

Article

Diversity and Community Composition of Labyrinthulomycetes Protists in the Coastal Zone of Hainan Island, South China Sea

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Abstract: The study of heterotrophic protists in the ocean is still in its early stages, compared to other microorganisms such as bacteria, archaea, and photoautotrophic protists. Labyrinthulomycetes protists (LP) are a type of unicellular protists that are widely distributed in global waters and have the potential to produce high-value products. In this study, the abundance, diversity, and community structure of LP in the coastal zone of Hainan Island in the South China Sea were investigated through quantitative PCR and high-throughput sequencing. The results showed that LP abundance varied by location and depth, with the highest levels (37.3×10^3 copies/L) found in the middle layer offshore and the lowest (0.386×10^3 copies/L) in the bottom layer offshore. The middle layer (chlorophyll maximum layer) had higher LP abundance both inshore and offshore than the surface and bottom layers. Interestingly, the highest LP richness and diversity was found in the inshore bottom. There was a significant difference in LP abundance between the offshore surface and bottom layers. The LP community was dominated by the genus *Aplanochytrium*, and four different ecotypes were identified. Additionally, the genus *Aurantiochytrium* had different cooperative and competitive strategies with bacteria in different habitats. This study sheds light on the abundance and community structure of LP in the coastal zone of Hainan Island, explores the potential interactions between LP and bacterial populations, and raises questions about the potential differentiation of LP ecotypes.

Keywords: coastal ocean; Labyrinthulomycetes; abundance; co-occurrence; ecotype



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1. Introduction

Heterotrophic microorganisms, such as Labyrinthulomycetes protists (LP), have been increasingly recognized as important players in marine ecosystems due to their role in material and energy cycling [1,2]. LP, known for their ability to degrade organic matter and secrete extracellular enzymes [3–7], have a significant impact on the marine carbon cycle [8–13]. There is evidence that LP may also contribute to the marine phosphorus cycle by secreting extracellular alkaline phosphatase [12,14]. However, compared to prokaryotes, microalgae, and microzooplankton, our understanding of LP in the ocean is still limited and requires further investigation.

The offshore marine habitat is known for its instability and richness in both primary productivity and biodiversity [15,16]. Studies on LP in these environments have shown that they are abundant and diverse. In the mangrove waters of southern China, over 200 LP strains were identified, with *Aurantiochytrium* being the dominant genus [17]. Similarly, more than 1000 LP strains were isolated and cultured in Osaka Bay and the Shukugawa River mouth in Japan, with over half being newly discovered strains [18]. High-throughput sequencing of the offshore waters of Qinhuangdao revealed thousands of LP operational taxonomic units (OTUs) across nine genera with high levels of diversity [3].

Furthermore, LP has been found in higher abundance levels in offshore marine waters and substrates [3,4,18,19]. On the other hand, studies on LP in open ocean waters are limited. In the East Indian Ocean, over 700 LP amplicon sequence variants (ASVs) were identified with a patchy distribution [20]. High-throughput sequencing in programs such as Tara Oceans and VAMPS have also discovered partial LP sequences in deep-sea waters [21]. LP have also been found in significant abundance in some open ocean waters above 200 m, reaching 6.30×10^5 cells/L in Hawaiian waters and 6.75×10^5 cells/L in Indian Ocean equatorial waters [5,22].

In this study, we aimed to explore the diversity and community structure of Labyrinthulomycetes protists (LP) in the eastern coastal zone of Hainan Island in the South China Sea. The objectives of the study were: to report the abundance, diversity, and community composition of LP; to analyze the factors affecting LP community composition; to investigate the differences in LP community composition across habitats and their ecological niche partitioning; and to analyze the potential interactions between LP and bacteria. The results of the study will provide insights into the community patterns, ecological niche partitioning, and interactions networks of LP in the South China Sea, and contribute to the advancement of our understanding of the ecology of marine heterotrophic protists communities.

