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Microbiome analysis of sponges *Callyspongia ramosa* and *Callyspongia confoederata*

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해면동물 *Callyspongia ramosa*와 *Callyspongia confoederata*의 마이크로바이옴 분석

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Diversities of prokaryotic symbionts were investigated in two sponge species, *Callyspongia ramosa* and *Callyspongia confoederata* collected from Jeju Island by 16S rRNA gene amplicon sequencing using. The V3-V4 and V5-V6 regions of the 16S rRNA gene of bacteria and archaea were amplified and sequenced using Illumina MiSeq platform. Gammaproteobacteria (85.40–87.50%), Bacteroidetes (8.51–10.71%), and Alphaproteobacteria (0.98–1.15%) were major taxa in *C. ramosa* while Gammaproteobacteria (25.06–40.05%), Bacteroidetes (26.99–27.38%), Tenericutes (8.55–16.20%), Betaproteobacteria (8.17–9.51%), Alphaproteobacteria (7.35–8.46%), Cyanobacteria (3.47–10.35%), Chloroflexi (0.51–1.71%), and Actinobacteria (0.45–1.18%) in *C. confoederata*. Prokaryotic diversity of *C. confoederata* was more complex than that of *C. ramosa* and unique prokaryotic communities differentiated the different species in the same genus. This study would provide additional evidence that the sponge symbiotic communities have host specificity and elucidate their diversity in detail.

Keywords: *Callyspongia*, amplicon sequencing, microbial diversity, prokaryotic symbionts, sponge

Sponges are one of the most diverse marine invertebrates in terms of number of species which are more than 8,550 species (Van Soest *et al.*, 2012). Sponges have been widely studied as sources of new marine natural products (NMNP), as more than 4,851 NMNP have been identified from sponges (Mehbub *et al.*, 2014). Those NMNP showed activities of anticancer, antibiotics, anti-inflammation, immune stimulation, and nerve protection. The metabolites from sponges were considered to have roles to protect sponges from threats such as predators, microbial infection, and attachment of other organisms (Paul and Puglisi, 2004).

Some of the metabolites were originated from symbiotic microorganisms of sponges (Faulkner, 2002). Various symbiotic microorganisms residue in mesohyl and many natural products from sponges showed similarities with microbial products (Wang, 2006). The metabolites produced by sponge symbionts have stability and activity in salty condition of sea (Mehbub *et al.*, 2014). Bacteria isolated from the sponge in genus *Callyspongia* showed strong activities of anti-infection agent against fungi and parasitic flagellates (*Rhodococcus* sp. UA13) and biosurfactant (*Bacillus subtilis* MB-7, *Bacillus amyloliquefaciens* MB-101, *Halomonas* sp. MB-30, and *Alcaligenes* sp. MB-I9) (Dhasayan *et al.*, 2015; Elsayed *et al.*, 2018). The application of symbiotic

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microbes in sponges in biotechnology is one of the main topics of marine microbiologists. Because large-scale cultivation of sponge symbionts that produce useful metabolites can be a solution for limitation of supplement of the metabolites (Duckworth, 2009), studies for sponge symbionts should be continued.

Another role of sponge symbionts is to supply metabolites to their host, allowing sponges to access intrinsic microbial properties such as nitrogen fixation (Mohamed *et al.*, 2008), autotrophy, and nitrification (Mohamed *et al.*, 2010). The exchange of metabolites between a sponge and symbionts via organic material absorption and transformation and nitrogen cycle, etc. supports their symbiotic relationship (Thomas *et al.*, 2016). Because the role of the microbial community is important in the physiology of sponges, it is essential to understand the stability and specificity of the symbiotic relationship between the host and the community (Reveillaud *et al.*, 2014).

Sponges have very diverse and complex microbial communities and are often divided into HMA (High Microbial Abundance) and LMA (Low Microbial Abundance) sponges according to the abundance of their symbionts (Gloeckner *et al.*, 2014). It is known that HMA group has a more stable microbial community than LMA group, and that LMA group has a distinct microbial community that varies according to sponge species. This difference is thought to be due to the seawater filtration capacity of sponges (Lurgi *et al.*, 2019), showing that sponges can maintain a highly diverse and specific microbial community despite of the constant influx of seawater microorganisms through filtration (Taylor *et al.*, 2013). The structure of sponge microbial community is markedly different from that of seawater or marine sediments and certain sponge species contain a higher density of microorganisms than their surroundings (Thomas *et al.*, 2016; Yang *et al.*, 2019). Although forty-one prokaryotic phyla have been discovered from sponges in recent studies, the community structure and their ecological functions are not completely understood (Moitinho-Silva *et al.*, 2017; Weigel and Erwin, 2016). Because it is hard to cultivate most of sponge symbionts, culture-independent methods are essential to elucidate the microbial diversity in sponges in detail (Weigel and Erwin, 2016). The next generation sequencing (NGS) has become the most effective approach to investigate the ecology of microorganisms by examining 16S rRNA sequences very deeply (Roh *et al.*, 2010). The NGS method has provided insight into the diversity of minor microbial populations, often referred to as the rare biosphere (Lynch and Neufeld, 2015).

