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Metagenomics analysis of marine eukaryotic community in water and sediments at Lang Co - Da Nang sea by throughput 18S rRNA gene sequencing

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ABSTRACT

The present study applied metagenomics to characterize the diversity and relative occurrence of eukaryotic organisms in the sea water (LC05.W and LCDN.W) and sediment (LC05.S and LCDN.S) samples collected at the Lang Co - Da Nang sea in two years 2016 and 2017. The marine DNA metagenomes from water and sediments were isolated and analyzed by using specific primer 18S V4: 528F-706R with the barcode for gene-based metagenomic approach. Total tags were 374,336 (92,864 in LC05.W; 95,742 in LCDN.W; 86,593 in LC05.S and 91,385 in LCDN.S samples) and clustered at a 97% similarity into 5,204 unique operational taxonomic units (936 in LC05.W; 1631 in LCDN.W; 2,259 in LC05.S and 1,631 in LCDN.S). The taxonomic profile obtained by comparison with SILVA SSU database showed predominance of the kingdom: Eukaryote domain (61% in LC05.W; 32% in LCDN.W; 43% in LC05.S and 69% in LCDN.S); Metazoa (26% in LC05.W; 22% in LCDN.W; 37% in LC05.S and 19% in LCDN.S). Fungi in samples collected in 2017 (31% in LCDN.W and 10% in LCDN.S) were dominant as compared to 2016 (6.0% in LC05.W and 0.6% in LC05.S). In addition, 0.4% and 10.0% in water and 19% and 2% in sediment sequences were unclassified. Protalveolata, Annelida, Chlorophyta, Nematoda, Arthropoda, Rotifera, Ascomycota, Diatomea were top ten at the phylum level in Lang Co - Da Nang sea water and sediments. The abundance distribution of 35 dominant genera among all samples was displayed in the species abundance heatmap. The taxonomic assignment based on 18S ribosomal sequences with the SSU base possibly showed the presence of eukaryotic species (191 in LC05.W; 320 in LC05.S; 278 in LCDN.W and 207 in LCDN.S) in the marine water and sediments collected at Lang Co - Da Nang sea.

Keywords: Eukaryotic community, Lang Co - Da Nang sea, marine water and sediments, 18S rRNA gene, high-throughput sequencing.

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INTRODUCTION

The ocean occupies about 71% of surface and 90% of biosphere on our planet and 97% of water on the Earth belongs to the ocean. The ocean is one of the richest biome habitats on our planet. Most recent estimations, all based on indirect approaches, suggest that there are millions of marine eukaryotic species. Moreover, a large majority of these organisms are less than 1 mm, cryptic and still unknown to science. Small and cryptic organisms, which play important ecological roles despite being inconspicuous, remain overlooked in biodiversity surveys. According to the World Register of Marine Species [1], there were 228,739 identified eukaryotic marine species as of September 2015 (among which Animalia constituted 195,702 species, Plantae - 9689 species, Chromista - 21,403 species, Protozoa - 589 species and Fungi - 1,356 species). This indicates that between 24 and 98% of all marine eukaryotic species have not been described and known. Taxonomic experts have estimated that fewer than 10% of total species might be formally described in the most cryptic taxonomic groups [2]. Even among well-known groups such as marine mammals, new species continue to be discovered [3]. For many groups, the absence of diagnostic morphological characters [4], the lack of taxonomic expertise and the time required to describe or identify species [5] have been major impediments to obtaining a comprehensive understanding of marine diversity.

At present, high-throughput sequencing (HTS) platforms became widely available; a technological revolution that now allows the detection of tens to hundreds of species simultaneously from whole-community samples in a matter. DNA is extracted from environmental water or sediments. Then a small fragment of a DNA marker gene is amplified by PCR using general primers, yielding thousands of sequences per sample. DNA sequences are then sorted informatically, low-quality reads and contaminants are removed, and remaining sequences are clustered into molecular operational taxonomic units (OTUs) [6]. This approach is also referred to as metagenetics which was first applied to study bacterial and archaeal diversity and it is also now a cost- and time-effective alternative for eukaryote community profiling.

