Vietnam Journal of Biotechnology **21**(4): 745-757, 2023

METAGENOMIC CHARACTERIZATION OF ARCHAEAL AND BACTERIAL COMMUNITIES ASSOCIATED WITH CORAL, SEDIMENT, AND SEAWATER IN A CORAL REEF ECOSYSTEM OF PHU QUOC ISLAND, VIETNAM

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Received: 07.11.2023
Accepted: 27.12.2023

SUMMARY

Recent advancements in metagenomics, particularly in the studies of conservative 16S rRNA sequences, have significantly accelerated our understanding of the relationship between corals and their associated microbial communities. While bacteria are known to be closely linked with corals, there is limited understanding of the connections between archaea and corals. Unlike previous 16S rRNA studies conducted in similar tropical coral reef ecosystems, we analyzed both the archaeal and bacterial communities associated with *Acropora* sp. and *Lobophyllia* sp. corals, as well as the surrounding sediment and water columns in Phu Quoc Island, Kien Giang Province, Vietnam. The data collected were sequenced using the 16S rRNA sequencing method and further analyzed using bioinformatics tools in the R programming language, employing DADA2 and phyloseq pipelines. We examined the compositions and diversity of bacteria and archaea in coral, sediment, and water column samples to establish potential connections between these two domains. The results revealed that archaea constituted a small percentage of all samples, averaging 3.18% in coral mucus and reaching an average of 7.49% in sediment samples. Among the most abundant archaeal taxa were Crenarchaeota and Nanoarchaeota, alongside bacterial taxa Gammabacteria, Cyanobacteria, and Desulfobacteria, which are associated with important metabolic processes within coral hosts. Alpha and beta diversity analyses confirmed the highest archaeal diversity in sediment samples and the distinct existence of microbial communities in each biotope. These findings complement our knowledge of archaea's presence and potential roles in the coral-associated microbiome.

Keywords: 16S rRNA, metagenomics, archaea, bacteria

INTRODUCTION

Coral reefs are regarded as one of the most diverse and complex marine ecosystems, encompassing a multitude of coral species (Wagner *et al.*, 2020; Zhang *et al.*, 2021a, 2021b). Millions of people depend on coral reefs for the development of industries such as fisheries, tourism, food, and medicine (Eddy *et al.*, 2021). Additionally, coral reefs provide a favorable habitat for numerous microbial species and play a crucial role in coastal protection against erosion (Elliff, Silva, 2017). However, coral reefs face the phenomenon of coral bleaching, which reduces their coverage (Gardner *et al.*, 2003; Bruno, Selig, 2007; Silverstein *et al.*, 2015; Hughes *et al.*, 2018; Harrison *et al.*, 2019). This phenomenon is primarily attributed to climate change-induced ocean warming, which negatively impacts the symbiotic relationship between corals and their associated microorganisms (Ritchie, 2006; Rosenberg *et al.*, 2009; Lesser, 2011; MacKnight *et al.*, 2021). This highlights the urgent need for research on the diversity and interactions of microbial communities in coral reef ecosystems.

Coral provides three habitat niches for bacteria: the surface mucus layer, coral tissue (including the gastrovascular cavity), and the calcium carbonate skeleton, each hosting distinct bacterial communities (Bourne, Munn, 2005; Koren, Rosenberg, 2006). Initially, research on coral-associated microbes focused on the surface mucus layer, utilizing traditional culture-based methods. These studies revealed that this layer harbors diverse and abundant beneficial bacteria, including nitrogen-fixing bacteria and chitin-degrading bacteria (Ducklow, Mitchell, 1979; Williams *et al.*, 1987; Shashar *et al.*, 1994; Lesser *et al.*, 2004). However, due to the limitations of culture-based approaches, only a small fraction of environmental microbes (0.001-0.01%) were isolated (Kogure *et al.*, 1979).

Recently, the continuous development of metagenomics has provided a broader overview of microbial communities in the environment, particularly enabling the study of environment-independent microbial communities using 16S rRNA sequences (Pootakham *et al.*, 2017). Next-generation sequencing technology (NGS) has been utilized to sequence and analyze the 16S rRNA gene of microbial communities in various coral species, aiming to assess microbial diversity and identify prevalent symbiotic taxa in different coral species (Meenatchi *et al.*, 2020). Overall, while there is extensive research on bacteria, studies on archaea are relatively scarce. Attempts to characterize 16S rRNA archaea have been proven challenging due to their low abundance in coral mucus, with many studies struggling to identify them and often detecting them at very low levels (<0.5%) (Kellogg, 2004; Littman *et al.*, 2011; Frade *et al.*, 2016). Nonetheless, archaea are present in coral reef ecosystems without forming direct relationships with corals due to their interaction with anaerobic bacteria in potential anaerobic microenvironments within coral mucus layers (Wegley *et al.*, 2004). Investigations of archaea's unique metabolic capabilities, particularly their participation in anaerobic metabolism, may expand our understanding of the scope of interactions between marine hosts and their associated microbial communities.