Previous studies showed the bacterial diversity of sponges from Korea (Jeong *et al.*, 2013, 2015) and Micronesia (Jeong *et al.*, 2014) using NGS methods. In this study, we investigated the diversity of bacteria and archaea, important inhabitants of sponges, in two different sponges belonging to genus *Callyspongia*. Two primer sets targeting V3-V4 and V6-V7 regions of 16S rRNA gene were used to implement more complete community structure of sponge symbiotic communities.

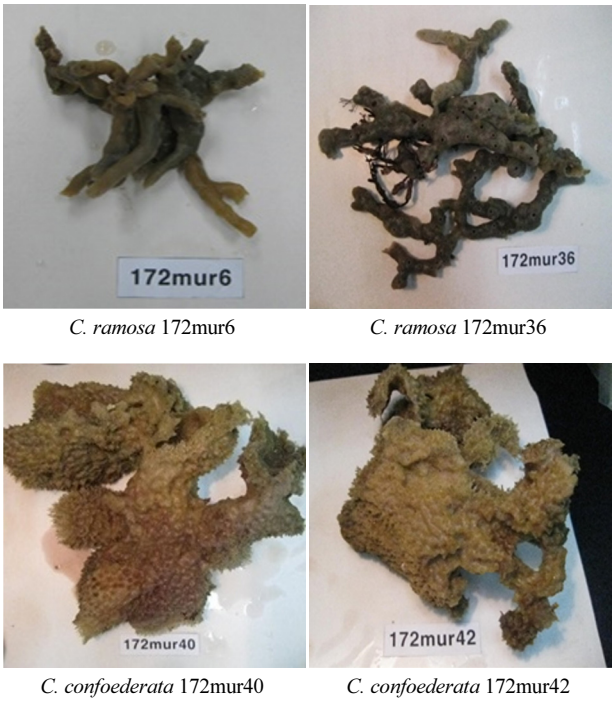
Materials and Methods

Preparation of samples

Sponge specimens were collected by scuba diving in February 2017 off a beach of Yeongnak-ri, Daejeong-eup, Seogwipo-si, Jeju Island, South Korea (Table 1). The average water temperature was 16.4°C and the salinity was 34.2 PSU. Two specimens per species of *Callyspongia ramosa* and *Callyspongia confoederata* (Fig. 1) were collected and washed three times with sterile artificial sea water (ASW) to investigate internal microbiome community of sponges. The specimens were then frozen at -20°C and transported to the laboratory within 24 h.

Table 1. Sponge samples used in this study

Collection no.	Sponge	Location	Collection date	Depth (m)	Salinity (PSU)	Temp. (°C)
172mur6	*Callyspongia ramosa*	N33° 15' 04.02" E126° 11' 44.99"	2017.02.22	20–25	34.2	16.4
172mur36	*Callyspongia ramosa*	N33° 15' 04.02" E126° 11' 44.99"	2017.02.22	20–25	34.2	16.4
172mur40	*Callyspongia confoederata*	N33° 15' 04.02" E126° 11' 44.99"	2017.02.22	20–25	34.2	16.4
172mur42	*Callyspongia confoederata*	N33° 15' 04.02" E126° 11' 44.99"	2017.02.22	20–25	34.2	16.4



C. ramosa 172mur6 *C. ramosa* 172mur36

C. confoederata 172mur40 *C. confoederata* 172mur42

Fig. 1. Photographs of the sponges used in this study.

Purification of DNA

The collected sponges were cut into pieces of approximately 1 cm^3 volume, washed with sterilized ASW, pre-frozen at -70°C for 24 h, and then dried in a freeze dryer at -50°C and 0.033 M bar pressure for 24 h. The freeze-dried sponge was ground using a sterile mortar, and DNA was extracted from the crushed sponge powder sample using PowerSoil® DNA Isolation Kit (MO BIO).

