stabilizing sponge skeleton, processing metabolic waste, and producing secondary metabolites are also roles of symbiotic microorganisms (Hentschel *et al.*, 2002). Microbes also mediate nutrient cycles such as carbon, nitrogen, and sulfur in sponge tissues (Taylor *et al.*, 2007).

Many studies have been performed to investigate bacterial diversity in sponges through culture-dependent (Wilkinson *et al.*, 1981; Webster *et al.*, 2011) as well as culture-independent approaches. Many unknown bacteria were detected from the PCR-cloning of 16S rRNA genes (Sfanos *et al.*, 2005; Hill *et al.*, 2006) and PCR-denaturing gradient gel electrophoresis (Webster *et al.*, 2004; Hardoim *et al.*, 2009). Massively parallel 454 pyrosequencing, a next generation sequencing method has been developed (Margulies *et al.*, 2005) and adapted to investigate microbial diversity in various ecosystems (Edwards *et al.*, 2006; Roesch *et al.*, 2007; Kim *et al.*, 2008). Dozens of samples can be analyzed at once and at reduced cost by being tagged or barcoded with specially-designed oligonucleotides (Binladen *et al.*, 2007; Hoffmann *et al.*, 2007; Parameswaran *et al.*, 2007; Roh *et al.*, 2010).

Tagged or barcoded pyrosequencing has been used to investigate the bacterial diversity of sponges from the Red Sea (Lee *et al.*, 2011), discriminate the vertical or horizontal transmission of bacteria through sponge generations (Webster *et al.*, 2010), identify the core, variable, and species-specific bacteria of sponges collected worldwide (Schmitt *et al.*, 2012) and the seasonal variation of bacterial communities in *Axinella corrugata* sponges (White *et al.*, 2012). Recently, the diversity of sponge-associated bacteria was investigated in 14 marine sponges collected around Chuja Island off the south coast of Korea (Jeong *et al.*, 2013).

About 8,500 species have been validly described and the total number of species is thought to be as many as twice that (Van Soest *et al.*, 2012). In this respect, a limited number of sponges were investigated so far, although the sponge is one of the most extensively-studied marine invertebrates. Many more sponges should be investigated to reveal their unexplored microbial diversity. As an effort to understand microbial roles in sponges in Micronesia, 13 sponge samples were collected from Chuuk Lagoon and Kosrae Island and analyzed to reveal their bacterial diversity in this study.

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Materials and Methods

Sample collection and DNA extraction

Sponge specimens were collected from the Chuuk lagoon in Chuuk State (n=6) and around the Kosrae Island (n=7) of the Federated States of Micronesia by scuba diving. The 2 sampling sites were 1,280 km distant from each other. Specimens were collected aseptically, frozen, and transported to a laboratory in South Korea. Sponge tissue was 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 tissue was homogenized in sterilized mortar. DNA was extracted using a G-spin™ genomic DNA extraction kit (iNtRON, Korea).

Barcoded PCR-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-WTTACCGCGGCTG CTGG-3′), in which X indicates the different oligomers comprised of 6 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

Reads were sorted into each sample according to barcode and the primer and barcode were removed. Sequences more than 300 bp in length and without any ambiguous bases, 'N', were used for further analysis. Chimera sequences were determined using the chimer.uchime command of the Mothur package (Schloss *et al.*, 2009). The QIIME package v1.7.0 was used for clustering and taxonomic assignment (Kuczynski *et al.*, 2012). Sequences were marked according to samples and merged into one file. OTU clustering and assignment of representative sequences were performed using the uclust approach in the QIIME package based on 97% similarity. Taxonomic assignment of representative sequences were performed 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). Some reads were found to be related to chloroplasts and mitochondria and were removed from further analyses. Diversity indices such as Chao, Shannon, and Simpson indices were calculated for subsamples composed of reads selected randomly and