The main goal of this work is to study the eukaryotic micro-organism diversity in the Lang Co - Da Nang sea by using the practical power of the metagenomics.

MATERIALS AND METHODS

Sample collecting

Samples were collected as part of two nearly simultaneous oceanographic expeditions in 2016 and 2017 in regions of the Lang Co - Da Nang sea (figure 1). Sea water (80 l) was collected through primary filter of 50 µm at each sample site. Then, the Millipore filter of 0.2 µm was used for biomass recovery. Marine sediments were collected at each sample site by special submerged equipment. The environmental characteristic of the samples at the Lang Co - Da Nang sea was shown in table 1.

Figure 1. The sample map at Lang Co - Da Nang sea

Table 1. Stations, depths and physical properties of samples at Lang Co - Da Nang sea

Sample	Coordinates for sampling	Depth, m	pH	DO, mg/l	Conductivity, Sm/cm
LC1	16°12'50.4"N, 108°08'27.6"E	10	8.10	5.83	47.77
LC5	16°16'01.2"N, 108°11'49.2"E	30	8.16	6.22	47.96
LC7 (Coral chain)	16°12'46.8"N, 108°10'44.4"E	15	8.14	5.30	48.31
LC9	16°14'38.4"N, 108°08'20.4"E	20	8.11	5.55	48.37
XLC	16°17'13.7"N, 108°13'18.1"E	50	8.15	5.79	47.96
DN1	16°12'01.6"N, 108°11'28.0"E	7	8.12	5.67	47.78
DN2	16°12'03.1"N, 108°11'09.6"E	6	8.12	5.60	47.82
LC6	16°11'33.6"N, 108°12'58.2"E	25	8.03	5.45	47.20

Extraction of metagenome DNA

Metagenome DNA of sea water samples was isolated by UltraClean MegaPrep Kit (MoBio Laboratories, Inc.). DNA - metagenome of sediments was isolated by G'NOME® DNA Extraction Kit (BIO101). DNA - metagenome samples of sea water and sediments after determination of concentrations were purified by the agarose electrophoresis and then were mixed together respectively to make temples for 18S rDNA gene application. The names of the metagenome samples were used in this study as follows: LC05.W: mix of water metagenome DNA; LC05.S: mix of sediment metagenome DNA collected in 2016; LCDN.W: mix of water metagenome DNA; LCDN.S: mix of sediment metagenome DNA collected in 2017; LC06.W: sample of water metagenome DNA collected in 2017.

Amplicon generation

Region V4 of 18S rDNA gene was amplified using specific primer 18S V4: 528F-706R with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

PCR product quantification and qualification

The same volume of 1X loading buffer (containing SYB green) was mixed with PCR products and then electrophoresis was operated on 2% agarose gel for detection. Samples with bright main strip between 400-450 bp were chosen for further experiments.

PCR product mixing and purification

PCR products were mixed in equidensity ratios. Then, mixture of PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The libraries were generated with NEBNext® Ultra™ DNA Library Prep Kit by Illumina Hiseq with 250 PE at First BASE Lab. Sdn. Bhd. - Singapore.

Sequencing data processing

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH [7]. It is a very fast and accurate analysis tool, which was designed to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end of the same DNA fragment, and the splicing sequences were called raw tags. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags [7] according to the QIIME [8] quality which controlled process.

Sequences analysis

Sequences analysis was performed by Uparse software [9] using all the effective tags. Sequences with ≥ 97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. Sequences analysis was performed by RDP classifier [10] and Silva database [11] for species annotation at each taxonomic rank (kingdom, phylum, class, order, family, genus, species) (Threshold: 0.6~1). To get the phylogenetic relationship of all OTUs representative sequences, the MUSCLE [12] can compare multiple sequences rapidly. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences.