Illumina Miseq amplicon library and sequencing

A library for paired-end sequence reads was prepared according to the Illumina 16S Metagenomic Sequencing Library protocols. The V3-V4 region of the 16S rRNA gene was amplified using the Bakt 341F/Bakt 805R (CCTACGGGNG GCWGCAG/ ACTACHVGGGTATCTAATCC) primer pair which amplifies bacterial and archaeal sequences (Klindworth *et al.*, 2013). The V5-V6 region was amplified using the ARC 787F/ARC 1059R (ATTAGATACCCSBGTAGTCC/ CCAT GCACCWCCTCT) primer pair that was designed to investigate archaeal diversity (Yu *et al.*, 2005). The PCR mixture was prepared by adding 12.5 μl of 2 X KAPA Hifi Hotstart ready mix (KAPA Biosystems), 2.5 μl of 5 pmol of each primer, 5 μl of D.W, and 2.5 μl of sample DNA to make a final volume of 25 μl. The PCR conditions were as follows: denaturation at 95°C for 30 sec, 25 times of amplification cycles (denaturation at 95°C for 3 min, annealing at 55°C for 30 sec, and elongation at 72°C for 30 sec) and finally elongation at 72°C for 5 min. A second amplicon PCR was performed using the Illumina Nextera XT Index Kit. The size of the amplified product was confirmed by 1.5% agarose gel electrophoresis and purified using QIAquick® PCR purification kit (Qiagen), and the concentration of the resulting amplified product was quantified using PicoGreen. Sequencing was performed using the MiSeq™ platform (Illumina) and sequence analysis was also done by Macrogen.

16S rRNA sequence data processing

The reads obtained through NGS were merged using the FLASH ver.1.2.11 program. Then, after filtering using the CD-HIT-OTUs program, operational taxonomic units (OTUs) were classified according to the 97% sequence similarity threshold based on the Ribosomal Database Project (RDP) database. The representative sequences of the OTUs were compared to the sequences in the database of the National Center for Biotechnology Information (NCBI) by BLAST search. Alpha diversity and beta diversity were analyzed using the QIIME ver.1.9.1 program. Shannon, Inverse Simpson, and Chao1 indices were derived as diversity indicators of alpha diversity. The OTUs assigned only to bacteria and archaea, respectively, were used to analyze the bacteria and archaea community separately. For analyzing beta diversity, principal coordinate analysis (PCoA) was done for the distances between samples was calculated using the unweighted and the weighted UniFrac methods in the QIIME.

Results

Alpha diversity

The primer set Bakt 341F/Bakt 805R is known to amplify archaeal sequences together (Klindworth *et al.*, 2013) but only bacterial sequences were obtained (proportion of archaea $< 10^{-4}$). After discarding unassigned and few archaeal sequences, 36,395 to 53,312 reads were obtained and 239 to 511 OTUs

Table 2. Richness and diversity estimation of microbial communities in sponge samples

	Species name	Sample name	Read count	OTUs	Chao1	Shannon	Inverse Simpson
Bacterial 16S rRNA gene primer set	*C. ramosa*	172mur6	49,017	239	269	2.19	0.51
		172mur36	46,789	281	330	2.42	0.60
	C. confoederata	172mur40	36,395	511	534	5.53	0.94
		172mur42	53,312	458	486	4.93	0.92
Archaeal 16S rRNA gene primer set	*C. ramosa*	172mur6	63,184	14	14	1.54	0.60
		172mur36	67,966	12	12	1.56	0.61
	C. confoederata	172mur40	78,267	29	29	1.62	0.59
		172mur42	81,349	23	23	1.78	0.63

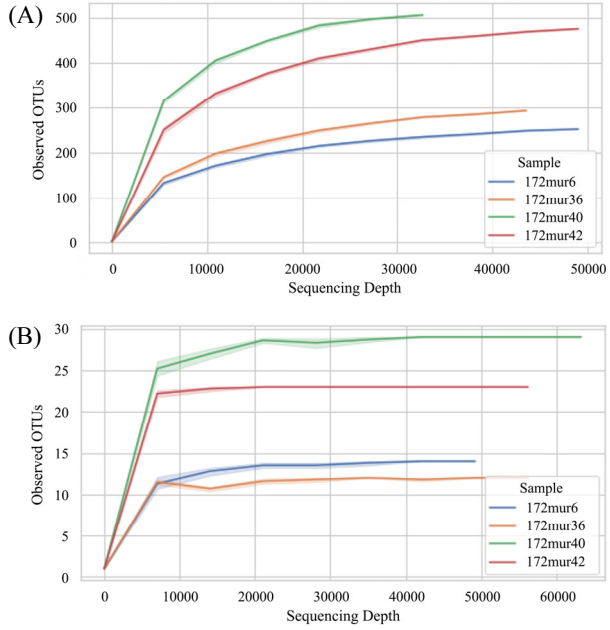


Fig. 2. Rarefaction curves for operational taxonomic units (OTUs) clustering at 97% sequence similarity of 16S rRNA sequences related to (A) bacteria and (B) archaea.

were binned from each sponge sample (Table 2). Like the number of OTUs, the Chao1, Shannon, and Inverse Simpson index all showed high values in the order of 172mur40, 172mur42, 172mur36, and 172mur6. Diversity indices of 172mur40 and 172mur42 were approximately twice higher than those of 172mur6 and 172mur36. The bacterial communities in *Callyspongia confoederata* sponges are more complex and diverse than those of *Callyspongia ramosa* sponges.