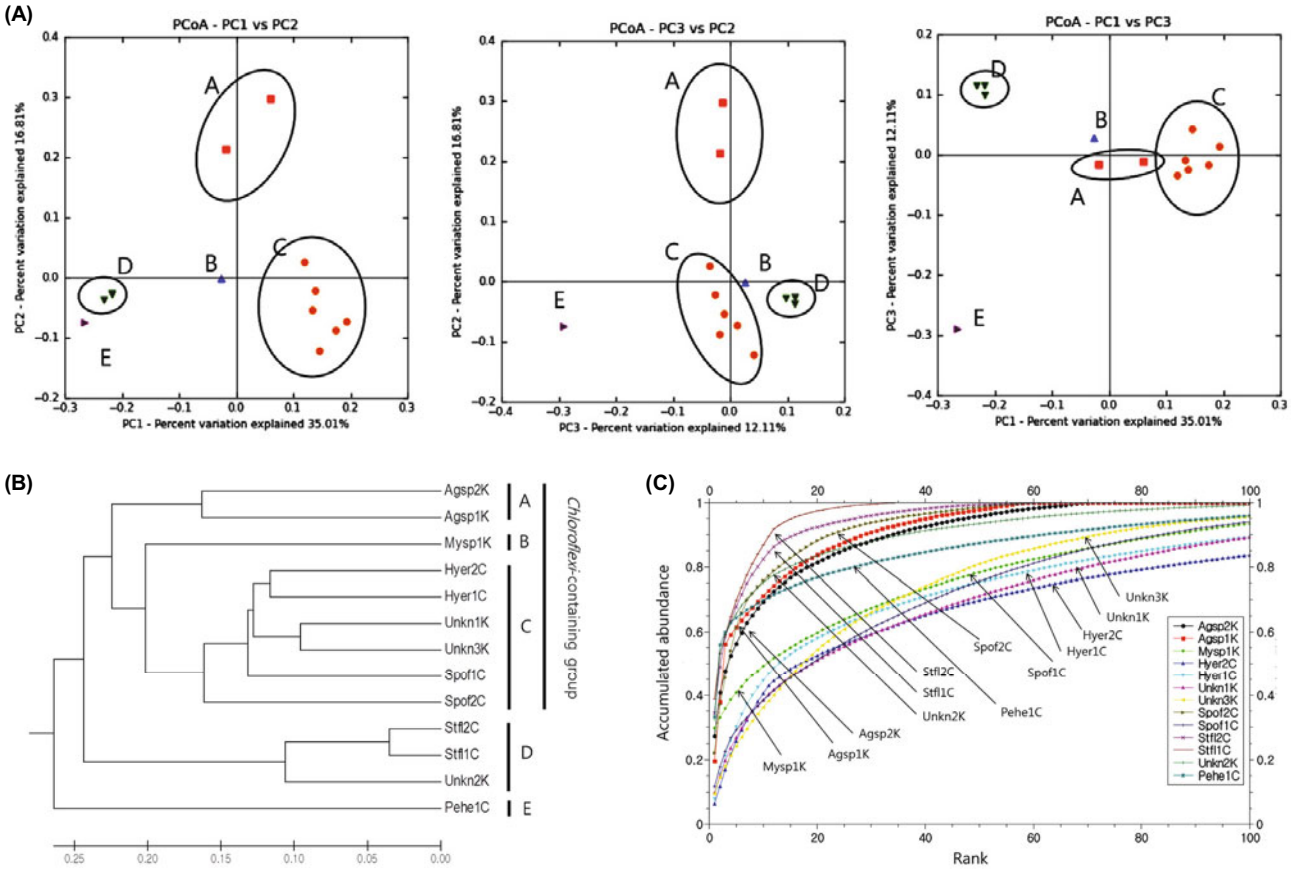

Fig. 1. Results of clustering and diversity analysis. (A) PCoA plot from weighted Unifrac analysis. OTUs were determined based on 97% similarity. (B) Weighted Unifrac UPGMA tree showing relationships between samples according to bacterial profile. OTUs were determined based on 91% similarity. (C) Cumulative rank abundance curve, in which the relative abundances of OTUs are accumulated sequentially in order of abundance from the largest. The first 100 OTUs were shown. OTUs were determined based on 97% similarity.