To gain a deeper understanding of microbiota in coral reef ecosystems, we conducted a metagenomic analysis of the diversity and composition of archaea and bacteria in samples collected from Phu Quoc

Island, Kien Giang, Vietnam. Samples were acquired from coral reefs inhabited by *Acropora* sp. and *Lobophyllia* sp., as well as from the sediment layer beneath the seabed and the water column above. By implementing established bioinformatics pipelines and employing appropriate statistical analysis methods, we aimed to elucidate the characteristics of archaea and bacteria within the coral-associated microbiomes.

MATERIALS AND METHODS

The 16S rRNA data in this study was provided by the Department of Bioinformatics, Institute of Biotechnology, Vietnam Academy of Science and Technology (VAST). Six samples were collected from healthy coral branches on Phu Quoc Island, Vietnam (9°55′20.6″N 104°01′16.4″E) in May 2020. These colonies hosted *Acropora millepora*, *Acropora formosa*, and *Lobophyllia* sp. (also known as brain corals). Additionally, five samples each from the sediment and the water column surrounding the coral colonies were gathered simultaneously. A total of 16 samples were utilized for DNA extraction and purification. Subsequently, polymerase chain reaction (PCR) amplification of the microbial 16S rRNA gene was carried out using the following primer set: 5'-CAGCMGCCGCGGTAA-3' (forward) and 5'-GTGCTCCCCCGCCAATTCCT -3' (reverse). The amplified libraries underwent sequencing using the Illumina MiSeq short read-sequencing system (San Diego, USA). The forward and reverse reads were 250 bases in length and were provided in FastQ format.

Processing and downstream analysis of 16S rRNA sequencing reads were performed in R using RStudio version 4.3.1. We implemented the Bioconductor's DADA2 pipeline (Callahan *et al.*, 2016) for quality control, trimming, and filtering of sequences. The first 10 bases containing primers and adapters were trimmed from all reads. Additionally, reads containing ambiguous bases or having a quality score lower than 20 were excluded from each sample. Subsequently, forward reads were truncated at position 240, while reverse reads were truncated at position 210. Following this, forward and reverse reads were merged and clustered into amplicon sequencing variants (ASVs) using a similarity threshold of 97%. Taxonomic classification was then assigned to each ASV using the SILVA database version 138.1 (https://www.arb-silva.de/). The average relative abundance of taxa was expressed as mean ± standard deviation. Further computational analysis, statistical tests, and visualization were performed using the R's phyloseq, vegan, and ggplot2 packages.

Alpha and beta diversity analyses were also conducted to evaluate the microbial diversity of the collected samples. Alpha diversity metrics assess the taxonomic richness within individual communities or samples, while beta diversity examines the diversity across distinct communities (Andermann *et al.*, 2022). Alpha analysis indices including Observed, Chao1, and Shannon were computed, followed by testing the differences between indices of different sample types using analysis of variance (ANOVA). For beta analysis, we employed principal coordinates analysis (PCoA), known as multidimensional scaling, which is a technique used to quantify and visualize the distance between observations or samples in a low-dimensional space (Zuur *et al.*, 2007). Bray-Curtis's dissimilarity method was

applied to measure sample distances (Bray, Curtis, 1957). Subsequently, analysis of similarities (ANOSIM) was used to assess statistically significant differences between groups of microbial communities with 1000 permutations.

RESULTS AND DISCUSSION

Taxonomic composition of archaea and bacteria

A total of 721,652 sequencing reads were produced from 16 samples. After filtering out low-quality reads, trimming, denoising, and removal of chimera, 301,399 reads remained and were clustered into 4,101 ASVs using the standard DADA2 pipeline. Using the SILVA database version 138.1 for taxonomic classification, taxa belonging to archaea and bacteria were identified in all samples with varying compositions (Table 1). The dominant component of all sample types was bacteria with an abundance higher than 90% in all samples. The microbiome associated with *A. formosa* and *A. millepora* exhibited a relatively low occurrence of archaea, (<2.0%) while brain corals contained a considerably high composition of archaea (6.65% to 7.39%). Sediment samples contained the highest abundance of archaea, averaging 7.49% per sample. The water column also harbored certain archaea, ranging from 3.57% to 8.10% in relative abundance.