In the case of the primer set ARC 787F/ARC 1059R, although majority of sequences were archaeal sequences, significant portions of them were identified as bacterial sequences (7.8–19.9%) including Cyanobacteria and Actinobacteria. After discarding bacterial sequences, 53,542 to 63,248 reads were obtained and 12 to 29 OTUs were binned from each sponge sample (Table 2). Similar to the cases of bacteria, the diversity indices of *Callyspongia confoederata* (172mur40, 172mur42) sponges showed higher values than those of *Callyspongia ramosa* (172mur6, 172mur36) sponges. However, since all of the measured values are much lower than those of bacterial cases, the bacterial species diversity is higher than that of the sponge archaea.

Rarefaction curve analysis showed whether the number of reads obtained from NGS was sufficient to identify OTUs and which sponge samples had more diverse microbial communities (Fig. 2). It was found that all curves reached plateaus, so a sufficient number of reads were used for the analysis. It coincided with the order (172mur40, 172mur42, 172mur36, and 172mur6) identified by the diversity indices.

Beta diversity analysis

Principal Coordinates analysis (PCoA) was performed to compare species diversity among sponge samples (Figs. 3 and 4). The distance between samples was calculated using the unweighted and the weighted UniFrac methods. Based on the both bacterial and archaeal diversities, samples were clustered into two groups, *C. ramosa* (172mur6 and 172mur36) and *C. confoederata* (172mur40 and 172mur42) as expected in the both UniFrac analyses.

Taxonomic composition of bacterial sequences

Among the various microbial taxa detected, only those

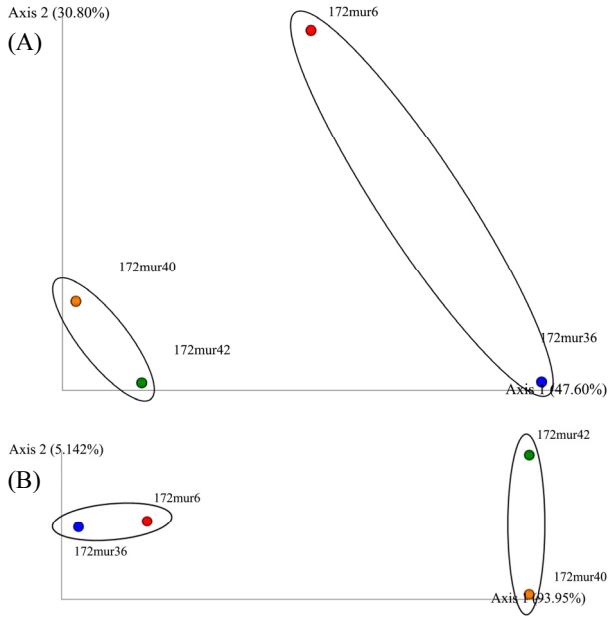


Fig. 3. Beta diversity analysis of bacterial sequences among four sponge samples. Two axes of three-dimensional principal Coordinates Analysis (PCoA) plot showed the beta diversity derived using bacterial 16S rRNA genes. (A) Unweighted UniFrac method, (B) Weighted UniFrac method.

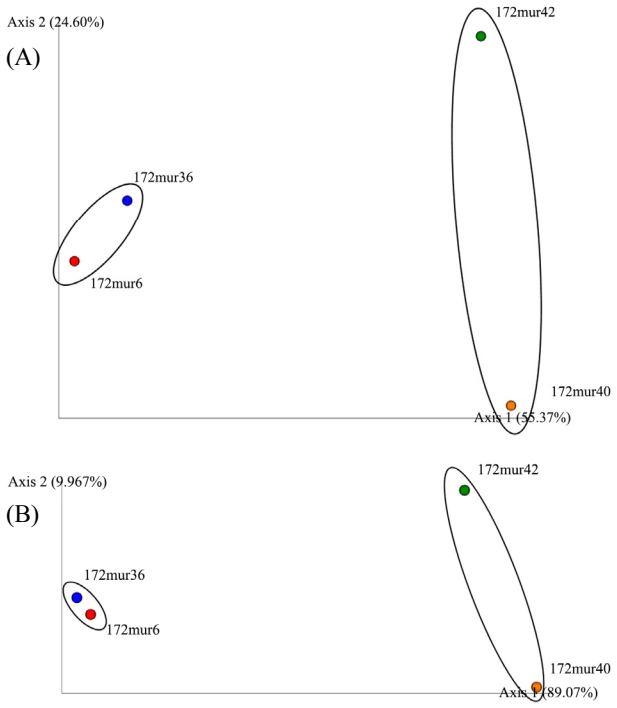


Fig. 4. Beta diversity analysis of bacterial sequences among four sponge samples. Two axes of three-dimensional principal Coordinates Analysis (PCoA) plot showed the beta diversity derived using archaeal 16S rRNA genes. (A) Unweighted UniFrac method, (B) Weighted UniFrac method.