RESULTS AND DISCUSSION

OTU analysis and species annotation

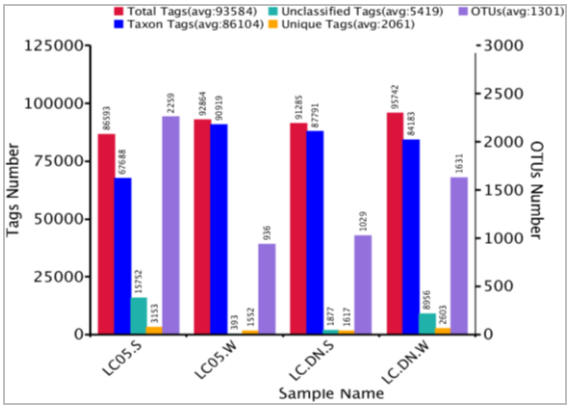

Figure 2. Statistical analysis of the tags and OTUs number of each sample used in this study

To investigate the diversity and relative abundance of eukaryotic species, the marine metagenomic DNA from water and sediment samples was sequenced using the Illumina machine. In order to analyze the species diversity in each sample, all effective tags were grouped using 97% DNA sequence similarity into OTUs (Operational Taxonomic Units). During the construction of OTUs, basic information from different samples had been collected, such as effective tag data, low-frequency tag data and annotation data of tags. The statistical dataset as shown in figure 2 indicated that the total tags were 92,864 in LC05.W; 95,742 in LCDN.W; 86,593 in LC05.S and 91,385 in LCDN.S samples and OTUs were 936 in LC05.W and 1631 in LCDN.W.

The taxonomic profiling of samples was depicted in Krona, which visually displays the analysis result of species annotation [13]. Circles from inside to outside stand for different taxonomic ranks, and the area of sector means respective proportion of different OTU annotation results (Figs. 3a–3d).

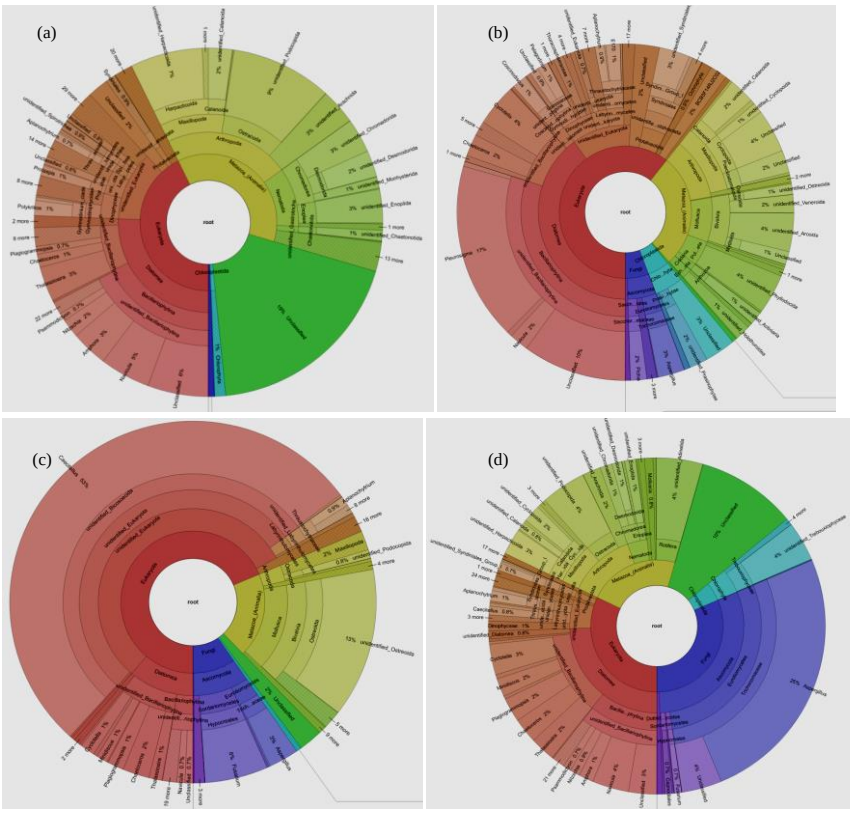

Figure 3. Krona displays of species annotation of mixed sediment and mixed water samples' metagenome DNA: (a)- (LC05.S); (b)- LC05.W; (c)- LCDN.S; (d)- LCDN.W

From the Krona diagrams, the 4 kingdoms and 1 Eukaryote domain were determined in the water samples collected at the Lang Co - Da Nang sea area (table 2). The results showed that Metazoa occupied 26% and 22% in water samples, and 37% and 19% in sediments collected in 2016 and 2017 respectively. Eukaryote domain was predominant in all samples with the level 61% and 32% in water, and 43% and 69% in sediments. The kingdom of Fungi was dominant in water (6.0% in LC05.W and 31% in LCDN.W) compared to the sediments (0.6% in LC05.S and 10% in LCDN.S).

