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COMPARATIVE ANALYSIS OF EUKARYOTIC MICROBIAL COMMUNITIES ASSOCIATED WITH *ACROPORA FORMOSA*, SEDIMENT, AND SEAWATER IN A CORAL REEF ECOSYSTEM OF WHALE ISLAND, NHA TRANG BAY, VIETNAM

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SUMMARY

In this study, eukaryotic microbial communities associated with coral *Acropora formosa* and its natural surroundings, sediment and seawater, in a coral reef ecosystem of Whale Island, Nha Trang Bay, Vietnam were investigated. First, genetic material was taken from *Acropora formosa*'s surface mucus layer (SML) as well as the sediment underneath and seawater above the colonies from four different sampling locations in a coral reef ecosystem. Subsequently, the data were sequenced using 18S rRNA gene amplicon sequencing method. Sequences (reads) were then analyzed in Rstudio version 4.2.0. Bioinfomatic tools such as DADA2 pipeline clustered the sequences into amplicon sequence variants (ASVs), to which the taxonomy was assigned using SILVA 132 database. The majority of the sequences was categorized at the kingdom and phylum levels, but fewer sequences were identified at genus and species level. The visualization of the results revealed changes in abundance and composition of the eukaryotic communities in all samples. The results demonstrated that phylum Dinoflagellata had the highest relative abundance in coral samples. Meanwhile, Ochrophyta was the most prevalent phylum in seawater samples. Notably, after filtering out the sequences with abundance less than 2%, only genus *Symbiodinium* appeared significantly in coral samples. The composition of samples from coral sampling sites was more consistent. The same was true for samples of seawater, whereas the composition of sediment samples varied more. Alpha and beta diversity indices confirmed that there were significant differences ($p < 0.05$) in abundance and composition of eukaryotic communities among three different habitats. These findings come as the first effort to explore the diversity of eukaryotic communities in different habitats and could be valuable for further study in functional profiling or metabolic functions of microbial communities in the coral ecosystem.

Keywords: eukaryotic microbials communities, coral *Acropora formosa*, sediment, seawater, R programing language

INTRODUCTION

Accounting for approximately a quarter of all marine biodiversity, coral reef ecosystem is one of the ecosystems with the greatest biodiversity on Earth, providing habitat for a variety of sea creatures (Wagner et al., 2020). Millions of people worldwide rely on coral reef ecosystems through services such as fisheries, tourism, coastal protection, food and medicine (Eddy *et*

al., 2021). However, tremendous declines in coral cover have resulted from several severe coral bleaching events that have been reported over the past few decades (Harrison *et al.,* 2019). Rising ocean temperatures due to global climate change disrupt the symbiotic relationship between the coral host and its endosymbiotic algae, and are widely acknowledged to be one of the main drivers of coral bleaching (Lesser, 2011, Rosenber *et al.,* 2009). This serves as an impetus for researches on coral and its microbiomes communities. However, the interaction between coral and eukaryotic microbials communities has received less attention, despite their significant relationship (Ainsworth *et al.,* 2017).

To gain a deeper understanding of the topic, we attempted to use metagenomics to get access to the functional gene composition of the microeukaryotic communities in samples taken from Whale Island, Van Phong Nha Trang, Vietnam. Metagenomics, which has emerged in the last decade, is believed to revolutionize the field of microbial ecology by allowing researchers to study the genetic material of microorganisms in their natural settings (Thomas *et al.,* 2012). *Acropora formosa* is a stony coral (Schoch *et al.,* 2020), which can establish mutualistic symbioses based on nutrient exchange with photosynthetic dinoflagellates of the genus *Symbiodinium* (Rosic *et al.,* 2015). Samples from coral *Acropora formosa* from 4 different samplings locations in a coral reef ecosystem were gathered in this research. Samples from the sediment below and the seawater above the coral colonies were also collected to study the biodiversity of microeukaryotic communities in the coral reef ecosystem.

The samples' genetic material was sequenced using Illumina's short-read sequencing technique, which is not only inexpensive but also offers higher sequence fidelity (Slatko *et al.,* 2018). In order to process this large amount of data, bioinformatics tools such as R programming language were used, with the hope to learn the underlying structure and extract meaningful information from the raw data. One of the most important tools that were used in this study is the DADA2 pipeline, which gave us an amplicon sequence variant table (ASV table) as an output. Then we assigned taxonomy to the sequence variants with the SILVA 132 database and visualized the results with different packages in R. This included plotting the composition and abundance of eukaryotic microbes in each sample at different levels. Eventually, alpha diversity and beta diversity analysis along with statistical tests were carried out to compare the richness and diversity of the microeukaryotic communities in our samples.