communities that were present in a ratio of 0.1% or more were compared and an abundance taxon and a rare taxon were classified based on 1% (Fig. 5). When the microbial community composition was analyzed at the pylum or class level (Fig. 5A), in samples 172mur6 and 172mur36 of *C. ramosa* sponge, two classes and one phylum, Gammaproteobacteria (85.40–87.50%), Bacteroidetes (8.51–10.71%), and Alphaproteobacteria (0.98–1.15%) were found as common major taxa. Acidobacteria (0.13%), Actinobacteria (0.32–0.35%), Chloroflexi (0.59–0.86%), Cyanobacteria (0.34–0.51%), Nitrospirae (0.48–0.82%), Betaproteobacteria (0.03–0.17%), and Deltaproteobacteria (0.27–0.38%) were found to be sparse taxa so the community structure of the two samples was consistent. The symbiotic bacterial community of *C. ramosa* sponges was composed of a total of 8 phyla and 10 classes. In the samples of *C. confoederata* sponges, Gammaproteobacteria (25.06–40.05%), Bacteroidetes (26.99–27.38%), Tenericutes (8.55–16.20%), Betaproteobacteria (8.17–9.51%), Alphaproteobacteria (7.35–8.46%), Cyanobacteria (3.47–10.35%), Chloroflexi (0.51–1.71%), and Actinobacteria (0.45–1.18%) were found as common abundant taxa. Deltaproteobacteria (0.75–0.81%), Nitrospirae (0.41–0.59%), Firmicutes (0. 22–0.52%), and Acidobacteria (0.11–0.21%) were found to be sparse taxa. The symbiotic bacterial community of *C. confoederata* sponges consisted of a total of 10 phyla and 12 classes.

At the genus level (Fig. 5B), *C. ramosa* sponges contain *Inmirania* (60.48–70.50%) and *Halioglobus* (10.77–20.33%) in class Gammaproteobacteria and *Polaribacter* (3.54–4.86%) and *Phaeocystidibacter* (1.79–3.15%) in phylum Bacteroidetes were found as common abundant taxa in both samples, while *Aquimarina* (4.46%) in phylum Bacteroidetes was found only in 172mur36. The other group except for major 14 genera, occupy only small portions (4.50–5.03%) in the *C. ramosa* sponges. In the samples of *C. confoederata* sponges, *Polaribacter* (12.42–15.96%) and *Phaeocystidibacter* (6.93–7.23%) in phylum Bacteroidetes, *Spiroplasma* (8.54–12.83%) in phylum Tenericutes, *Stanieria* (2.09–6.02%) in phylum Cyanobacteria, *Oceanicella* (2.08–3.96%) and *Pelagibacter* (1.83–1.87%) in class Alphaproteobacteria, *Thauera* (6.07–8.11%) and *Formosimonas* (1.24–1.78%) in class Betaproteobacteria, *Alkalimarinus* (4.25–18.22%), *Endozoicomonas* (4.37–12.11%), *Paraferrimonas* (1.17–6.77%), and *Aestuariibacter* (1.84–2.34%) in class

