

Next-generation sequencing-based Actinobacteria community associated with *Callyspongia* sp. from Kepulauan Seribu Marine National Park, Jakarta, Indonesia

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Abstract. Retnowati D, Solihin DD, Ghulamahdi M, Lestari. 2021. Next-generation sequencing-based Actinobacteria community associated with *Callyspongia* sp. from Kepulauan Seribu Marine National Park, Jakarta, Indonesia. *Biodiversitas* 22: 3702-3708. Sponges are sessile benthic filter-feeding animals, which harbor numerous microbes. The enormous community and abundance of sponge-associated actinobacteria envisage sponges as hot spots of microbes. The metagenomic approach with Next Generation Sequencing (NGS) technology was used to investigate the actinobacteria community of *Callyspongia* sp. collected from Kepulauan Seribu Marine National Park, Indonesia. Fourteen actinobacteria families were found associated with the sponge. Among them, Microbacteriaceae was the most dominant family. The community of actinobacteria was higher than that of the culturable method from our previous study. The research showed that a metagenomic approach using NGS technology reveals better information regarding the actinobacteria community associated with *Callyspongia* sp.

Keywords: *Callyspongia*, metagenomic approach, sponge-associated actinobacteria

INTRODUCTION

Sponges are the most ancient Metazoa and generally have strong symbiotic relations with the complex communities of microbes. Actinobacteria were found as one of the bacterial groups associated with 3569 sponge samples obtained from almost all over the world with a relative abundance of 5% (Silva et al. 2017). Recent evidence suggests that various genera of culturable actinobacteria were obtained to be associated with 50 types of sponges (Abdelmohsen et al. 2014). Intriguingly, members of the phylum actinobacteria have been identified as abundant members of sponge-associated microbial communities.

Research on sponge-associated actinobacteria communities has been widely used culture-dependent and several of them using metagenomic approach (Bibi et al. 2019). A metagenomic approach can better reveal all community members of the association because culturable microbes are thought to constitute less than 1% while the other 99% are unculturable microbes. On the basis of the metagenomic approach, several methods such as the Denaturing Gradient Gel Electrophoresis (DGGE) and the Terminal Restriction Fragment Length Polymorphism (TRFLP) have been widely applied to determine the community of sponge-associated actinobacteria (Simamora

et al. 2016). However, reports on the sponge-associated actinobacteria community by using Next-Generation Sequencing (NGS) technology are still limited. The NGS technology allows gene sequencing from complex environmental samples such as bacterial communities associated with marine sponges, including the analysis of actinobacteria communities in a comprehensive manner (Bokulich et al. 2012). This paper attempts to show the existence of *Callyspongia* sp. sponge-associated actinobacterial communities using a NGS approach.

Callyspongia sp. is one of the common sponges found in the Kepulauan Seribu Marine National Park, Indonesia. Several reports have shown that *Callyspongia* sp. has the capability to produce various secondary metabolites, and the active compounds showed cytotoxic activity and acyltransferase inhibitor (Gelani and Uy 2016; Kapojos et al. 2018). Bioactive compounds produced by sponges are thought to be the result of their interactions with associated bacteria. However, the community of actinobacteria associated with this marine sponge from Kepulauan Seribu Marine National Park has not yet been studied further. This study aimed to reveal the actinobacteria community associated with *Callyspongia* sp. based on a metagenomic approach using a NGS technology.

MATERIALS AND METHODS

Sponge collection

Specimens of the marine sponge *Callyspongia* sp. were collected at a depth of 16 m below sea surface of Panggang island Kepulauan Seribu Marine National Park (5°38'00"-5°45'00" S, and 106°33'00"-106°47' E), Jakarta, Indonesia. The samples were rinsed with sterile seawater and placed in sterile plastic trays. All sponges were stored in a freezer box before being examined. All sponges were identified by the Fisheries Diving Club of the Faculty of Fisheries and Marine Science (IPB University).

DNA extraction

One gram of sponge sample was crushed into powder in liquid nitrogen. The DNA extraction methods utilized Zymobiomics DNA Isolation Mini Kit, following the manufacturer's protocol. The DNA was stored at -20°C until further investigation.