MATERIALS AND METHODS

Sample collection

The data utilized in this study were provided by the Department of Bioinformatics (Vietnam Academy of Science and Technology (VAST). Samples were taken in May, 2020 from 4 different sampling locations within an area of 600 m^2 (20 m x 30 m) in a coral reef ecosystem in Whale Island, Van Phong Nha Trang, Vietnam (12°39.1'N, 109°23.9'E). Four samples were gathered from the mucus layer of visually healthy *Acropora formosa* coral colonies. Four samples of the sediment below the colonies and four samples of the seawater above the colonies were taken, 12 samples in total.

Sample sequencing

The sample's genomes were extracted by viral nucleic acids extraction kit (Roche, Diagnostics, Meylan, France). Then they were sequenced using Miseq short-read sequencing platform (Illumina Inc., San Diego, USA), and were provided as FastQ files. Composition and abundance of coral-associated microeukaryotes were investigated using their 18S rRNA gene amplicon sequences.

Sequence processing and taxonomic assignment

The data were then processed through DADA2 pipeline (Callahan *et al.,* 2016) in

RStudio version 4.2.0. DADA2 generates fewer false-positive sequences while detecting real biological variation that OTU methods miss (Callahan *et al.,* 2016). After inspecting the read quality profile, we truncated the forward reads at position 240 and reverse read at position 200, removing any reads with a QC less than 25. Next step involves learning the error rate for our dataset, dereplication and applying the sample inference algorithm to the data. The filtered and denoised sequences were then merged to construct a sequence table. After chimeras were eliminated, the finished product is an ASV table. We assigned taxonomy to the sequence variants using the SILVA 132 database (Quast *et al.*, 2013). This database offers an excellent resource for high-throughput data classification, which was retrieved using next-generation sequencing methods (Quast et al., 2013).

Microeukaryotic composition and abundance analysis

The data were then imported into the phyloseq package (McMurdie, Holmes, 2013) for further analysis. Our ASV table, the sample data, and the taxonomy were all combined into a single phyloseq object. Microbiomes that do not belong to the kingdom Eukaryota were removed using the subset function in order to investigate only the microeukaryotes in the samples. Then we analyzed the microeukaryotic composition and abundance, in which the metagenomic data were accessed and visualized with stacked bar plots using the package ggplot2, as well as heatmaps using the package pheatmap.

Alpha diversity analysis

A rarefaction curve was generated using the vegan package (Oksanen *et al.,* 2016). Then, using the phyloseq package, alpha diversity analysis was performed. The indices Observed, Chao1 and Shannon for each sample were calculated, which represent the degree of species richness and diversity in that sample. Kruskal–Wallis test, a non-parametric test, was carried out to determine whether there is a significant difference in richness among three sample groups (coral, seawater and sediment). Statistical significance is considered to exist when $p < 0.05$.

Beta diversity analysis

Beta diversity analysis was conducted to assess the species composition differences amongst our samples. First, we plotted the hierarchical clustering of the samples to get a quick overview of how they are related to each other. We normalized across samples using variance stabilizing transformation with the DESeq2 package. Euclidean distance matrix was computed (with the Ward method) and visualized by utilizing the ggdendro package. This is followed by an ordination method, a visual representation of sample relatedness on the basis of dimension reduction. Initially, NMDS (non-metric multidimensional scaling) method based on the dissimilarity matrix between samples was used. However, limited information was gained because of the substantial sample overlap. Instead, Principal Coordinates Analysis (PcoA), a type of multidimensional scaling, was generated using phyloseq package. The PCoA was made with the DESeq2 transformed table and plotted with the plot ordination function. Data transformation was performed to avoid the slight overlap between seawater samples.

RESULTS

After chimera were eliminated, the original sequencing table lost around 8% of the data. The final ASV table consists of 2110 sequences. With the SILVA 132 database we were able to categorize the sequences into 4 kingdoms, 62 phyla, 104 classes, 153 orders, 145 families, 252 genera, and 8 species. The database performed strongly at the kingdom and phylum levels. Few species were identified due to the fact that only 0.33% of the sequences was assigned at species level.