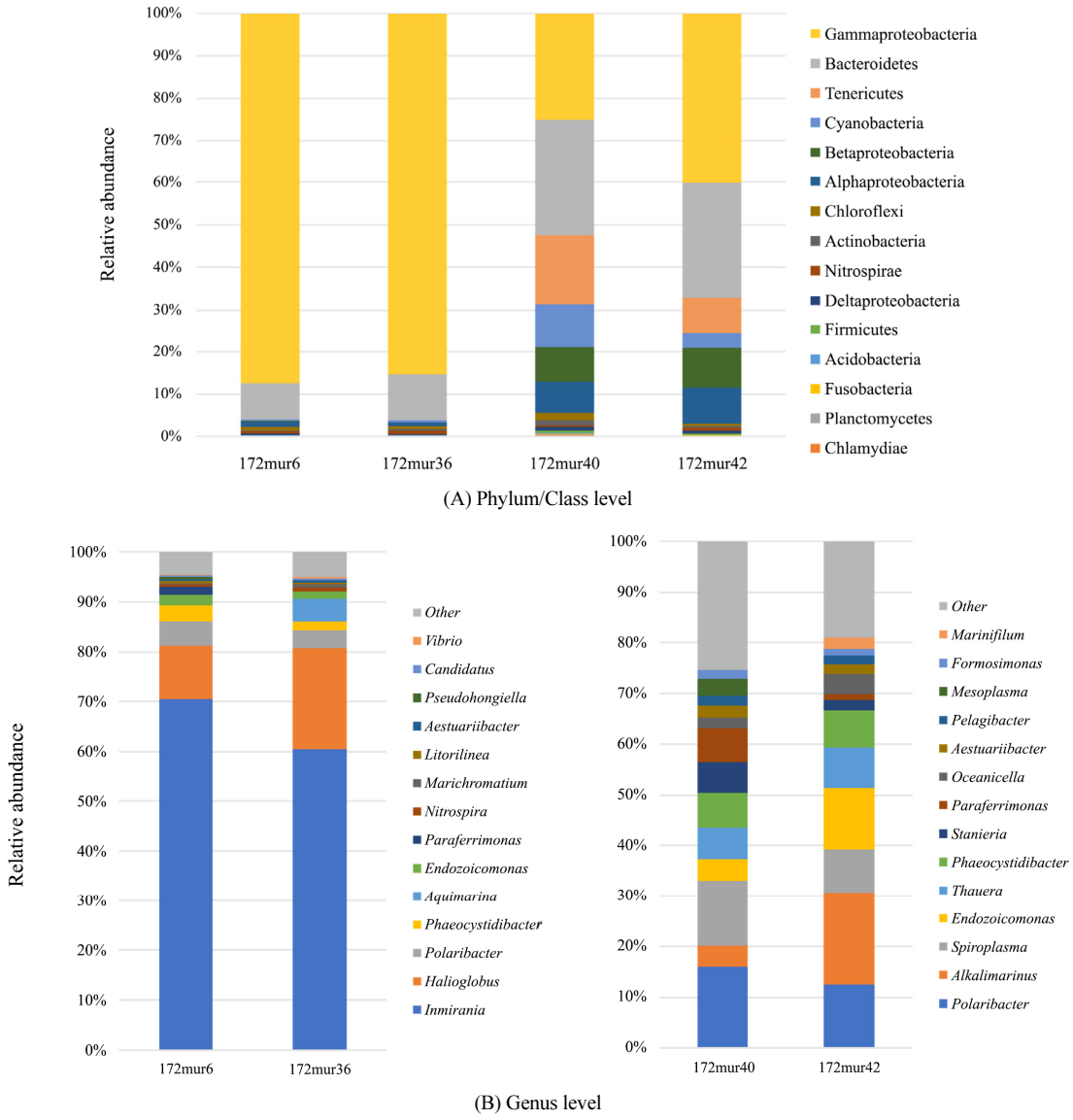


Fig. 5. Relative abundances of different microbial taxa in each sample identified as bacterial 16S rRNA genes (primer pair for V3-V4 region). (A) Phylum or class (only for *Proteobacteria*) level, (B) genus level. Phyla less than 0.1% in all samples and genera except for 14 most abundant genera were summed to the 'Other' group.

Gammaproteobacteria were common abundant taxa in both samples. *Mesoplasma* (3.36%) in phylum Tenericutes was found only in 172mur40. The other group except for major 14 genera, occupy significant portions (18.99–25.34%) in the *C. confoederata* sponges.

Taxonomic composition of archaeal sequences

The V5-V6 region was analyzed using the Archaeal 16S rRNA gene primer set. Although the set targets archaeal sequences significant portion of the sequences assigned to bacterial sequences (Fig. 6). Five bacterial phyla were also detected in *C. confoederata*: Cyanobacteria, Actinobacteria, Deltaproteobacteria, Firmicutes, and Fusobacteria (17.1–19.9% in total). When considering only archaeal sequences, at the phylum or class level, Thaumarchaeota ware the most predominant phylum in the *C. ramosa* (99.2–99.6%) and *C. confoederata* sponges (96.7–98.1%). Euryarchaeota was the second phylum but the proportions are very small in the *C. ramosa* (0.3–0.8%) and *C. confoederata* sponges (1.9–3.1%).