Next-generation sequencing analysis

The bacterial 16S rRNA gene was used as DNA template for amplification of the V4 and V5 domains, with primers: 515F-907R modified to contain MiSeq Illumina platform (Illumina, Inc., San Diego, CA, USA) according to customer service by Axil Scientific, Singapore. PCR reactions were conducted using the Q5 Hot Start High Fidelity PCR 2X Master Mix (New England Biolabs, Ipswich, Massachusetts, USA) according to the two-step cycling standard protocol recommended by the manufacturer. The corresponding reaction began with the initial denaturation at 98°C for 30 seconds followed by 30 cycles of 98°C for 10 seconds, 55-65°C of annealing temperature for 30 seconds, 72°C for 30 seconds, a final extension at 72°C for 2 minutes and, lastly, hold at 4°C. Furthermore, the second stage PCR reaction that involved the addition of dual indices (Nextera XT i7 index and Nextera XT i5 index) was prepared with total volume of 50 µL. This particular reaction was started with an initial denaturation at 98°C for 3 minutes, 8 cycles of 98°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes was done prior to putting on hold at 4°C.

Analysis of the sequence data

Raw fastq files were de-multiplexed, quality filtered, and analyzed with QIIME V1.7.0. The tags obtained from the previous step were compared to the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences. Operational Taxonomic Unit (OTU) clustering and analysis were conducted by UPARSE v.7.0.1001 (<http://drive5.com/uparse/>). Moreover, the sequences were clustered into the OTUs with ≥97% similarity. Sequence frequently existed within each OTUs was chosen and screened to obtain representative sequence. This research focused on actinobacteria at the level of class, order, and family.

RESULTS AND DISCUSSION

DNA purity of 16S rRNA gene

DNA purity of *Callyspongia* sp. produced using the mini DNA Zymobiomics extraction method was 1.85 (UV 260/280). Interestingly, the amplification results discovered several DNA bands that appeared, indicating the isolation process was completely carried out and the sample contained more than one organism (Figure 1). There were more than one band found in the 300 bp molecular weights.

Diversity and taxonomic rank of bacteria associated with *Callyspongia* sp. sponges

A total of 107,223 raw sequences was found from *Callyspongia* sp. after trimming, denoising, and removing chimera sequences with 67,885 sequences were obtained and subjected to downstream analysis. It was discovered 97% similarities and were clustered into 500 OTUs. They consisted of were 7 different bacterial phyla, i.e. Proteobacteria (82%), Acidobacteria (12%), Planctomycete (2%), Actinobacteria (2%), Bacteroidetes (1.08%), Firmicutes (0.61%), and Cyanobacteria (0.09%) (Figure 2).

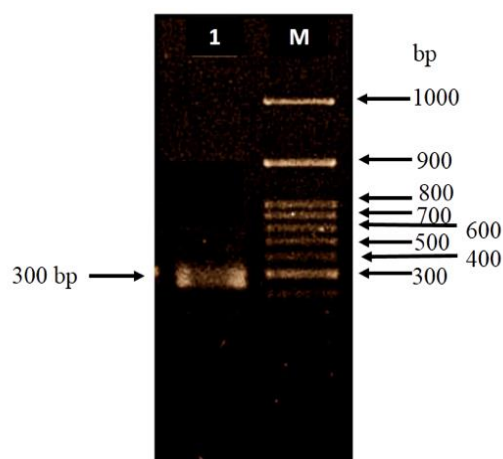


Figure 1. The amplified of 16S rRNA gene using V4-V5 domains from marine sponge *Callyspongia* sp. DNA sample (1: sample, M: Marker).

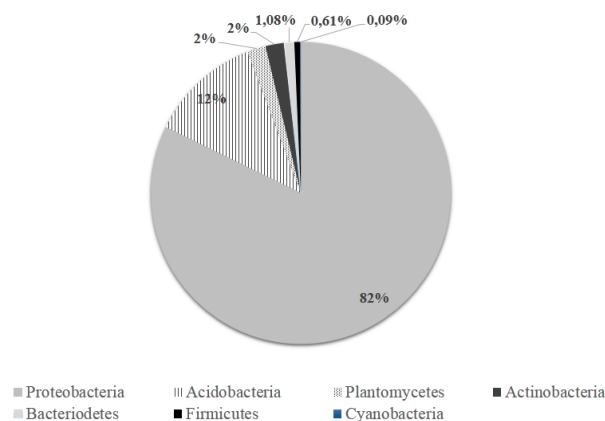


Figure 2. Diversity of bacteria community associated with marine sponge *Callyspongia* sp. at the phylum level