2. Materials and Methods

2.1. Water Sampling and Environmental Data

In June of 2020, seawater samples were collected from three distinct depths along the eastern coast of Hainan Island: the surface layer (3 m), the middle layer (at the chlorophyll maximum), and near the bottom (40–150 m). Sampling was performed at six stations, with three stations (1, 2, and 3) located inshore, and three stations (4, 5, and 6) located offshore (as depicted in Figure 1). The relevant environmental information for each sample, including the location, depth, temperature, and salinity, as well as nutrient data, was obtained from previous studies conducted in our laboratory [23].

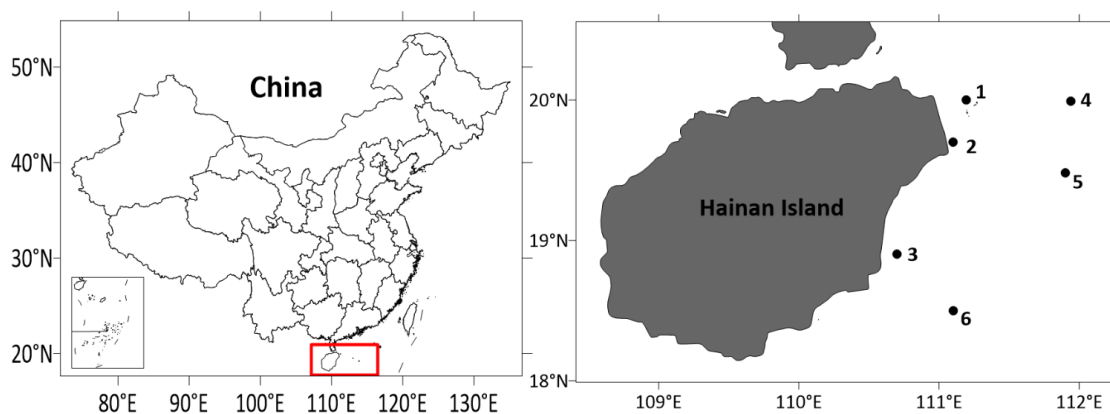


Figure 1. Map of sampling stations in the South China Sea. Stations 1, 2, and 3 represent inshore areas, and stations 4, 5, and 6 represent offshore areas.

2.2. Quantitative PCR Analysis

Total seawater DNA was extracted using an aqueous DNA kit (OMEGA, Norcross, GA, USA) according to the protocol provided by the manufacturer and stored at $-20\text{ }^{\circ}\text{C}$ for subsequent manipulation. The abundance of the 18S rRNA gene of LP was determined by qPCR using primers specific for LP (LABY-A and LABY-Y) [24]. A quantitative standard curve was established using purified plasmid DNA inserted with the target sequence as template. Q-PCR reactions were performed on a CFX Connect Sequence Detection System (Bio-Rad, Richmond, CA, USA) in a volume of 10 μL of reaction mixture: 1 $\times$ SYBR Green Mix, 0.25 μL of forward and reverse primers (5 μM), approximately 5 to 45 ng of DNA template and sterile water. The specific reaction conditions were: pre-denaturation at $95\text{ }^{\circ}\text{C}$ for 2 min; denaturation at $95\text{ }^{\circ}\text{C}$ for 5 s, annealing at $50\text{ }^{\circ}\text{C}$ for 30 s, extension at $72\text{ }^{\circ}\text{C}$ for

1 min, detection of fluorescence signal and recording of fluorescence threshold for 40 cycles. Finally, the melting curve was constructed. The linearity coefficient of the standard curve was 0.997, and the amplification efficiency was 96.3%.