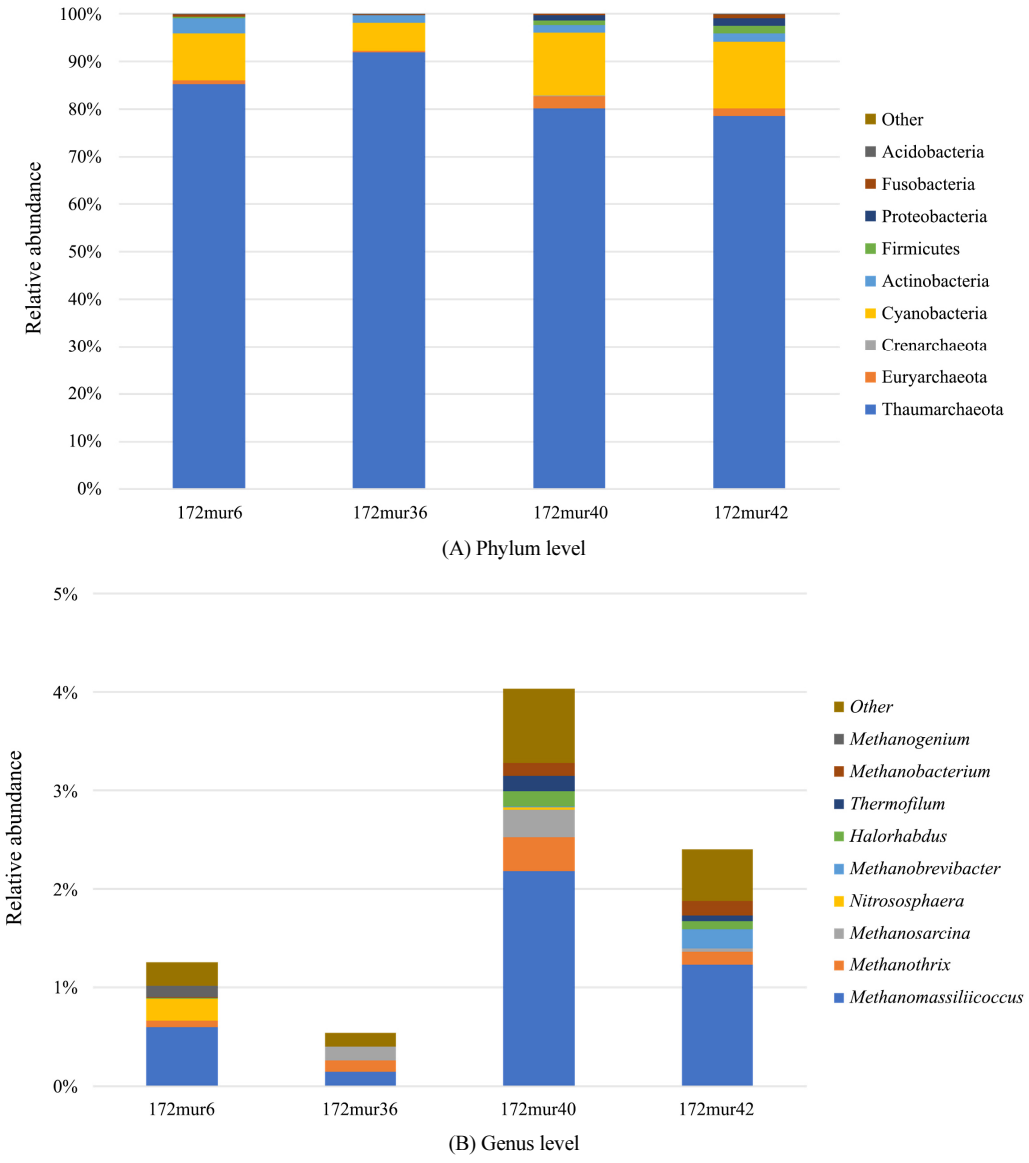


Fig. 6. Relative abundances of different microbial taxa in each sample identified as bacterial 16S rRNA genes (primer pair for V4-V5 region). The taxa less than 0.1% in all samples were summed to the 'Other' group. (A) Phylum level. Bacterial sequences obtained using primer pair for V4-V5 region were also shown at phylum level. (B) Genus level. Only the minor archaeal genera except for the most dominant genus *Nitrosopumilus* (96.7–99.6%) were shown at genus level.

At the genus level, in the samples of *C. ramosa* sponges, *Nitrosopumilus* in phylum Thaumarchaeota was the most predominant genus in both sponges (98.9–99.6% for *C. ramose* and 96.6–98.1% for *C. confoederata*). Minor genera except for *Nitrosopumilus* were shown for comparison (Fig. 6B). *Methanomassiliicoccus* is a common genus in both sponges (0.15–0.60% for *C. ramose* and 1.23–2.18% for *C. confoederata*). *Methanothrix* is also a common genus (0.06–0.12% for *C. ramose* and 0.13–0.35% for *C. confoederata*). *C. confoederata* sponges showed more archaeal genera and more proportion of minor or sparse genera.

Discussion

In this study, bacterial and archaeal diversities in the two different sponge species in the same genus *Callyspongia* were compared by amplicon sequencing based on the Illumina

Miseq platform. We used two kinds of primer pairs that target to amplify the V3-V4 and V5-V6 regions of the 16S rRNA gene of bacteria and archaea, respectively.