Table 2. The kingdom level (%) in the sediments and water of Lang Co - Da Nang sea

No.	Kingdom	Water samples		Sediment samples	
		LC05.W	LCDN.W	LC05.S	LCDN.S
1	Metazoan (Animalia)	26.0	22.0	37.0	19.0
2	Eukaryote domain	61.0	32.0	43.0	69.0
3	Chloropastia	5.0	5.0	1.0	0.5
4	Fungi	6.0	31.0	0.6	10.0
5	Discoba	0.004	0.002	0.006	0.04
6	Unclassified	0.4	10.0	19.0	2.0

At phylum level, there were 2 identified phyla that belong to Chloropastia; 9 phyla to Eukaryote domain; 6 phyla to Fungi; 9 to Metazoan. In addition, 10.9% (2016) and 5.3% (2017) sequences were unidentified belonging to eukaryote. Species relative abundance in top ten phyla in sea water at the Lang Co - Da Nang region in 2016 and 2017 was shown in table 3 and figure 4.

To study the similarity among different samples, clustering analysis was applied and clustering tree was constructed. Unweighted pair group method with arithmetic mean (UPGMA) was a type of hierarchical clustering methods which is widely used in ecology for the classification of samples. The average distance between the newly created "sample" and other samples was calculated and the two nearest samples could be found again to repeat above steps. A complete clustering tree could be obtained until all samples were clustered together.

Table 3. The top ten phylum level (%) in the sediments and water of Lang Co - Da Nang sea

No.	Phylum	Water samples		Sediment samples	
		LC05.W	LCDN.W	LCDN.S	LC05.S
1	Protalveolata	4.0	1.0	0.3	1.0
2	Annelida	4.6	0.1	0.2	0.2
3	Chlorophyta	5.3	4.5	0.5	1.0
4	Nematoda	0.1	3.7	0.6	10.6
5	Mollusca	8.3	0.8	15.0	0.5
6	Arthropoda	11.5	12.4	2.8	22.4
7	Rotifera	0.0	4.5	0.0	0.0
8	Ascomycota	5.3	30.9	9.5	0.3
9	Diatomea	40.0	24.5	11.2	25.6
10	Unidentified Eukaryote	10.9	5.3	56.0	12.5
11	Other	0.4	9.6	2.1	18.9