Table 1. Description of samples of sponges

No	Sample ID	Sponge species	Taxonomy (Class; Order; Family)	Site[a]	Latitude/ longitude
1	Agsp2K	*Agelas* sp.	*Demospongiae; Agelasida; Agelasidae*	K	N05°17′15.56″/E163°01′49.70″
2	Agsp1K	*Agelas* sp.	*Demospongiae; Agelasida; Agelasidae*	K	N05°17′15.56″/E163°01′49.70″
3	Mysp1K	*Myrmekioderma* sp.	*Demospongiae; Halichondrida; Desmoxyidae*	K	N05°21′23.04″/E162°55′48.48″
4	Hyer2C	*Hyrtios erectus*	*Demospongiae; Dictyoceratida; Thorectidae*	C	N07°31′09.34″/E151°58′05.58″
5	Hyer1C	*Hyrtios erectus*	*Demospongiae; Dictyoceratida; Thorectidae*	C	N07°30′48.96″/E151°57′52.06″
6	Unkn1K	Not determined	Not determined	K	N05°21′10.41″/E162°56′49.77″
7	Unkn3K	Not determined	Not determined	K	N05° 16′24.7″/E 162°57′34.56″
8	Spof1C	*Spongia officianlis*	*Demospongiae; Dictyoceratida; Spongiidae*	C	N07°28′21.73″/E151°51′51.16″
9	Spof2C	*Spongia officianlis*	*Demospongiae; Dictyoceratida; Spongiidae*	C	N07°31′09.34″/E151°58′05.58″
10	Stfl2C	*Stylissa flabeliformis*	*Demospongiae; Halichondrida; Dictyonellidae*	C	N07°30.48.96″/E151°57′52.06″
11	Stfl1C	*Stylissa flabeliformis*	*Demospongiae; Halichondrida; Dictyonellidae*	C	N07°30′48.96″/E151°57′52.06″
12	Unkn2K	Not determined	Not determined	K	N05°21′10.41″/E162°56′49.77″
13	Pehe1C	*Pericharax heteroraphis*	*Calcarea; Clathrinida; Leucettidae*	C	N07°26′39.92″/E151°51′09.44″

[a] K, Kosrae Island; C, Chuuk Lagoon

adjusted to the same numbers. The Mann-Whitney (Mann and Whitney, 1947) test was performed to compare diversity indices between two groups. Representative sequences were aligned with the PyNAST program (Caporaso *et al.*, 2010) and used for the construction of a phylogenetic tree with the FastTree program (Liu *et al.*, 2011). The Unifrac analysis with the phylogenetic tree (Lozupone *et al.*, 2011) was performed for a beta diversity comparison.

Results and Discussion

Clustering of sponges according to bacterial diversity

Among the 13 specimens, 9 are siliceous sponges (the class *Demospongiae*) and 1 is a calcareous sponge (the class *Calcarea*) while 3 specimens were unidentified (Table 1). Sponges were identified primarily based on morphological characteristics such as shapes of spicules by a sponge taxonomist. A total 24,729 reads were used for diversity analyses and taxonomic comparison. Sponges can be divided into 5 groups based on principal coordinate analysis (PCoA) analysis and the UPGMA tree from Unifrac analysis (Fig. 1A and 1B). Four pairs of specimens were collected from the same species, (Table 1) and each pair was clustered in a clade except for the species *Spongia officianlis*. Among them, 3 groups can be clustered into only a loose group that contained *Chloroflexi* as a major phylum (Fig. 2).