Microeukaryotic abundance and composition

Microeukaryotic diversity was analyzed in detail by phylum, class, order and family compositions **(Figure 1. A-D).** Dinoflagellata

phylum dominated coral samples, with the highest relative abundance in *Acropora formosa* 3 sample (96.4%) and the lowest in the *Acropora formosa* 4 sample (75,7%), as shown in **Figure 1A.** Ochrophyta was the most prevalent phylum in seawater samples (49.1% in seawater 1, 45.1% in seawater 2, 38.3% in seawater 4, and 32.0% in seawater 3). Seawater samples were predominated by Dinoflagellata phylum and Arthropoda phylum. Dominant phyla in sediment samples included Annelida (64.7% in sediment 4, 15.0% in sediment 2, 12.7% in sediment 3, 10.8% in sediment 1), Arthropoda (44.3% in sediment 3, 23.8% in sediment 2, 12.0% in sediment 4, 6.8% in sediment 1). Seawater, sediment, and coral samples all contained members of phylum Dinoflagellata.

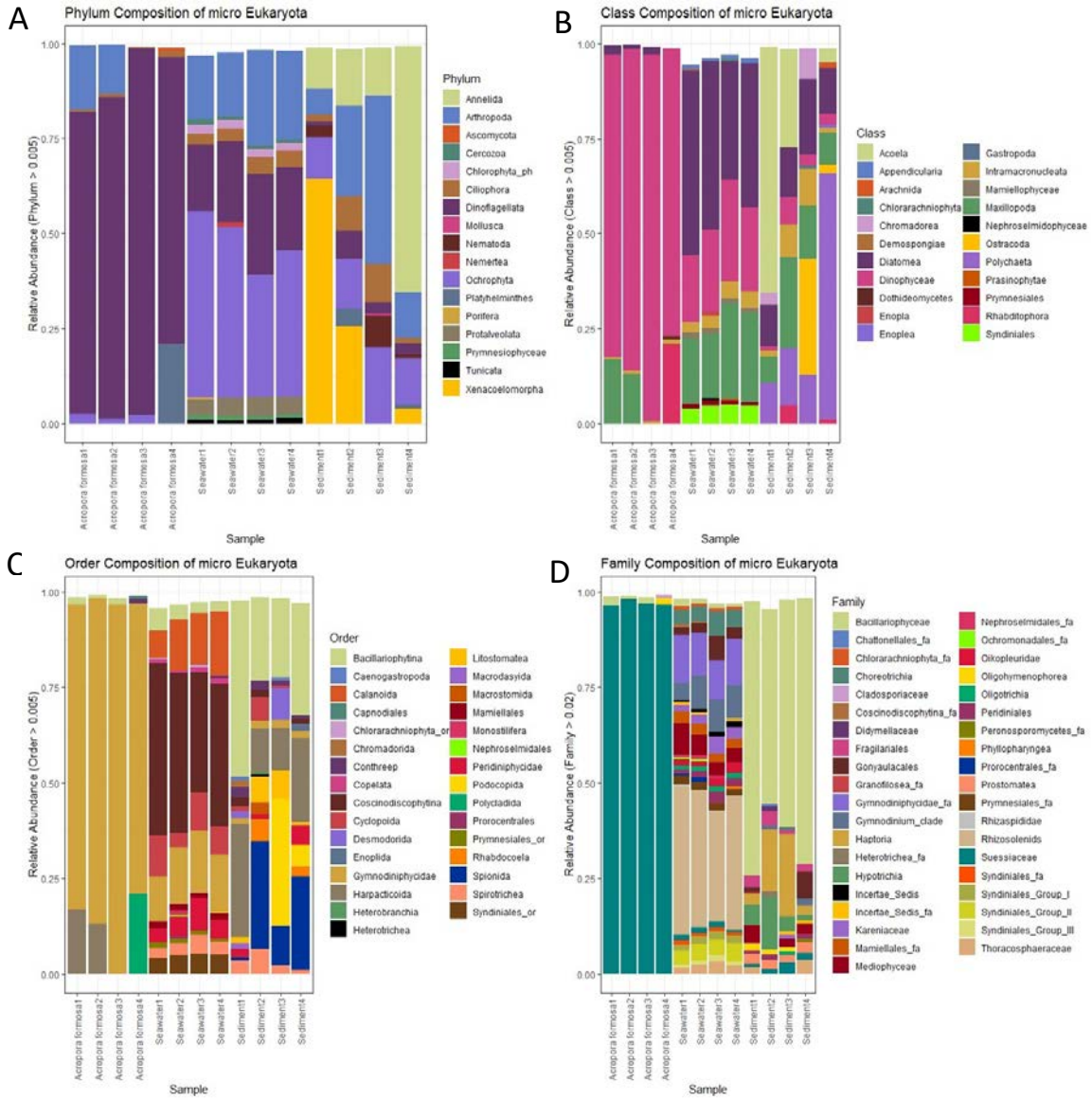

Figure 1. Microeukaryotic taxonomic classification in *Acropora formosa*, seawater and sediment. The stacked bar plot showed composition and abundance of microeukaryotes at phylum level (A), class level (B), order level (C) and family level (D). Sequences with abundance less than 0.5% were filtered out.

Symbiodinium (Family Suessiaceae, Order Gymnodiniphycidae, Class Dinophyceae, Phylum Dinoflagellata) was the only genus with a relative abundance higher than 2% in *Acropora formosa* samples, as shown in **Figure 2.** The majority of the species in seawater samples belonged to genus *Guiardia* (41.5% in seawater 1, 41.3% in seawater 2, 41.1 % in seawater 4, 32.1% in seawater 3) and genus *Gyrodinium* (14.3% in seawater 4, 14.0% in seawater 1, 13.3% in seawater 2, 11.1% in seawater 3). Bacillariophyceae family, which included the genera *Amphora* and *Navicula*, was highly abundant in sediment samples (average abundance: 22.9% and 15.4%, respectively).