Sponge-associated actinobacteria community

The results showed that actinobacteria community structure associated with *Callyspongia* sp. varied in the class levels, i.e Actinobacteria, Coriobacteria, and Thermoleophilia with the abundance of 83%, 16%, and 1%, respectively (Figure 3). Furthermore, on the basis of the order level, Actinobacteria was divided into three groups, i.e Actinomycetales (83%), Coriobacteriales (16%), and Gaiellales (1%). Actinobacteria observed were also classified into 14 different families with the most dominant family was Microbacteriaceae (71%), followed by Coriobacteriaceae (16%), Micrococcaceae (7%), Corynebacteriaceae (6%), Propionibacteriaceae (5%), Dermabacteriaceae (4%), Geodermatophilaceae (1%), Mycobacteriaceae (1%), Nocardiaceae (1%), Nocardioidaceae (1%), and Gaiellaceae (1%). The other phyla with a relative abundance below 1% were Streptomycetaceae (0.90%), Williamsiaceae (0.70%), and Pseudonocardiaceae (0.45%) (Figure 4).

Discussion

Successful DNA extraction for obtaining good DNA purity was achieved. Sponge samples had high moisture content with concentrated liquid colors, thus it can reduce the reagents sensitivity and effectiveness used during the extraction process. The recovery of high quality and high purity of the representative DNA from environmental samples is very important. Therefore, the extraction efficiency of microbial DNA from the initial samples is certainly important for determining its quality (Kennedy et al. 2014; Fang et al. 2015).

The results showed that there were 7 different phyla sponge-associated and Proteobacteria known as a dominant phylum. A previous study indicated that Proteobacteria was the dominant phylum associated with *Callyspongia*

vaginalis (Bibi et al. 2019). *Callyspongia* sp. is included as Low Microbial Abundance sponges (LMA), recognized by the same group of Proteobacteria (Weisz et al. 2007; Giles et al. 2013). Proteobacteria perform different functions in host such as nitrogen fixation, host defense mechanism, and nitrification process (the oxidation of ammonia to nitrite) (Mohamed et al. 2010).

Acidobacteria has an important ecological role and extensive metabolic versatility in marine environment. The other phylum was found associated with *Callyspongia* was *Planctomycetes*. According to Sanchez et al. (2014), members of the genus *Acidobacteria* are very difficult to be cultured and currently available information is based on the 16S rRNA gene sequence. The genus *Acidobacteria* was also found to be associated with the sponge *Palthoa australiae* by 6% (Sun et al. 2014). They have important ecological functions in different marine environments, as shown to perform anaerobic ammonia oxidation (anammox). However, the anammox process is important in biological nitrogen removal from water containing high nitrogen and carbon ratios (Jeske et al. 2013).

Planctomycetes are a group of bacteria with the ability to produce various bioactive compounds. For instance *Streptomyces*, this genus has a large genome for a prokaryotic organism and a complex life cycle (Jeske et al. 2013). Planctomycetes are slow-growing bacteria, in their adaptation to various habitats including extreme environments this group of bacteria has developed an adaptation mechanism that is still unknown (Graca et al. 2016). Members of the Planctomycetes group have quite important ecological functions in the marine environment, namely through anaerobic ammonium oxidation (anammox). This anammox process is important to reduce the availability of biological nitrogen from water with high carbon and nitrogen content.