Table 1. Relative abundance of archaea and bacteria in corals, sediment, and water column.

Location	Sample	Archaea (%)	Bacteria (%)
Corals	AF1	0.51	99.49
	AF2	0.83	99.17
	AM1	1.75	98.25
	AM2	1.97	98.03
	BR1	7.39	92.61
	BR2	6.65	93.35
Sediment	SD1	9.15	90.85
	SD2	8.76	91.24
	SD3	6.87	93.13
	SD4	6.36	93.64
	SD5	6.33	93.67
Water column	WC1	3.57	96.43
	WC2	6.25	93.75
	WC3	6.02	93.98
	WC4	5.47	94.53
	WC5	8.10	91.90

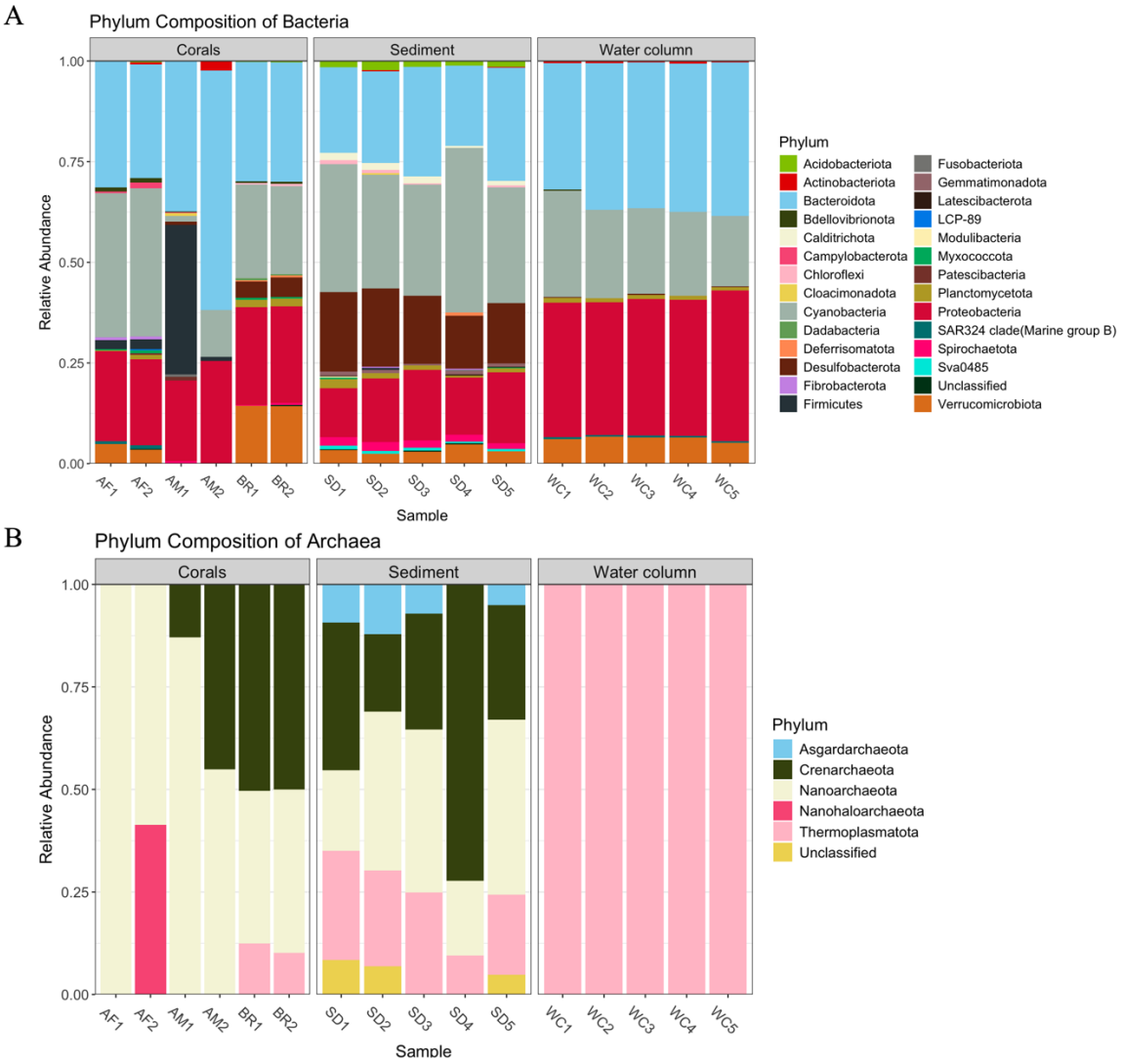

Figure 1. Composition at phylum level of bacteria (A) and archaea (B) in coral, sediment, and water column samples (AF: *A. formosa*, AM: *A. millepora*, BR: brain corals). The abundance threshold was set to 0.2% to filter out all low-abundant phyla.