Diversity estimators were determined from the subsamples, adjusted to have the same number of reads, and compared in Table 2. Diversity in the C group is generally higher (except for Spof2C) than in other groups. The estimated numbers of OTUs and Shannon indexes were higher than in other groups. However, Agsp 2K and Agsp1K showed low diversities compared to the C group, even if they contained the *Chloroflexi* phylum, which was different from the CF group known to have high diversity in a previous study (Jeong *et al.*, 2013).

Shannon and Simpson index difference between the A/B/C and the D/E groups is significant ($p=0.011$) and highly significant ($p=0.003$), respectively, from statistical analysis with the Mann-Whitney test. The C and D group showed significant ($p<0.05$) differences from each other for all indexes,

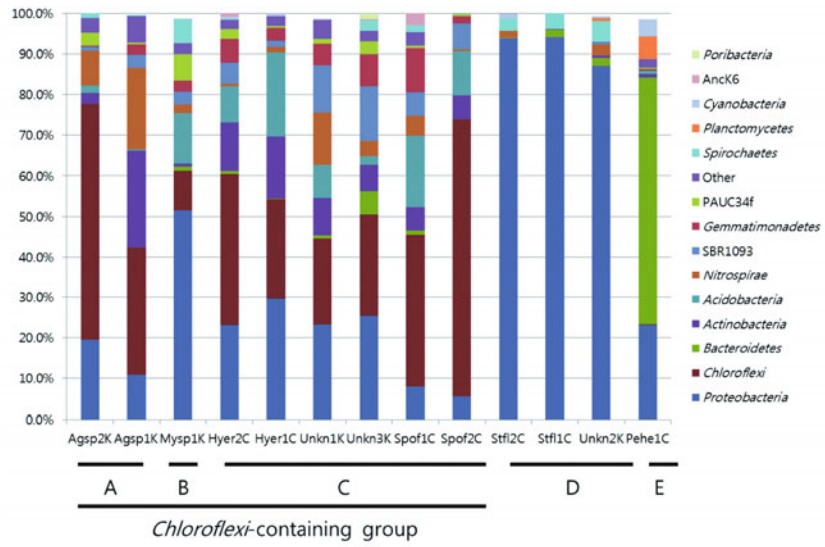

Fig. 2. Taxonomic classification of reads at the phylum level. Only phyla with abundance of more than 1% in at least one sample were shown in the Figure.

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

Sample ID	Total reads	Total OTUs	Reads subsampled[a]	OTUs	Chao	Shannon	Simpson	Group1[b]	Group2[c]
Agsp2K	1136	105	780	93	140	4.66	0.901	Cf	A
Agsp1K	783	81	780	84	105	4.46	0.897	Cf	A
Mysp1K	2030	248	780	166	268	5.66	0.913	Cf	B
Hyer2C	3347	425	780	218	382	6.63	0.979	Cf	C
Hyer1C	2741	317	780	185	296	6.33	0.974	Cf	C
Unkn1K	1500	281	780	200	325	6.59	0.978	Cf	C
Unkn3K	1458	186	780	150	251	6.25	0.977	Cf	C
Spof1C	1387	203	780	161	256	6.28	0.972	Cf	C
Spof2C	955	78	780	72	96	4.35	0.905	Cf	C
Stfl2C	1811	72	780	54	72	3.71	0.839	Non-cf	D
Stfl1C	974	43	780	39	55	3.39	0.813	Non-cf	D
Unkn2K	3379	180	780	97	153	4.19	0.852	Non-cf	D
Pehe1C	3228	269	780	141	239	4.51	0.847	Non-cf	E

[a] Reads were subsampled from each sample and used to calculate diversity estimators.
[b] Groups were differentiated based on presence and absence of phylum *Chloroflexi*: Cf, *Chloroflexi*-containing group; Non-cf, groups with no *Chloroflexi*.
[c] Groups were clustered based on the UniFrac analysis, as shown in Fig. 1. OTUs were determined based on 97% of 16S rRNA gene similarity.