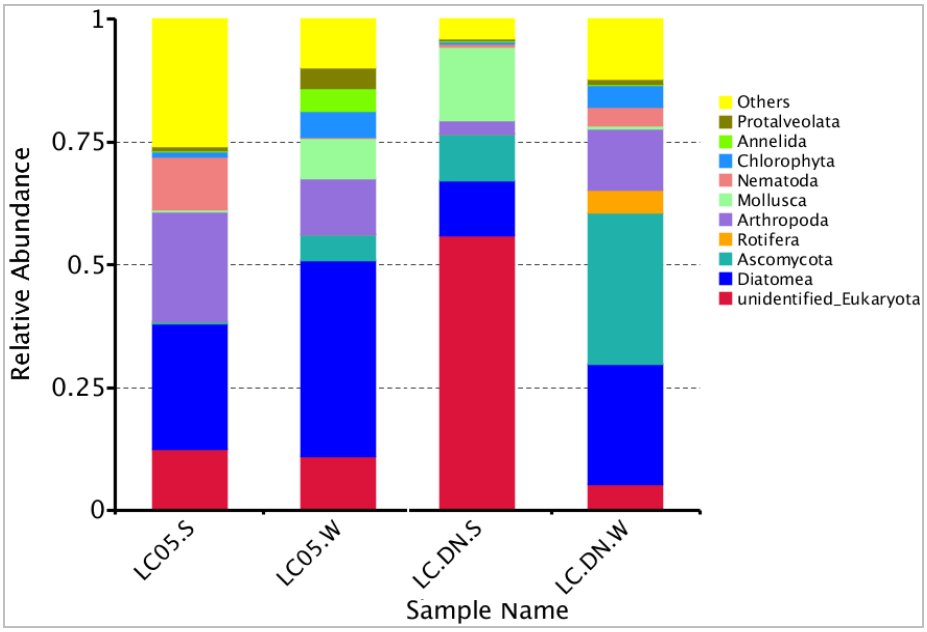

Figure 4. Species relative abundance in top ten phyla in sea water and sediment samples collected at the Lang Co - Da Nang region in 2016 and 2017

Weighted Unifrac distance matrix and unweighted Unifrac distance matrix were calculated before used for UPGMA cluster analysis. They were displayed with the integration of clustering results and the relative abundance of each sample by phylum was shown in figure 5.

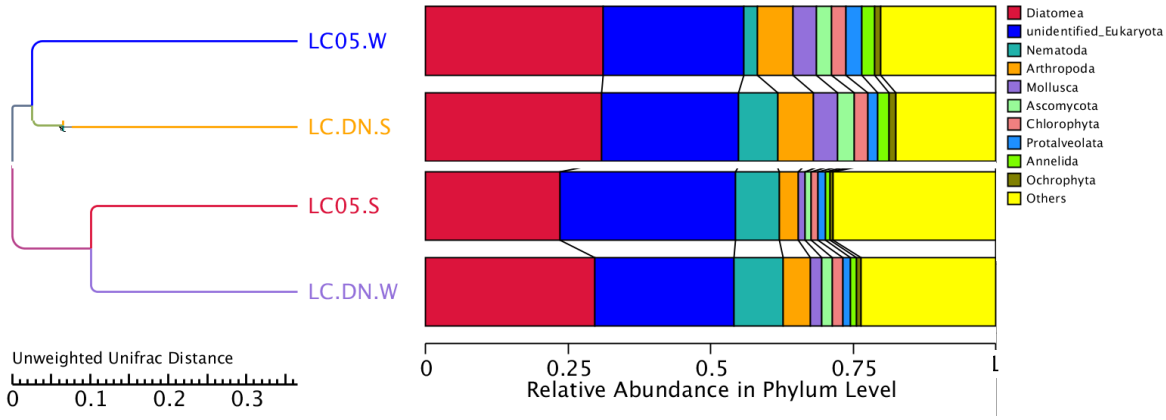

Figure 5. UPGMA cluster tree based on unweighted Unifrac distance

At the genus level, the abundance distribution of 35 dominant genera among all samples was displayed in the species abundance heat map. Based on the information of clustering results of samples as well as taxa, we could check whether the samples with similar processing are clustered or not, and the similarity and difference of samples can also be observed. The obtained result was shown in figure 6.

According to species annotation results (table 4.), 191 eukaryotic species were identified in the LC05.W; 278 in LCDN.W; 320 in LC05.S; and 207 in LCDN.S samples.

The average number of identified species was in the range of about 25 to more than 50%. It means that there are high quantities of unidentified microbial eukaryote in the studied sea habitat.