Using the SILVA database, we identified a total of 5 phyla, 7 classes, 6 orders, 4 families, and 2 genera of archaea in all samples. However, no archaeal species were characterized. Regarding the bacterial community, 27 phyla, 44 classes, 95 orders, 112 families, 167 genera, and 12 species were found. Figure 1 depicts the phylum-level composition of both archaeal and bacterial communities.

Archaea were considerably more diverse in sediment compared to other specimens, hosting four out of five phyla detected in all samples. These include Asgardarchaeota, Crenarchaeota, Nanoarchaeota, and Thermoplasmatota, averaging 8.4±3.0%, 36.7±20.8%, 31.8±11.8%, and 20.8±6.8% in abundance respectively, with Nanoarchaeota and Crenarchaeota consistently making up more than 56% of archaea across all

sedimentary samples. This could also be observed in coral samples, where the abundance of Crenarchaeota ranged from 12.5% to 45.1% in *A. millepora* samples, and 50.2% in brain corals, but they were absent in *A. formosa.* Our result aligned with previously described observations by Littman *et al.* (2011) that Crenarchaeota might not be present significantly nor play a dominant role in *A. millepora*. However, it has been recorded that Crenarchaeota sequences increased by 40% after natural bleaching events, suggesting a potential adaptation of these species to temperature and pH-related stress (Thurber *et al.*, 2009; Littman *et al.*, 2011).