Chao estimation, estimation OTUs, Shannon index, and Simpson index in the Mann-Whitney test. The curves with rank versus cumulative relative abundance showed more detailed shapes of OTU diversity (Fig. 1C). All members of the C group except for Spof2C showed similar curves; the OTUs in the first rank were less than 15% in abundance and the curves showed less steep slope than other groups. Mysp1K, the sole member of the B group showed a slow increase of cumulative abundances, which was similar with the C group but contained the OTU of high abundance (about 30%) in the first rank, which was different from the C group. Pehe1C, the sole member of the E group showed a unique curve with two OTUs of high abundance more than 20% for each and the slowest increase of cumulative abundance. The A/D groups showed similar curves with few OTUs of high abundance and a saturation of abundance up to 100% before the first 100 OTUs.

Bacterial diversity at the phylum level

Several different patterns of bacterial composition were clearly distinguished in the comparison at the phylum level, as shown in Fig. 2. The phylum *Chloroflexi* was the major phylum (21.4–68.2%) in the A, and C groups and was rarely detected (< 0.2%) in the D and E groups. A moderate amount (9.7%) of the phylum *Chloroflexi* was found in the group B. The phylum *Proteobacteria* was detected in the all samples but its proportion varied. Almost all reads were related to *Proteobacteria* (87.0–94.2%) in the D group and about half of the reads in the B group. The phylum *Actinobacteria* was detected in the A (2.6–24.0%) and C groups (5.8–15.3%) at a significant proportion. The phylum *Acidobacteria* was generally detected in the B/C group as a dominant phylum (2.0–20.6%). The phylum *Nitrospirae* was detected in the A group as a dominant phylum (8.6–20.0%) but its proportion varied between the B/C group (0.5–12.7%) and other groups (0.0–2.7%). The phyla SBR1093 (0.9–13.4%), PAUC34f (0.4–6.5%), and *Gemmatinonadetes* (0.3–10.9%) were frequently detected phyla in the A/B/C groups. A candidate phylum AncK6 was detected only in the C group (0.2–2.9%) except for Unkn1K. The phyla PAUC34f and AncK6 are candidate phyla that were originally recognized in sponges *Theonella swinhoei* (Hentschel *et al.*, 2002) and *Ancorina alata* (Kamke *et al.*, 2010), respectively. Another candidate phylum SBR1093 was originally detected from marine basalts (Mason *et al.*, 2009), but frequently detected in sponges (Hentschel *et al.*, 2002; Schmitt *et al.*, 2012). The phylum *Poribacteria* was detected in only one sample, Unkn3K (1.3%) collected from the Chuuk Lagoon, and was not detected in other samples. *Poribacteria* is a sponge-specific phylum discovered from sponges frequently and exclusively (Fieseler *et al.*, 2004; Lafi *et al.*, 2009). Most of sponges in this study belonged to taxa such as the genus *Agelas* and the orders *Halichondrida* and *Dictyoceratida* that were reported not to harbor *Poribacteria* (Lafi *et al.*, 2009). The phylum *Spirochaetes* was detected in small proportions in the A/C/E groups (0.0–2.6%) and in significant proportions in the B/D groups (3.0–5.1%). The phylum *Spirochaetes* is one of phyla detected from sponges frequently (Taylor *et al.*, 2007; Isaacs *et al.*, 2009) and some of them are specific to sponges (Neulinger *et al.*, 2010; Simister *et al.*, 2012). Although the roles of spirochetes are little understood, they are cosmopolitan residents of sponges and might have significant influence on sponges.

Table 3. Relative ratios of OTUs in terms of the frequency observed in the samples of sponges

Detection frequency	Ratio of OTUs detected at each frequency		
	In A/B/C/D/E groups (n=13)	In A/B/C groups (n=9)	In C group (n=6)
1	66.7%	63.5%	59.3%
2	18.2%	19.6%	21.3%
3	6.7%	6.8%	10.0%
4	3.3%	3.7%	4.8%
5	2.2%	3.3%	2.3%
6	1.5%	2.1%	2.3%
7	0.9%	0.6%	
8	0.2%	0.1%	
9	0.1%	0.3%	
12	0.1%		

OTUs were determined based on 94% of 16S rRNA gene similarity to compare OTUs at the level of genus and to reduce the number of OTUs for comparison.