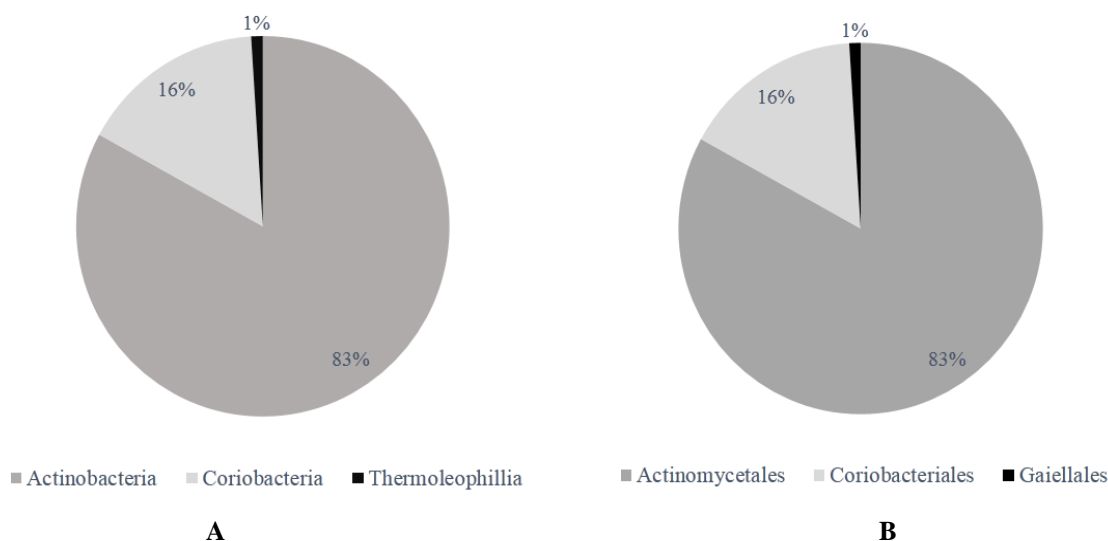


Figure 3. The existence of actinobacteria community associated with marine sponge *Callyspongia* sp.; A. At the Class level, B. At the Order level.

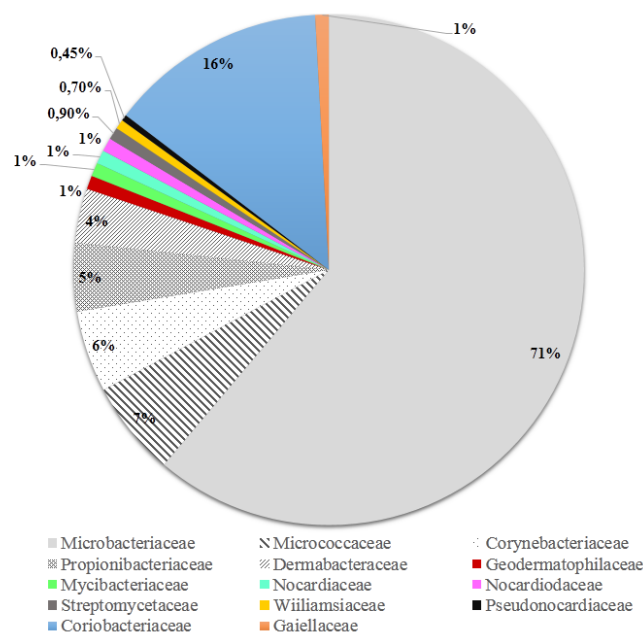


Figure 4. Diversity of the actinobacteria community associated with marine sponge *Callyspongia* sp. at the family level

The association of bacteria with the sponge plays an important role in the metabolism of carbon, nitrogen, and the production of secondary metabolites from the sponge as their host (Steinert et al. 2019; Fieth et al. 2015; Kennedy et al. 2014). Moreover, the role of associated bacteria plays important in the growth and development of sponges such as UV protection, defense against toxic compounds, stabilizing their skeletons, and decomposing residuals metabolites (Shick and Dunlap 2002). The different sponge contains bacterial communities consisting of mainly different bacterial species. This was suspected due to vertical transfer of species-specific bacterial communities.

Sponges are sessile and lack other anti-predator defense, secondary metabolites of bacteria can provide them with chemical defense the expression of secondary metabolites during predation may be independent of antibiotic expression in pure culture, the latter to have evolved for mutualism with higher animal of plant hosts (Harir et al 2018; Van der Meij et al. 2017). Sponges are a reservoir of exceptional microbial diversity and a major contributor to the total microbial diversity found in the world's ocean. Microbial communities exhibit little similarity in species composition or structure although a number of emerging properties related to community organization are evident (Thomas et al. 2016). The sponge host provides a special habitat for its symbiotic microbiota, and the microbial processes in the sponge holobiont have an impact on the health of the host and the surrounding ecosystem.