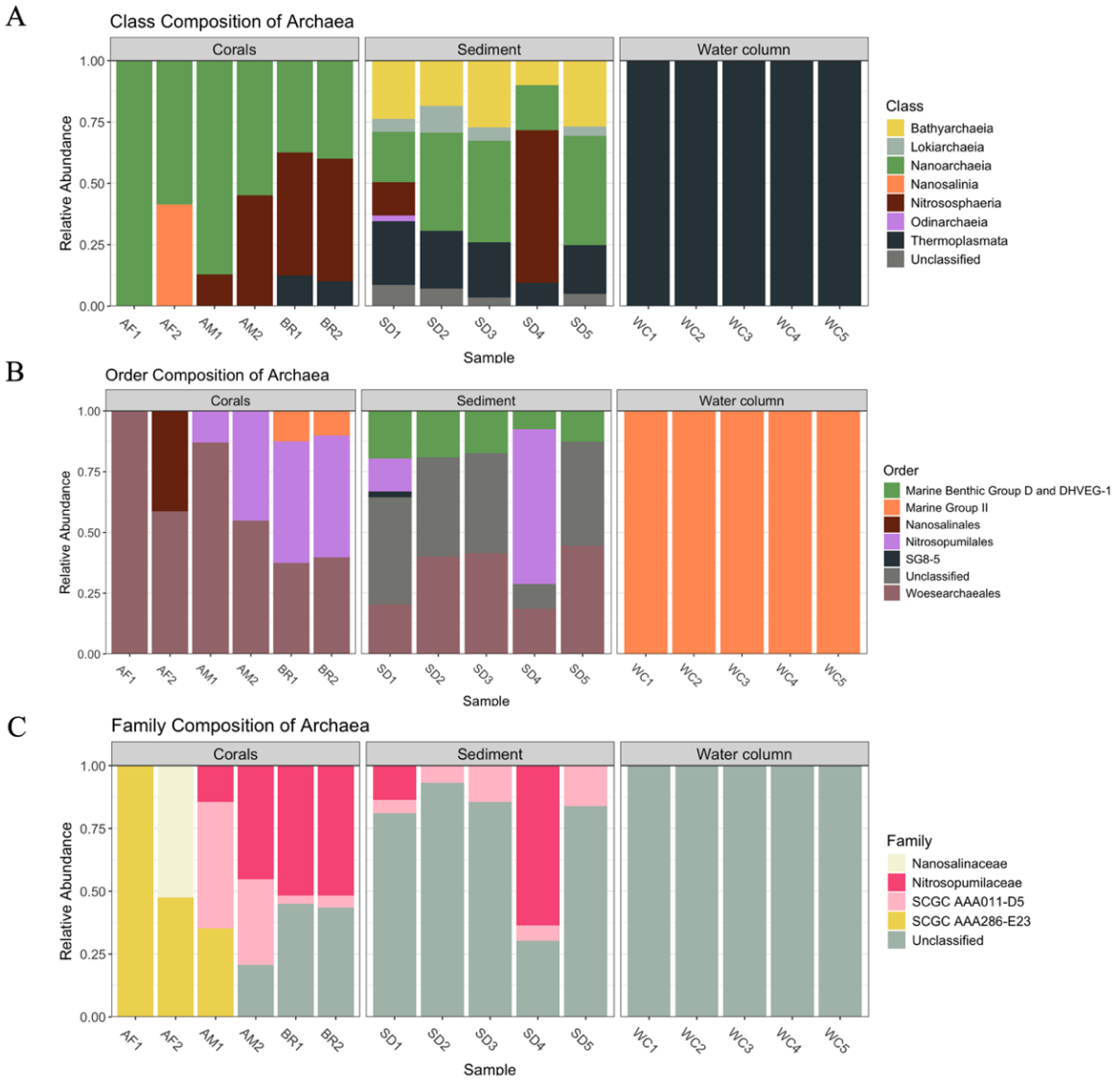

Figure 2. Composition of archaea at class level (A), order level (B), and family level (C) in coral, sediment, and water column samples.

Further investigations of the archaeal composition at more specific taxonomic levels i.e., class, order, and family revealed that Woesearchaeales, an order in the Nanoarchaeota phylum, along with Nitrosopumilales which is a member of the Crenarchaeota phylum, predominated coral samples (Fig. 2). Unlike Nitrosopumilales, Woesearchaeales were more widely distributed in all corals as well as in the nearby sediment. They accounted for 75.1±21.9% of archaea inhabiting *Acropora* sp., 38.6±1.7% in brain corals, and 33.0 ± 12.4% of all sedimentary archaea. Members of Nanoarchaeota have been known for their ability to survive in extreme habitats (Shu, Huang, 2022). Their environmental tolerance may be due to their symbiotic relationships with other archaea. They carry genes associated with glycolysis and gluconeogenesis but lack genes related to nitrogen pathways, suggesting interactions with archaea such as Nitrosopumilales, which are involved in nitrogen metabolism (Baker *et al.*, 2020; Chen *et al.*, 2023).

All archaea found in water column samples belonged to the Marine group II order of Thermoplasmata class, which was also present in brain coral samples and sediment. Unique archaeal taxa were found in sediment biotopes. Marine Benthic Group D (MBG-D) accounted for 15.3±5.1% of the total archaea in sediment samples. MBG-D is associated with carbon remineralization from organic-rich sediments, producing compounds that can be utilized by other microorganisms (Zhou *et al.*, 2019).