Table 4. List of OTUs observed most frequently in the sponges according to group

OTU ID	Taxonomy	Detection frequency in group(s)		
		A/B/C/D/E (n=13)	A/B/C (n=9)	C (n=6)
1190	*p_Nitrospirae;c_Nitrospira;o_Nitrospirales;f_Nitrospiraceae;g_;s_*	12	9	6
261	*p_SBR1093;c_EC214;o_;f_;g_;s_*	9	9	6
506	*p_Chloroflexi;c_SAR202;o_;f_;g_;s_*	8	8	5
757	*p_Nitrospirae;c_Nitrospira;o_Nitrospirales;f_Nitrospiraceae;g_;s_*	8	6	6
1163	*p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_;g_;s_*	7	6	6
1056	*p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_koll13;g_;s_*	7	7	4
315	*p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_wb1_P06;g_;s_*	7	7	6
114	*p_Chloroflexi;c_Anaerolineae;o_Caldilineales;f_Caldilineaceae;g_;s_*	7	6	6
1338	*p_Chloroflexi;c_Anaerolineae;o_Caldilineales;f_Caldilineaceae;g_;s_*	7	6	6
911	*p_PAUC34f;c_;o_;f_;g_;s_*	7	7	4
1137	*p_Proteobacteria;c_Deltaproteobacteria;o_[Entotheonellales];f_[Entotheonellaceae];g_;s_*	7	7	6
423	*p_Proteobacteria;c_Gammaproteobacteria;o_Chromatiales;f_;g_;s_*	7	6	5
1169	*p_;c_;o_;f_;g_;s_*	6	6	6
1236	*p_Acidobacteria;c_Acidobacteria-6;o_iii1-15;f_;g_;s_*	6	6	4
1070	*p_Acidobacteria;c_Sva0725;o_Sva0725;f_;g_;s_*	6	6	6
389	*p_Acidobacteria;c_Sva0725;o_Sva0725;f_;g_;s_*	6	6	6
764	*p_Chloroflexi;c_Anaerolineae;o_SBR1031;f_A4b;g_;s_*	6	6	6
938	*p_Chloroflexi;c_SAR202;o_;f_;g_;s_*	6	6	5
611	*p_Chloroflexi;c_TK17;o_mle1-48;f_;g_;s_*	6	6	4
550	*p_Gemmatimonadetes;c_Gemm-2;o_;f_;g_;s_*	6	6	5
1390	*p_Gemmatimonadetes;c_Gemm-2;o_;f_;g_;s_*	6	5	5
1143	*p_Gemmatimonadetes;c_Gemm-2;o_;f_;g_;s_*	6	5	5
596	*p_Proteobacteria;c_Deltaproteobacteria;o_[Entotheonellales];f_[Entotheonellaceae];g_;s_*	6	5	4
78	*p_Proteobacteria;c_Gammaproteobacteria*	6	5	5
1257	*p_Proteobacteria;c_Gammaproteobacteria;o_Chromatiales;f_Ectothiorhodospiraceae;g_;s_*	6	6	3

The prefix p means phylum; c, class; o, order; f, family; g, genus; s, species. No word after '_' at each taxon level means that no name was assigned taxonomically in the classification system of Greengenes ver. 13_5. OTUs were determined based on 94% of 16S rRNA gene similarity similarity to compare OTUs at the level of genus and to reduce the number of OTUs for compariso.

Pehe1C, the sole member of the E group, had a very different composition of bacterial community. It had a high proportion of *Bacteroidetes* (60.9%), *Planctomycetes* (5.6%), and *Cyanobacteria* (4.2%), which are figures that distinguish it sharply from all the other samples. It is notable that the sponge of Pehe1C belongs to calcareous sponges, the class *Calcarea*, whereas the other sponges belong to siliceous sponges, the class *Demospongiae*.