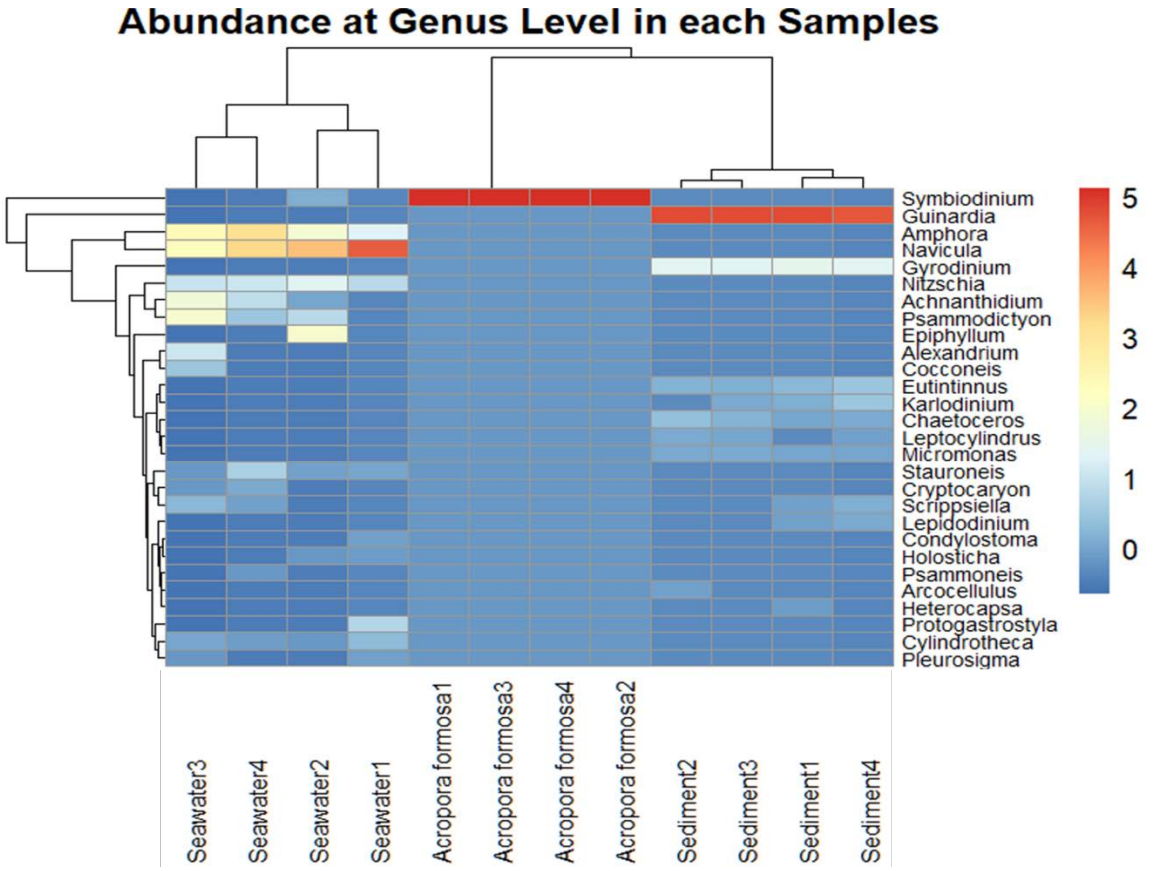

Figure 2. Microeukaryota taxonomic classification in *Acropora formosa*, seawater and sediment at genus level. The heatmap showed composition and abundance of microeukaryotes at the genus level. Sequences with abundance less 2% were filtered out. The x-axis represents the samples, while the y-axis represents the genera.

Alpha diversity analysis

A rarefaction curve of the total number of sequences (sample size) against the number of ASVs was plotted. **Figure 3** illustrated how the quantity of ASVs and sample size from the samples from one sample groups (coral, seawater or sediment) were quite consistent and did not differ greatly from one another. *Acropora formosa* samples had the fewest unique sequences recovered (number of ASVs), indicating that samples acquired from seawater and sediment have a greater diversity, despite the fact that coral possessed the most number of sequences.

This is likewise depicted in the Observed values of **Table 1** and **Figure 4**. The values in

Table 1 displayed the calculated alpha diversity indices, such as Observed, Chao1, a richness indicator and Shannon, a diversity indicator, for our 12 samples. The values of Chao1 and Shannon indices were highest in seawater samples (Average: 352.6242 and 4.013185, respectively), implying that they were the most diversified. In sediment samples, these values were a little lower (Average: 291.1261 for Chao1 and 3.08402 for Shannon). These indices from seawater and sediment samples were substantially greater compared to *Acropora formosa* samples (Average: 84.65164 for Chao1 and 1.079441 for Shannon). **Figure 4** displayed that the diversity indices of sediment samples fluctuated more compared to the indices of coral and seawater samples. Lastly, Kruskal-Wallis test was applied to the Observed, Chao1 and Shannon indices. P values less than 0.05 were found in all cases (0.0154 for Observed, 0.01832 for Chao1 and 0.00971 for Shannon), indicating that there was a substantial difference in richness and diversity among coral, seawater and sediment samples. In conclusion, samples from coral sampling sites were the least diverse, the alpha diversity indices from sediment samples were less consistent than the indices from seawater and coral samples, and there were significant differences in richness and diversity among *Acropora formosa*, seawater and sediment samples.