The investigation of *Callyspongia* sp. sponge-associated actinobacteria has successfully been conducted using NGS technology and identified 14 actinobacterial families, i.e. Microbacteriaceae, Micrococcaceae, Corynebacteriaceae, Propionibacteriaceae, Dermabacteriaceae, Geodermatophilaceae, Mycobacteriaceae, Nocardioideaceae,

Coriobacteriaceae, Gaiellaceae, Nocardioideaceae, Streptomycetaceae, Williamsiaceae, and Pseudonocardioaceae. The most dominant *Callyspongia* sp. sponge-associated actinobacteria family is Microbacteriaceae. The results of this study indicated that by using a metagenomic approach, the diversity of actinobacteria associated with the sponge *Callyspongia* sp. is more diverse when compared to cultivation techniques. The NGS technique has successfully described the diversity of actinobacteria that may not be detected using other molecular methods.

In this study, 14 families of microbes were obtained, being the dominant family was Microbacteriaceae. A previous report using Restriction Fragment Length Polymorphism (RFLP) method in *Callyspongia* sp. showed that this sponge has closed association with actinobacteria communities originated from the Micrococcaceae family (Qian et al. 2006). On the contrary, on the basis of DGGE method conducted by Yang et al. (2013), actinobacteria associated with *Callyspongia* sp. from Botany Bay (Sydney, Australia) were undetected. However, *Callyspongia* sp. from South Pacific Ocean resulted in, 4 bands that belonged to actinobacteria phylum. Sequencing results of the four bands were uncultured actinobacteria EU816864, uncultured actinobacterium FN556247, uncultured actinobacterium FN556246, and uncultured actinobacterium FN556248 (Park 2010). The DGGE method only describes the dominant species found in the sample, whereas RFLP result may be interfered with a PCR bias and gives complicated bands. Meanwhile, using NGS technology will read all the genomes of the DNA template randomly, thereby that the entire bacterial community contained in the sample will be completely detected. The use of different methods will affect to the community of sponge-associated actinobacteria.

Xestospongia sp. which is the same order as *Callyspongia* i.e. Haplosclerida was reported to have an association with Micromonospora, Streptomyces, Saccharomonospora, and Verrucosipora (Vicente et al. 2013). Actinobacteria found in *Halicondra* sp. which is the same family as *Callyspongia* sp. was Demospongia. There are five genera of actinobacteria associated with that sponge i.e. *Actinoalloteichus*, *Micrococcus*, *Micromonospora*, *Nocardiopsis*, and *Streptomyces*. Furthermore, Simamora et al. (2016) found five genera of sponge-associated actinobacteria with *Neofibularia* sp. detected by DGGE method which was identified as rare actinobacteria, i.e. *Ferrihrix*, *Lamia*, *Aciditerrimonas*, *Thermobispora*, and *Propionibacterium*.

The difference in actinobacteria diversity in sponge samples is caused by numerous factors, such as nutrients and environmental factors. Sponge-associated actinobacteria required simple nutrients available in the environment. Notably, the sponge contains a diverse actinobacteria group, however, their ecological functions are hardly known. Sponge-associated actinobacteria were reported could produce bioactive substances that may play an important role in the chemical ecology of sponge hosts (Sun et al. 2015). In addition to environmental factors, nutrition also affects the diversity of actinobacteria associated with certain sponges. Actinobacteria have the ability to break down complex compounds into simpler compounds to be used as nutrients for their growth. The existence of anthropogenic interventions will cause changes in the actinobacterial community associated with certain sponges (Undabarrena et al. 2016).

Actinobacteria might play a major role in the defense system of the life cycle in marine sponges (Bibi et al. 2019). A total of 74% of sponge-associated actinobacteria showed antagonism against assayed pathogenic bacteria (Liu et al. 2019). In addition, members of actinobacteria phylum might also be able to synthesize polyketides (bioactive compounds) in a marine sponge, indicating that they also played an important role in sponge defense systems against predators. The sponge is limited to innate immunity. They only employ physiochemical barriers, such as mucous layers, which act as sponge cellular defense. Interestingly, a lot of sponge-associated actinobacteria are known to have the potential symbiotic relations and/or could produce secondary metabolite productions. Several genera of actinobacteria i.e. *Acidimicrobium*, *Brachbacterium*, *Corynebacterium*, *Leucobacter* were found in various sponge species (Khan et al. 2012).