A notable group, designed CF group, that contained *Chloroflexi* was defined in a previous paper (Jeong *et al.*, 2013). This group also contained phyla of *Gemmatimonadetes*, AncK6, PAUC34f, *Acidobacteria*, and SBR1093 exclusively or almost exclusively and showed higher diversity than other groups. The A, B, and C groups showed similar bacterial compositions, but, some differences were observed among them. The A and B groups contained no AncK6. The B group has a significantly lower proportion (9.7%) of *Chloroflexi* than the A and C groups (21.4–68.2%). It also has a higher proportion of *Proteobacteria* (51.5%) than the A and C groups (5.7–29.6%). The members of the A group also showed low diversities than the B and C groups (Table 2).

Analysis of core OTUs and individual- and species-specific OTUs

OTUs were determined based on 94% 16S rRNA gene similarity and were compared through samples to identify core OTUs (Table 3). OTUs were highly specific to each sample and core OTUs were not recognized well. None of the 889 OTUs was observed in all samples (total n=13) and only one OTU was detected in 12 samples. One additional OTU was observed in 9 samples and was the second most frequently-detected OTU. In the case of the A/B/C groups, 2 OTUs were detected in all samples (n=9) and 12 OTUs in the C group (n=6). The core OTUs detected most frequently in

Table 5. Individual-specific and species-specific OTUs

	Total (n=13)	*Agelas* sp. (n=2)	*Hyrtios erectus* (n=2)	*Spongia officianlis* (n=2)	*Stylissa flabeliformis* (n=2)
No. of OTUs in any samples	889 (100%)	102 (100%)	326 (100%)	170 (100%)	56 (100%)
No of OTUs in only one sample	593 (67%)	64 (63%)	129 (40%)	61 (36%)	12 (21%)
No. of OTUs in both samples in the same species	-	18 (18%)	103 (32%)	19 (11%)	14 (25%)
No. of OTUs in only two samples in the same species	-	8 (8%)	37 (11%)	0 (0%)	4 (7%)

OTUs were determined based on 94% of 16S rRNA gene similarity similarity to compare OTUs at the level of genus and to reduce the number of OTUs for comparison.

the samples are listed in Table 4. One OTU that belongs to family *Nitrospiraceae* was the most-frequently detected OTU in the sponges. The second one was an OTU related to candidate phylum SBR1093. OTUs belonging to the phylums *Chloroflexi*, *Actinobacteria*, *PAUC34f*, *Proteobacteria*, *Acidobacteria*, and *Gemmatimonadetes* in the various taxonomic ranks such as class, order, and family were observed as common OTUs.

Moreover, more than half of OTUs (67%) were detected only once in the samples, implying highly-specific and exclusive connections of bacteria with sponges (Table 5). When 95% 16S rRNA gene similarity was applied, 65% of sequences were recognized as species-specific OTUs in a previous paper with 32 sponge species (Schmitt *et al.*, 2012). Considering that 4 pairs of sponges were the same species as each other, this specificity can be referred to as individual specificity. The ratios of OTUs which are common in the same species were only 11–32% of the total OTUs in that species, which implies that variety exists even in the same species. The numbers of species-specific OTUs that were detected only in that species and not in other specimens were reduced greatly; no species-specific OTUs were detected in the case of *Spongia officianlis*. This observation means that many bacterial OTUs don't exist commonly even in the same species and can be individual-specific OTUs. Sponges have very diverse bacterial residents according to species and individuals.

Micronesia is located in the western Pacific Ocean and harbors a variety of marine organisms and microorganisms which are not investigated yet. In this study, we investigated microbial diversity in the sponges collected from Micronesia and revealed unknown diversity of the bacterial communities of the sponges. Much more efforts should, however, be taken to reveal to the unexplored resources of microorganisms including Archaea, Eukarya as well as Bacteria in further studies.

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