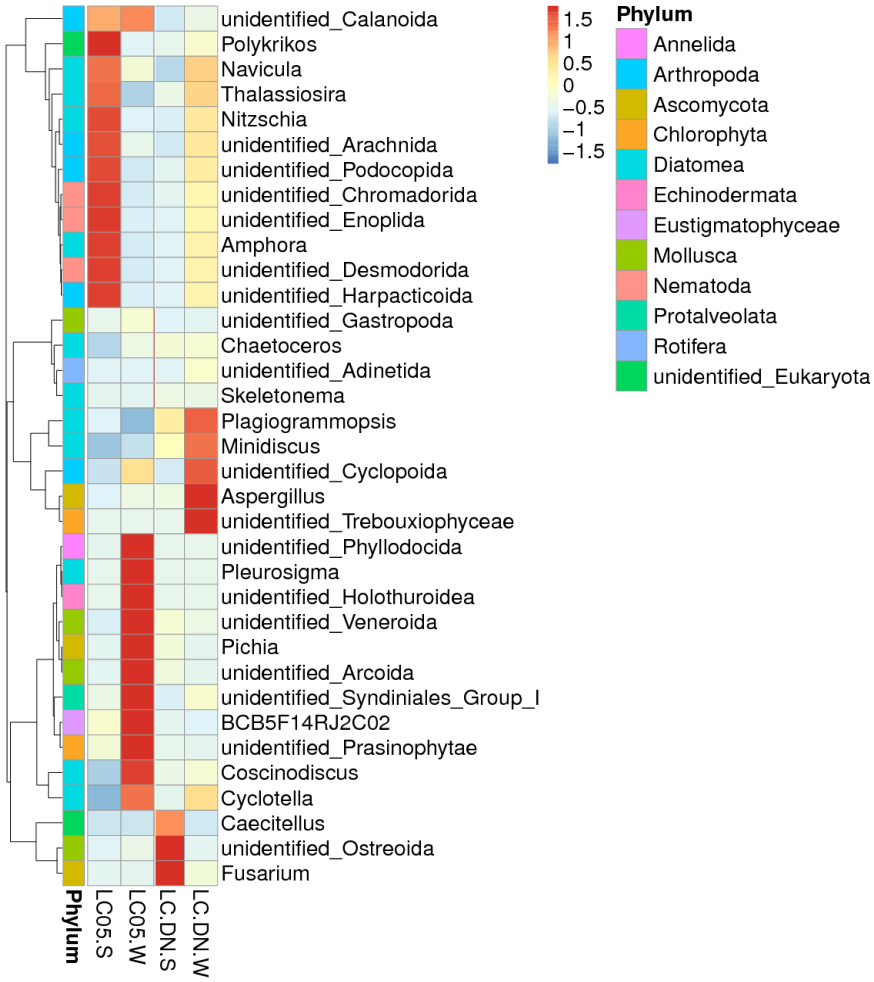

Figure 6. The genus abundance heatmap in Lang Co - Da Nang sea water and sediment samples

Notes: Sample name is plotted on the X-axis and the Y-axis represents the genus. The absolute value of 'z' represents the distance between the raw score and the mean of the standard deviation. 'Z' is negative when the raw score is below the mean, and vice versa.

Table 4. Quantity and level of identified eukaryote species in water and sediments of Lang Co - Da Nang sea

No.	Sample	Identified species	Level (%) of identified species/total taxon tags
1	LC05.W	191	34.96
2	LC05.S	320	30.02
3	LCDN.W	278	53.94
4	LCDN.S	207	25.02

The species at top level in sea water and sediment samples collected at Lang Co - Da Nang were shown in the tables 5 and 6.

Table 5. Predominant species in the marine water at Lang Co - Da Nang sea

No.	Species in LC05.W (2016)	Level (%)	Species in LCDN.W (2017)	Level (%)
1	*Alitta succinea*	3.704458	*Aspergillus versicolor*	24.780681
2	*Anadara antiquata*	3.674497	*Adineta vaga*	4.488255
3	*Aspergillus versicolor*	2.562320	*Coccomyxa simplex*	3.793145
4	*Eukaryote clone* OLI11011	2.517977	*Navicula radiosa*	2.423298
5	*Junceella aquamata*	1.812081	*Canuella perplexa*	2.043384
6	*Chaetoceros* sp.	1.797699	*Oithona* sp. 2 New Caledonia-RJH-2004	1.670662
7	*Pycnococcus* sp. MBIC10637	1.762943	*Thalassiosira profunda*	0.822148
8	*Navicula radiosa*	1.740173	*Chaetoceros* sp.	0.816155
9	*Pichia occidentalis*	1.538830	*Labyrinthuloides yorkensis*	0.801774
10	*Labidoplax digitata*	1.478907	*Ptycholaimellus* sp. 1092	0.794583
11	*Coscinodiscus radiatus*	1.410594	*Psammodictyon panduriforme*	0.735858
12	*Bartholomea annulata*	1.027085	*Chaetoceros calcitrans*	0.564477
13	*Saccostrea glomerata*	0.968360	*Karlodinium veneficum*	0.553691
14	*Oithona* sp. 2 New Caledonia-RJH-2004	0.957574	*Hortaea werneckii*	0.535714
15	*Labyrinthuloides yorkensis*	0.837728	*Coscinodiscus radiates*	0.456616