The richness of the bacterial and archaeal communities of *C. confoederata* sponges was about twice as high as those of *C. ramosa* sponges in both types of primers. The differences between individuals within the same sponge species were not significant. Beta diversity analysis confirmed that the samples were clustered according to the species of sponges in the both primer sets. Based on these results, the *Callyspongia* species possess a unique symbiotic microbial community that is distinguished in different sponge species in the same sponge genus. Since the sponges of the genus *Callyspongia* are classified as LMA sponges by containing an average of $4.0 \times 10^6 \pm 2.0 \times 10^6$ cells/g sponge tissue (Gloeckner *et al.*, 2014), the difference in microbial diversity might be observed more clearly.

Gammaproteobacteria, Bacteroidetes, and Alphaproteobacteria are common major taxa in both species. This is consistent with the findings of the study that the Proteobacteria is generally abundant in LMA sponges (Gloeckner *et al.*, 2014). Although most major phyla/classes were found in both species, the quantitative composition of the high-rank taxa were quite different. Phylum-level diversity of their bacterial community was higher than that of *C. ramosa*. Tenericutes and Firmicutes were found only in *C. confoederata* sponges and the high proportion of Tenericutes might imply its specialized and/or significant role in the sponges.

Phylum Bacteroidetes is known as a bacterial group that has a large number of protein and glycolytic enzymes to decompose polymer organic matter (Fernández-Gómez *et al.*, 2013). Phylum Cyanobacteria is also one of the common bacterial groups, living in tropical and temperate regions. It has been detected in more than 100 species of sponges and has various roles such as photosynthesis, nitrogen fixation, and toxin production for UV protection and defense purposes (Burgsdorf *et al.*, 2015; Gao *et al.*, 2017). Phylum Tenericutes was first recorded in sponges by NGS analysis (Webster *et al.*, 2010) and was only detected in *C. confoederata* sponges in the study. Phylum Thaumarchaeota, which dominates both species of sponges, is a group of archaea generally abundant in sponges and is known to be closely involved in the carbon and nitrogen cycle in the marine environment (Hentschel *et al.*, 2012; Hu *et al.*, 2013).

Genus-level diversity has different aspects compared to phylum-level one. Only *Polaribacter* and *Phaeocystidibacter* in phylum Bacteroidetes were common abundant taxa in both species. *Polaribacter haliotis* was isolated from the gut of abalone (Kim *et al.*, 2016). *Phaeocystidibacter marisrubri* was isolated from Red Sea sediment (Zheng *et al.*, 2015).

The diversity of genera except the two genera are very different between two sponges. *Inmirania* and *Halioglobus* were two major species detected in *C. ramosa* sponges existing in high proportions. *Inmirania thermothiophila* is known to be autotrophs associated with sulfur-oxidizing ability in nitrate-reducing condition (Slobodkina *et al.*, 2016) and its high proportion in *C. ramosa* sponges implies that the taxon might be important in organic carbon supplement from chemosynthetic symbionts to the host. *Halioglobus japonicus* is proposed based on a strain isolated from seawater (Park *et al.*, 2012). More diverse and different species were detected in *C. confoederata* sponges. Interestingly, among them, a *Spiroplasma* species was isolated from hemolymph of rootworm beetle that is an insect while sponges are the most primitive metazoa (Seimiya *et al.*, 1994). *Nitrosopumilus* is known as an archaeon with ammonia oxidation capacity (Qin *et al.*, 2017) and it might be important in nitrogen cycle of the sponges because it occupies most of the archaea community in both species of sponges.

적 요

제주도에서 채취된 두 해면동물 종 *Callyspongia ramosa*와 *Callyspongia confoederata*의 원핵생물 공생체의 다양성을 16S rRNA 유전자 amplicon sequencing 기법을 이용하여 분석하였다. 이를 위해 세균과 고균의 16S rRNA 유전자의 V3-V4와 V5-V6 부분을 각각 증폭하였고 Illumina MiSeq을 이용하여 서열분석하였다. Gammaproteobacteria (85.40–87.50%), Bacteroidetes (8.51–10.71%), and Alphaproteobacteria (0.98–1.15%)가 *C. ramosa*의 주요 분류군이었으며, 반면에 *C. confoederata*에서는 Gammaproteobacteria (25.06–40.05%), Bacteroidetes (26.99–27.38%), Tenericutes (8.55–16.20%), Betaproteobacteria (8.17–9.51%), Alphaproteobacteria (7.35–8.46%), Cyanobacteria (3.47–10.35%), Chloroflexi (0.51–1.71%), Actinobacteria (0.45–1.18%)가 주요 분류군들이었다. *C. confoederata*의 원핵생물 군집의

다양성이 *C. ramosa* 에 비하여 더 복잡하였으며 같은 속 내에 다른 해면동물 종에 따라서 독특한 군집으로 구분되었다. 본 연구는 해면동물의 공생군집이 숙주 특이성을 가지고 있다는 추가적인 증거이며, 그 다양성을 자세히 보여주고 있다.

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Conflict of Interest

There are no financial or non-financial interests that are directly or indirectly related to the work.

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