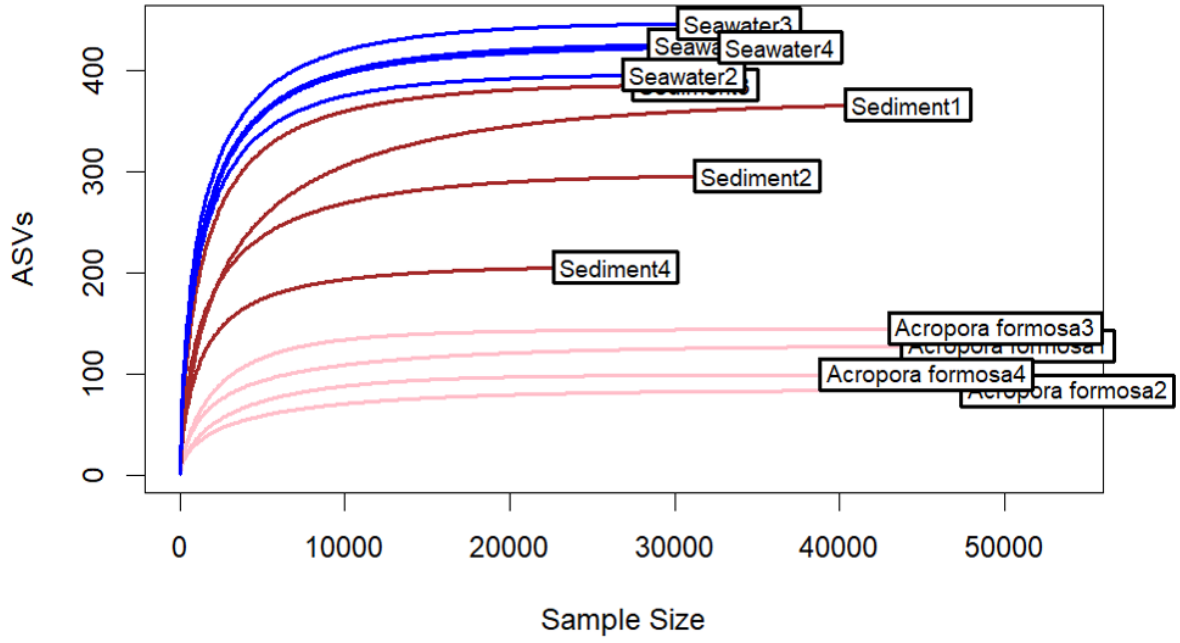

Figure 3. Rarefaction curve (plot of number of ASVs on y-axis against the sample size on x-axis).

Beta diversity analysis

Figure 5A depicted the hierarchical clustering of our 12 samples, where the vertical axis represented the clusters, and the horizontal scale on the dendrogram reflected distance or dissimilarity. In **Figure 5B**, the x-axis and y-axis were the two principal coordinates of PCoA, and their percentage values of 29.8% and 16.9% respectively, served as interpretations of variations in sample composition. Both graphs displayed how closely samples within coral

sampling sites and within seawater sampling sites clustered together. The greater distance among sediment samples indicated a greater degree of compositional dissimilarity. Seawater samples and coral samples were more similar to one another than the samples of sediments, as can be observed in both of the figures. Adonis test was ultilized to quantify the differences between the samples. The R-square value of 0.9998 indicates that 99.98% of the variation in distances could be accounted for by the grouping being tested. According to the p-value of 0.003, there was a 0.3% probability that we falsely concluded that there was a difference between the groups when there was none (the null hypothesis). R-square value and p value indicated that the differences in composition among the three sample groups (*Acropora formosa*, seawater, and sediment) were significant.

Table 1. The number of observed ASVs, the species richness indicator Chao 1 and the diversity indicator Shannon obtained for each sample.

	Observed ASVs	Chao1	Shannon
Acropora.formosa1	97	97.54545455	1.358100503
Acropora.formosa2	71	72.11111111	0.955685233
Acropora.formosa3	100	100.2	0.974919324
Acropora.formosa4	68	68.75	1.029060221
Sediment1	321	334.6764706	2.15417491
Sediment2	276	277.4	3.484891064
Sediment3	356	356.8823529	3.936915235
Sediment4	195	195.5454545	2.76009965
Seawater1	348	348.1578947	3.938027782
Seawater2	325	325.0666667	3.860058907
Seawater3	381	382.6470588	4.290607641
Seawater4	354	354.625	3.964044208