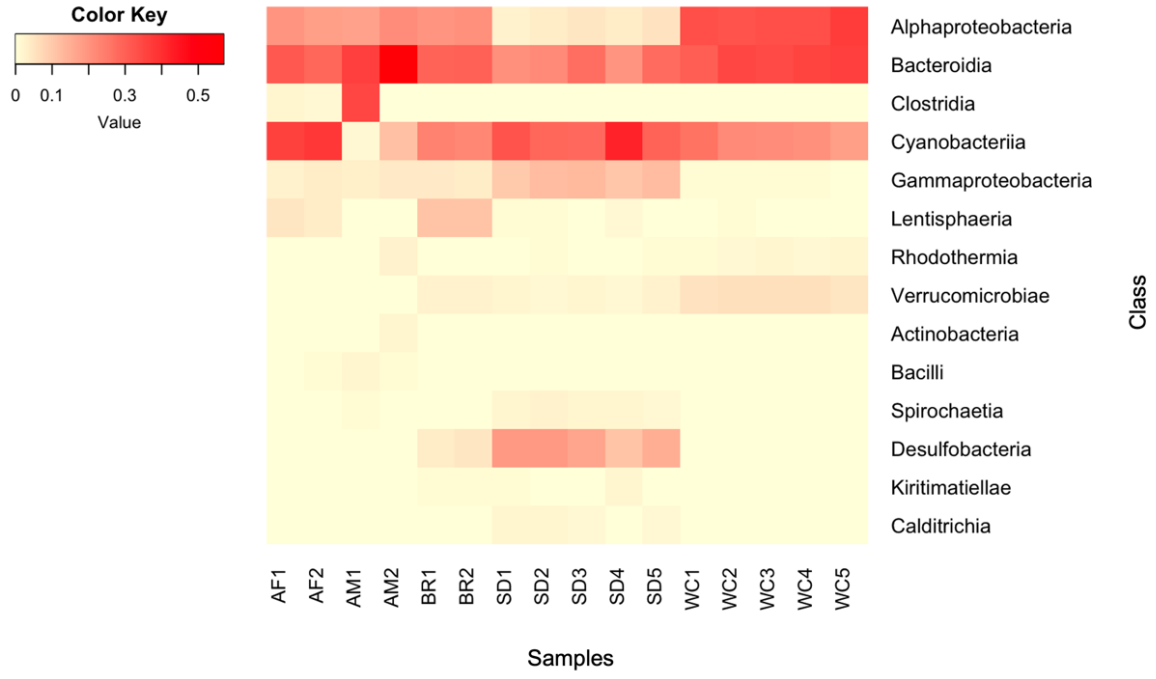

Figure 3. A heatmap illustrating the relative abundance of the most common bacterial classes. A yellow-to-red gradient was displayed with red indicating higher abundance. Only bacterial classes with abundances exceeding 1.5% in all samples were included.

Defining and assigning taxonomic classification to archaea remains an ongoing area of research. In sediment samples, an average of 6.7% of phyla and 35.8% of orders remained unclassified. This proportion tends to increase at more specific classification levels in all three biotopes (Fig. 2), highlighting the gaps in our current understanding of archaeal taxonomy.

The presence of bacteria alongside archaea is crucial to understanding the functions of microbiome in coral reef ecosystems. With regards to bacteria, all samples were dominated by Bacteroidota, Proteobacteria, and Cyanobacteria phyla, which collectively accounted for over 90% of the bacterial community (Fig. 1A). Cyanobacteria is responsible for photosynthesis-dependent nitrogen fixation in scleractinian coral holobionts (Lesser *et al.*, 2004). Sequences of these nitrogen-fixing species are homologous to *Synechococcus* sp., which was also detected in our analysis (data not shown). A more detailed examination of bacterial classes was presented in Figure 3, which shows that the most prevalent Proteobacteria members included Alpha- and Gamma-proteobacteria, with Alphaproteobacteria dominating coral (19.4±1.6%) and water column samples (34.0±2.0%), and Gammaproteobacteria being present abundantly in sediment (11.6±1.3%). This is similar to the results of Frade *et al.* (2016), showing a great abundance of these two Proteobacteria classes in reef-building coral systems. In sediment, Desulfobacterota was present with relatively high abundance (16.9±2.8%) but was barely detected in water column and corals (<0.05%). Bacteria exhibiting sulfur-consuming characteristics including Desulfobacterota and Proteobacteria are often abundant in sulfur-rich intermediate and deep layers and exist in distinct niches from archaea in extreme water environments (Chen *et al.*, 2023).

Taxonomic richness and diversity of archaea and bacteria

Alpha and beta analyses were conducted to examine the archaeal and bacterial diversity in the collected samples. Figure 4 shows a significant difference in Observed, Chao1, and Shannon diversity for bacterial and archaeal communities. We observed that the diversity of bacteria was most significant in coral branches, but less prevalent in the surrounding sediment. Interestingly, archaea seem to increase in abundance in sediment environments as indicated by the highest diversity measures (p-value < 0.0001) even though they only accounted for no more than 9.15% of the total microbial composition in a single sample (Table 1). Further investigation might be needed to elucidate how the biological interaction between these two kingdoms influences their respective presence in the coral-associated microbial community.

Beta analysis via PCoA demonstrated that samples formed distinct clusters, indicating differences in composition or structure (Fig. 5). ANOSIMS tests yielded significant p-values of 0.002 and 0.001 for archaea and bacteria, respectively. Samples located closer together in the plot exhibited greater similarity, while those further apart were more dissimilar. Notably, archaeal clusters of coral and sediment samples were relatively distinct with minor overlap. This result supports previous observations about composition (Fig. 2) where sediment samples and coral samples shared several archaeal orders.