Table 6. Predominant species in the marine sediments at Lang Co - Da Nang sea

No.	Species in LC05.S (2016)	Level (%)	Species in LCDN.S (2017)	Level (%)
1	*Canuella perplexa*	5.204938	*Saccostrea glomerata*	13.267018
2	*Navicula radiosa*	2.715724	*Aspergillus versicolor*	2.521572
3	*Ptycholaimellus* sp. 1092	2.132071	*Chaetoceros* sp.	0.770614
4	*Acartia pacifica*	1.412991	*Labyrinthuloides yorkensis*	0.692713
5	*Oncholaimidae* sp. MHMH-2008	1.241611	*Navicula radiosa*	0.536913
6	*Thalassiosira profunda*	1.040268	*Septifer bifurcatus*	0.436242
7	*Spinileberis quadriaculeata*	0.854506	*Chaetoceros calcitrans*	0.400288
8	*Psammodictyon panduriforme*	0.732263	*Canuella perplexa*	0.397891
9	*Bacillariophyta* sp. 1 MAB-2013	0.677133	*Thalassiosira profunda*	0.383509
10	*Coquimba ishizakii*	0.671141	*Coscinodiscus radiatus*	0.373921
11	*Bicornucythere bisanensis*	0.669942	*Anadara antiquata*	0.328380
12	*Chaetoceros* sp. SS628-11	0.610019	*Candida tropicalis*	0.321189
13	*Heterolepidoderma loricatum*	0.574065	*Psammodictyon panduriforme*	0.308006
14	*Thalassiosira concaviuscula*	0.528523	*Ptycholaimellus* sp. 1092	0.172579
15	*Chromadorita tentabundum*	0.419463	*Bellerochea yucatanensis*	0.171381

Discussion

Sequencing of the 18S rDNA amplicon was widely used for microbial community comparison among samples from various natural or endozoic environments including sea habitat. Charting the true dimensions of eukaryotic diversity is essential to fully understand evolution and, by extension, the ecological complexity of microbial food webs. Molecular surveys provide a primary route towards this understanding, and each new environment studied has yielded new insights into particular aspects of eukaryotic diversity and evolution. To date these studies have revealed new lineages and unexpected diversity within previously known lineages in open oceans, coastal areas, and deep sea vents. At present, HTS has been used to study benthic meiofauna diversity in shallow [14, 15], deep-sea [16] and estuarine sediments, macro- and meiofaunal diversity in

seagrass beds [17] and oyster reefs [18], as well as planktonic diversity across the globe [19], particularly the diversity of picoplanktonic size fractions (less than 3 μm).

Our study has shown that the level of eukaryote diversity at the industry level is equivalent to the published results of many authors reviewed in relation to marine eukaryotes [1, 14, 16, 19–22]. In Vietnam, there are no studies on metagenomics of marine eukaryotes except for one research on the diversity of fungi in sea water by Tran Dinh Man et al., (2018) [23] and surveyed by conventional sampling methods on the component of some phyla belonging to eukaryotes like Nematoda, Diatomea,... Our results show that metagenomics methods applied in our study give a richer picture of marine eukaryote diversity of the Vietnam sea.

CONCLUSION

The metagenomics study revealed the marine eukaryotic organisms present in water and sediment samples collected at Lang Co - Da Nang sea in two years 2016–2017. The eukaryote community at top ten phylum level, genus abundance headmap and lists of species were prevalent in both sea water and sediment habitats. The present study provides a good data for understanding the diversity of the marine microbial eukaryote ecology of the sea in Vietnam. Eukaryotic microbial plays important roles in regulating sea environment.

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