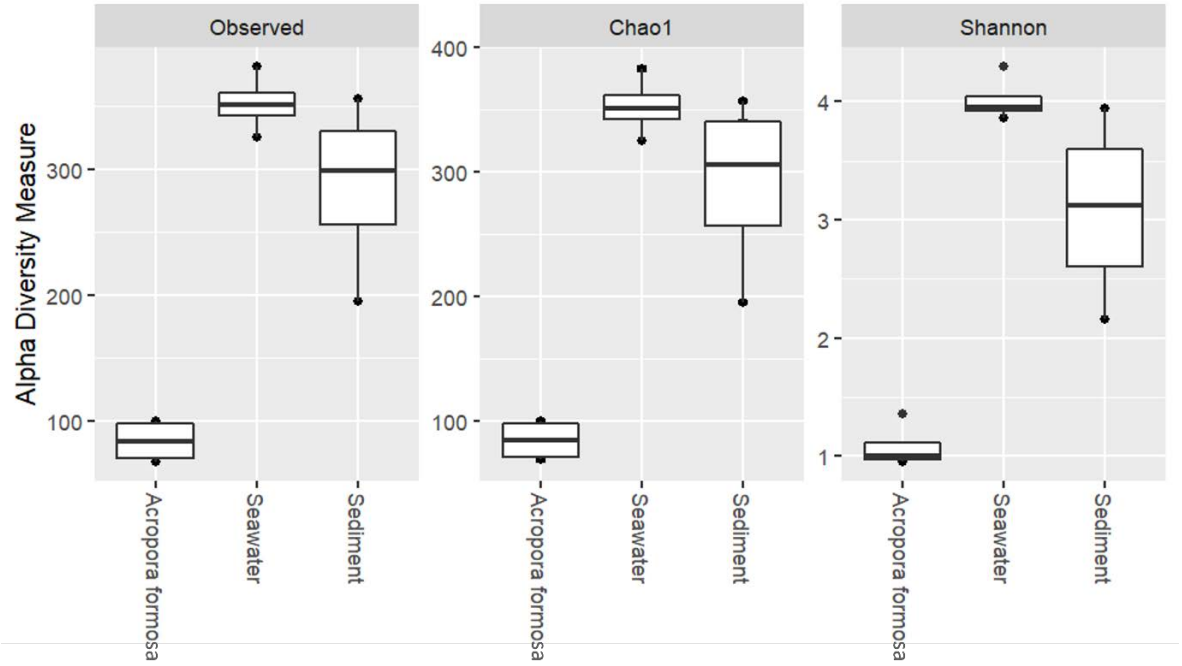

Figure 4. Alpha diversity of microeukaryota. Alpha diversity, measured by Observed, Shannon and Chao1 diversity indices, is plotted for *Acropora formosa*, seawater and sediment samples in boxplots. The line inside the box represented the median.

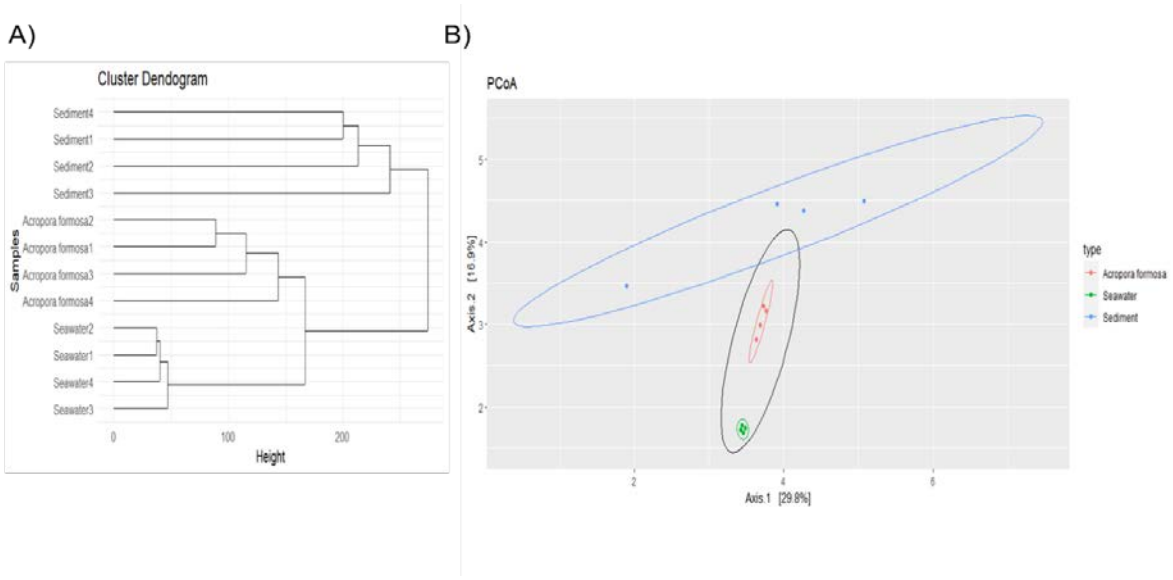

Figure 5. Hierarchical clustering (A) and Principal Coordinates Analysis (PCoA) (B) of microeukaryotic communities. Hierarchical clustering was performed using DESeq2 package and Ward method. PCoA was generated with the DESeq2 transformed table and phyloseq package. Statistical significance was assessed using Adonis test.