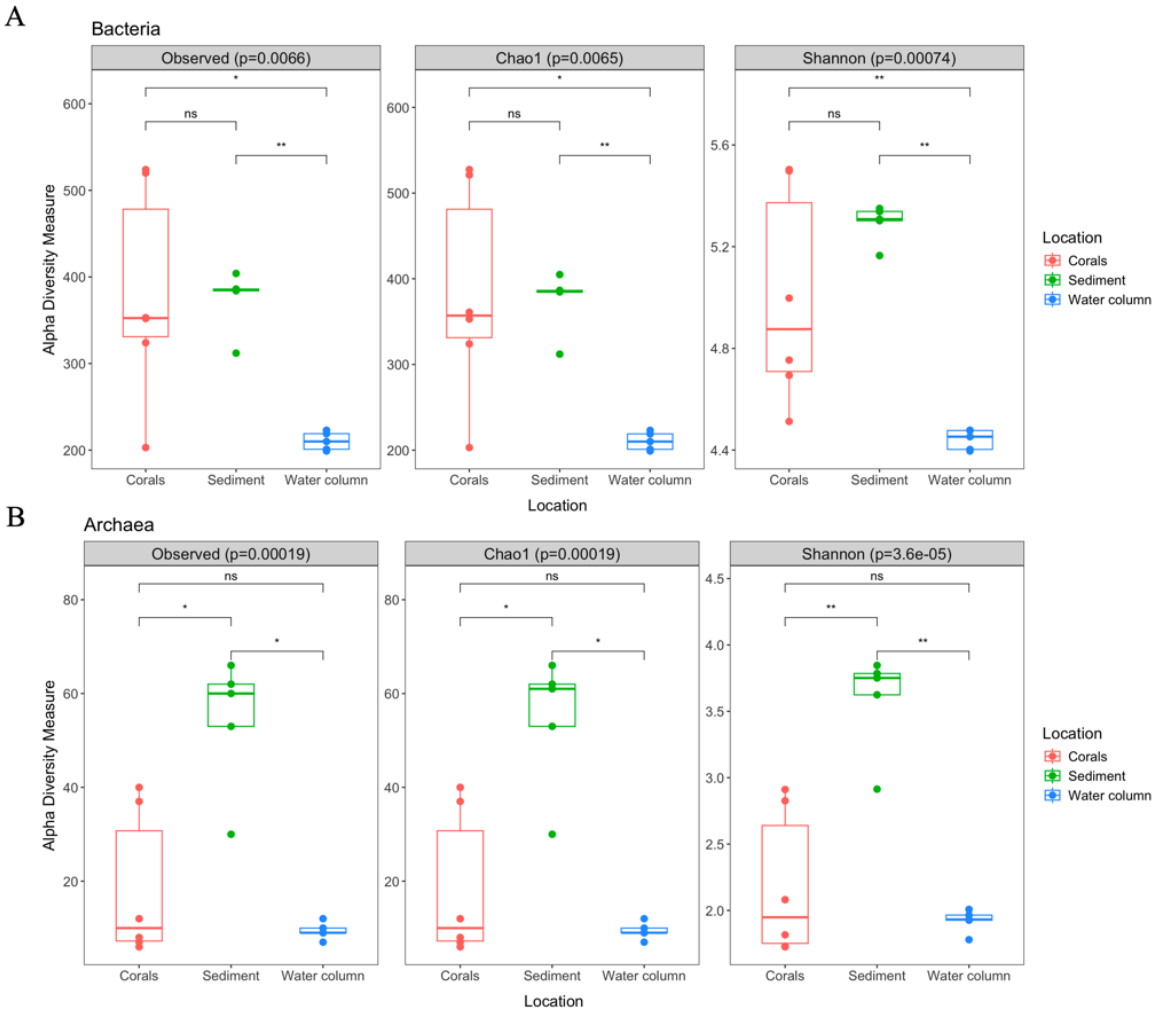

Figure 4. Alpha diversity estimations including Observed, Chao1, and Shannon calculated for the bacterial (A) and archaeal (B) communities. ANOVA test was used to evaluate whether the difference in diversity between samples was statistically significant, and the non-parametric Wilcoxon test was applied for pairwise comparison (*: p-value<0.05, **: p-value<0.01, ns: not significant).

Previous research has established that, unlike bacteria, archaeal communities do not respond to environmental factors such as host species, site location, or geographic distance, and potentially form their distinct microbiome in the coral holobionts after being transported into coral mucus from the water column (Kellogg, 2004; Frade *et al.*, 2016). Due to the considerable variation of coral samples, our analysis showed no significant difference in alpha diversity metrics of coral samples and water column samples. Increasing the sample size to account for the characteristics of each coral species is essential to draw further conclusions on the origins of archaea in coral hosts.