In 2013, the diversity of actinobacteria associated with sponges was constructed using a phylogenetic tree with a sequence length of 1300 bp. There were 60 different actinomycete genera known to be associated with sponges. The suborder Micrococccineae is known to be the most suborder found in sponges. The most common genera are *Microbacterium*, *Micrococcus*, and *Arthrobacter*. Marine sponges are also known to have the ability to associate with rare actinomycetes such as *Actinokineospora*, *Actinomadura*, *Amycolaptosis*, *Knoellia*, *Nonomuraea*, *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora*, *Verrucosipora* which are thought to have the ability to

produce novel metabolites. Several new species of actinomycete strains associated with sponges have been found, such as *Streptomyces axinellae* sp. nov. associated with the sponge *Axinella polypoides*, *Saccharopolyspora cebuensis* sp. nov. isolated from the sponge *Haliclona* sp. from the waters of Cebu, Philippines, and *Actinoalloiteichus hymeniacidonis* sp. nov. from the sponge *Hymeniacidon perleve* from Chinese waters. Three new actinomycetes species namely *Streptomyces tateyamensis* sp. nov., *S. marinus* sp. nov., and *S. haliclona* sp. nov., were successfully isolated from the sponge *Haliclona* sp. located in Tateyama City, Japan (Hentschel et al. 2012).

Culturable sponge-associated actinobacteria from *Callyspongia* sp. have been reported by (Retnowati et al. 2018). A total of 18 actinobacterial isolates were obtained by using a HVA medium. The largest number of actinobacteria members which was successfully isolated was *Streptomyces*. Phylogenetic analysis based on 16S rRNA gene sequence indicated that five culturable actinobacterial isolates were highly similar to *Streptomyces* sp., *Streptomyces rubrogriseus* AJ781373, *Streptomyces tendae* D63873, *Streptomyces violaceorubidus* AJ781374, and *Streptomyces tritolerans* DQ345779 (Retnowati et al. 2019). Intriguingly, in comparison of the findings with those of our recent studies confirms, the diversity of *Streptomyces* was only 0.9 % of the total diversity (Figure 3). This study supports the evidence from previous studies (Abdelmohsen et al. 2010, 2014), that *Streptomyces* is a common genus of sponge-associated actinobacteria. This means that *Streptomyces* is the most actinobacterial genus often found associated with various types of sponges observing by both culturable and metagenomic techniques.

Streptomyces spp. are not only benefiting from the resources of their hosts, and this interaction controls the expression of secondary metabolites produced by the actinobacteria. The exposure of the endosymbiont *Streptomyces* ACT-52A to *Applysilla resoa* promoted production of bioactive compounds with antibacterial activity (Mehbub et al. 2016). The factors involved in controlling the expression of these smbGCs are likely to be quite diverse, given the large degree of variability in the habitats and potential hosts, and how they are presumably influencing the secondary metabolite biosynthetic potential of *Streptomyces* symbionts (Adnani et al. 2017). Moreover, secondary metabolites from marine actinomycetes usually show distinct and often complex chemical structures, which help the development of further therapeutic agents efficiently (Elsayed et al. 2021). Molecular-based culture methods by adding nutrient supplements that mimic the marine conditions in nutrients content, oxygen gradient, pH, etc. have been highly efficient in overcoming technical bias and maximize the cultivable efficiency in recent times (Trindade et al. 2015).

The research showed that NGS technology can successfully reveal rare actinobacteria groups associated with the *Callyspongia* sp. that might have been undetected by culturable approach. Among all taxa, bacteria from the order actinomycetes are the active substance producers of marine microbial communities that form a stable and persistent population across different marine ecosystems

(Mahapatra et al. 2019). For years, actinobacteria were isolated from aquatic sources with limited usages. However, the introduction of metagenomic techniques in recent times has considerably boosted the genomic sequencing of marine actinobacteria exploring their huge biosynthetic potential from marine and other sources (Prichystal et al. 2016).

The use of NGS technology for describing microbial diversity has greatly enhanced the knowledge of community structure in sponge-associated actinobacteria. Supporting these results, the use of V4 and V5 domains showing the conservation and the hypervariable region of this NGS method could provide comprehensive importance on microbial community in sponges. The Miseq Illumina platform will produce more reads with a shorter sequence length of 250-300 bp. The length of the resulting sequence is not as long as the sequence produced by conventional sequencing, but the length of the sequence is quite feasible to be used in the taxonomic analysis (Vasileiadis et al. 2012). In addition, the quality of the resulting sequence is also higher than other platforms, so that the nucleotide that is read is more reliable.

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