DISCUSSION

In this research, a metagenomic approach and the R programming language were used to study the small eukaryote communities associated with coral *Acropora formosa* and the seawater above and sediment beneath the coral colonies. Our first effort to understand the implicit structure of the microeukaryotic communities in our samples is by plotting their composition and abundance at different taxonomic levels. We observed that microalgae appeared abundantly in our samples, especially phylum Dinoflagellata (genus *Symbiodinium)* in coral samples and phylum Ochrophyta (green algae; Barbosa *et al.,* 2020) in seawater and sediment samples. Microalgae are microorganisms that are capable of performing photosynthesis, a process where light energy is used to extract carbon from CO_2 and produce oxygen as a byproduct (Laamanen et al., 2020). Microalgae are found in seawater or fresh water environments (Ruane *et al.,* 2010), which explains why they were found in our samples. The two main groups of microalgae are diatoms and dinoflagellates (Peltomaa *et al.,* 2019*)*. Dinoflagellates of the genus *Symbiodinium* can form mutualistic symbioses with stony corals (Scleractinia) (Rosic *et al.,* 2015), including coral *Acropora formosa* (Schoch *et al.,* 2020). Therefore, the fact that coral samples were dominated by genus *Symbiodinium* confirms the accuracy of our computational data processing and analysis. The interaction between this genus and stony coral, in which inorganic waste metabolites from coral are exchanged for organic nutrients produced by *Symbiodinium*, underlies the development, formation and metabolism of coral reefs (Stat *et al.,* 2008). Thus, the loss of dinoflagellate symbionts and/or photosynthetic pigments from corals due to environmental stress such as heating, microplastic can result in coral mortality and reef damage (Syakti *et al.,* 2019). Despite this profound coral-dinoflagellate relationship, the complete molecular mechanisms remain poorly understood and further research is required (Liu *et al.,* 2018).

Aside from microalgae, metagenomic approaches revealed the existence of phylum Arthropoda and Annelida, two animal kingdom members, in seawater and sediment samples. Arthropoda is the largest phylum in the animal kingdom, with a jointed skeleton covering composed of chitin (a complex sugar) coupled to protein (Robert, 2022). Annelida is a phylum of invertebrates distinguished by the presence of a body cavity (or coelom), moving bristles (or setae), and a body split into segments by transverse rings, or annulations. They can be found all over the world in a variety of settings, including oceans, freshwater, and wet soils (Donald, 2022).

Next, alpha diversity analysis was performed, revealing significant differences in richness and diversity among our groups of samples. The Chao and Shannon indices of eukaryotic microbes in seawater and sediment samples were higher than those in coral samples. This is consistent with the findings of Kusdianto *et al.* (2021). One possible explanation for this difference could be that eukaryotes in sediment and seawater are less reliant on surface area (Kusdianto *et al.,* 2021). The seawater microbiome also retains the highest diagnostic accuracy for detecting changes in the nearby reef ecosystem (Glasl *et al.,* 2019). According to Glasl *et al.* (2019), the seawater microbiome retains the highest diagnostic accuracy for detecting changes in the nearby reef ecosystem. Beta diversity analysis displayed that the samples were grouped into distinct clusters based on whether they were collected from *Acropora formosa,* seawater or sediment, suggesting that the small eukaryotic communities were unique to each site. The seawater and coral samples exhibited greater similarity to each other than to the sediment samples.

Understanding the microbial communities on coral reef ecosystems is critical for coral reef conservation because these microorganisms play a key role in the nutrition and disease resistance of healthy corals (Rosenberg *et al.,* 2007). Environmental stress can trigger microbial population changes, which can have an impact on

coral health (Rosenberg *et al., 2*007). In addition, coral-associated microorganisms, its bioactivity mostly focused in the areas of antibacterial, cytotoxic, antifouling, and α-glucosidase inhibitory activity, have been acknowledged as possible sources of pharmaceutical compounds (Sang *et al.,* 2019). Thus, the additional knowledge of eukaryotic microbiome communities gained from the findings of this study may be significant for conservation efforts as well as possible medical applications.

In conclusion, the study conducted a comparative analysis of eukaryotic microbial communities in *Acropora formosa*, sediment, and seawater samples collected from a coral reef ecosystem in Whale island, Nha Trang Bay, Vietnam, and the results may provide valuable insights for future studies on the functional profiling or metabolic roles of microbial communities in coral ecosystems.

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