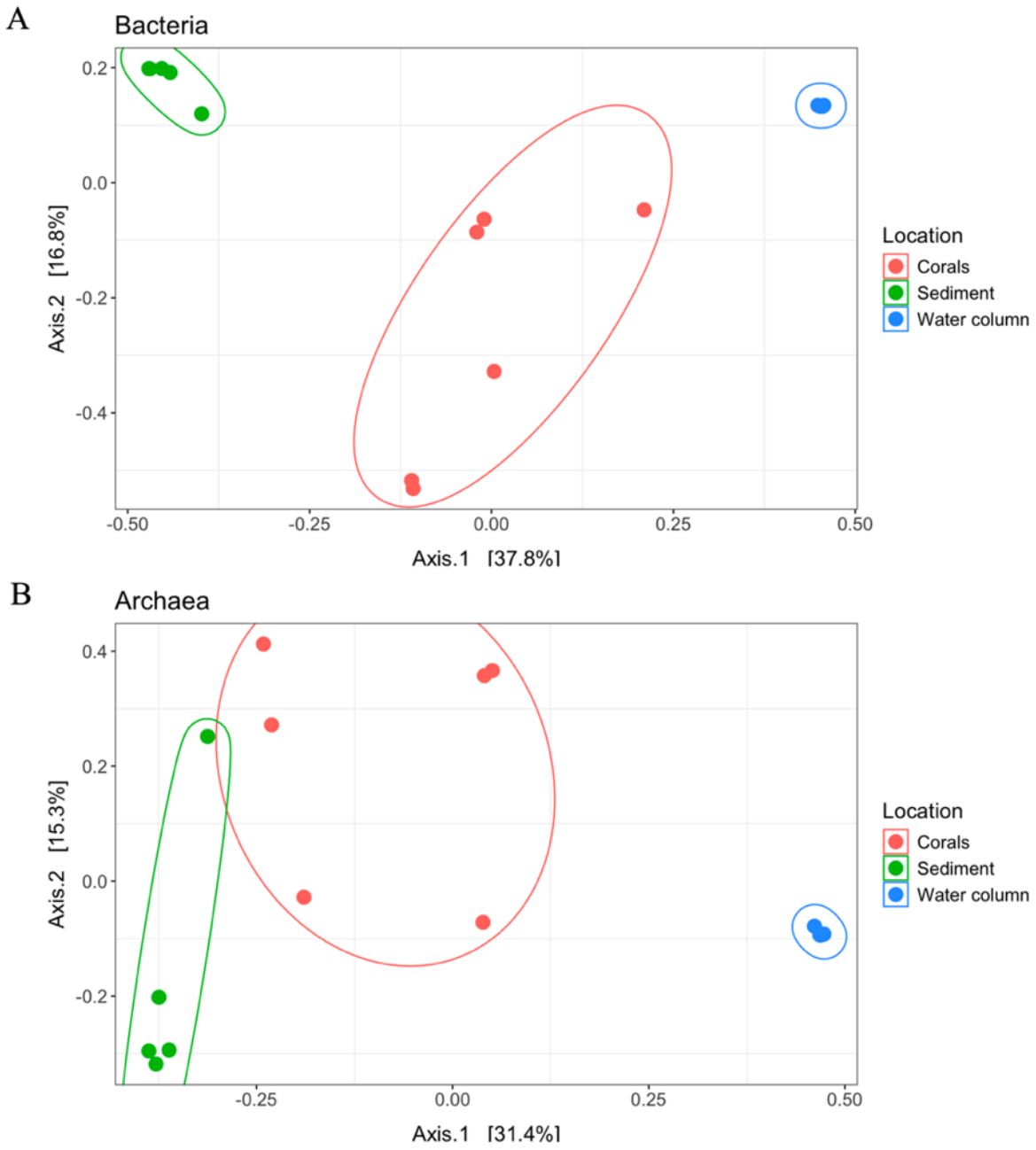

Figure 5. Principal Coordinates Analysis (PCoA) of the bacterial (A) and archaeal (B) communities. The plot's axes represent the maximum variation among samples in the data in orthogonal directions.

CONCLUSION

Our metagenomic analysis provided a preliminary overview of archaeal and bacterial compositions and diversity within a coral reef ecosystem in Phu Quoc Island, Kien Giang Province, Vietnam. However, the insufficient number of samples used in

our studies, particularly for different coral species, has yet allowed for a definitive conclusion regarding microbial differences among coral samples, despite the identification of several dissimilarities between species of corals. Each coral species may harbor a unique community of archaea and bacteria, exhibiting diverse functions and adaptability to the environment. Further exploration, including co-occurrence analysis of archaea and bacteria in corals and their surrounding environment, could yield deeper insights into the significance of the microbiome and its metabolic processes, potentially contributing to coral health.

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