2.3. High-Throughput Sequencing and Bioinformatics Analysis

The total environmental DNA of seawater samples was extracted using the Water DNA Kit (OMEGA, Norcross, GA, USA) according to the manufacturer's protocol and stored at $-20\text{ }^{\circ}\text{C}$ for subsequent manipulation. The 18S rRNA gene fragment was amplified using the primers LABY-A (5'-GGGATCGAAGATGATTAG-3') and LABY-Y (5'-CWCRAACTTCCTTCCGGT-3') targeting the labyrinthulomycetes [5,25]. The 25 μL PCR amplification system consisted of 12.5 μL Taqmix dye, 1 μL (10 μM) forward and reverse primers, 1 μL DNA template, and 9.5 μL sterile water. The PCR program included pre-denaturation at $95\text{ }^{\circ}\text{C}$ for 15 min; 31 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for 30 s, annealing at $50\text{ }^{\circ}\text{C}$ for 1.5 min, extension at $72\text{ }^{\circ}\text{C}$ for 1.5 min; and a final extension at $72\text{ }^{\circ}\text{C}$ for 10 min. The product was purified using the TIAN quick Midi Purification Kit. The PCR products were stored at $4\text{ }^{\circ}\text{C}$, and the amplicons were sequenced using Illumina NovaseqTM 6000 sequencing platform at Lianchuan Biotechnology Co. (Hangzhou, China).

The downstream processing of the sequencing reads was performed using the QIIME 2TM bioinformatics platform [26]. Cutadapt software was used to trim the raw sequences and remove the splice portion [27]. Then, DADA2 was used to denoise, including the removal of primers, low-quality sequences ($Q < 25$), and potential chimeras [28]. The generated ASVs were filtered by removing the singleton ASVs. Each sample was rarefied to 10,592 sequences, and the 192 LP ASVs were annotated using the BLAST+ classifier [29] and the SILVA SSU rRNA database (release 138) [30].

2.4. Statistical Analyses

The alpha diversity indices (richness, evenness, and Shannon diversity) of the LP community were calculated using the vegan package in R (version 4.1.3). The differences in diversity among different habitats were tested by ANOVA (IBM SPSS Statistics 25). The variation in the community composition was illustrated using principal coordinate analysis (PCoA) using Canoco 5. The correlation analysis was used to reveal the main environmental factors influencing the diversity of LP. The differences in the community composition of LP between different habitats were examined by PERMANOVA using R (version 4.1.3). To reveal the influence of environmental factors on the community composition of LP, redundancy analysis (RDA) was performed using Canoco 5. The 40 LP ASVs with the highest mean abundance ($\geq 0.25\%$ of relative abundance) were extracted and clustered based on log-transformed relative abundance data and Ward's hierarchical aggregation method [31]. Their distribution patterns and potential ecological partitioning was illustrated in the form of a heatmap using the OmicStudio tool at <https://www.omicstudio.cn/tool> (accessed on 24 January 2023). The Spearman's rank correlation coefficient (ρ) between the variables was calculated after integrating and processing the LP and bacterial ASV data. Using " $|\rho| > 0.5$, adjusted p (false discovery rate) < 0.05 " as the threshold, pairwise combinations of ASVs with strong correlation were screened, and a network based on the correlation was drawn using the OmicStudio tool at <https://www.omicstudio.cn/tool> (accessed on 30 January 2023). The igraph package (version 1.2.6) in R (version 3.6.3) was used for network construction.

3. Results

3.1. Abundance Pattern of Labyrinthulomycetes Protists

Our results showed that there was no significant (ANOVA, $p > 0.05$) difference in the abundance of LP between inshore and offshore habitats (Figure S1). However, there were significant differences (ANOVA, $p < 0.05$) within the inshore and offshore habitats, with depth being the strongest factor influencing LP abundance. The highest LP abundance was found in the middle layer of the offshore habitat (37.329×10^3 copies/L), while the lowest was in the deep layer (0.386×10^3 copies/L) (Figure 2). The mean abundance of surface

and middle layers in the offshore habitat was higher than that in the inshore habitat, while the deep layer was the opposite (Figure S1). Although there were some weak correlations between LP and environmental factors (for e.g., chlorophyll a, total nitrogen, silicate, etc.), they were not statistically significant (Table S1).

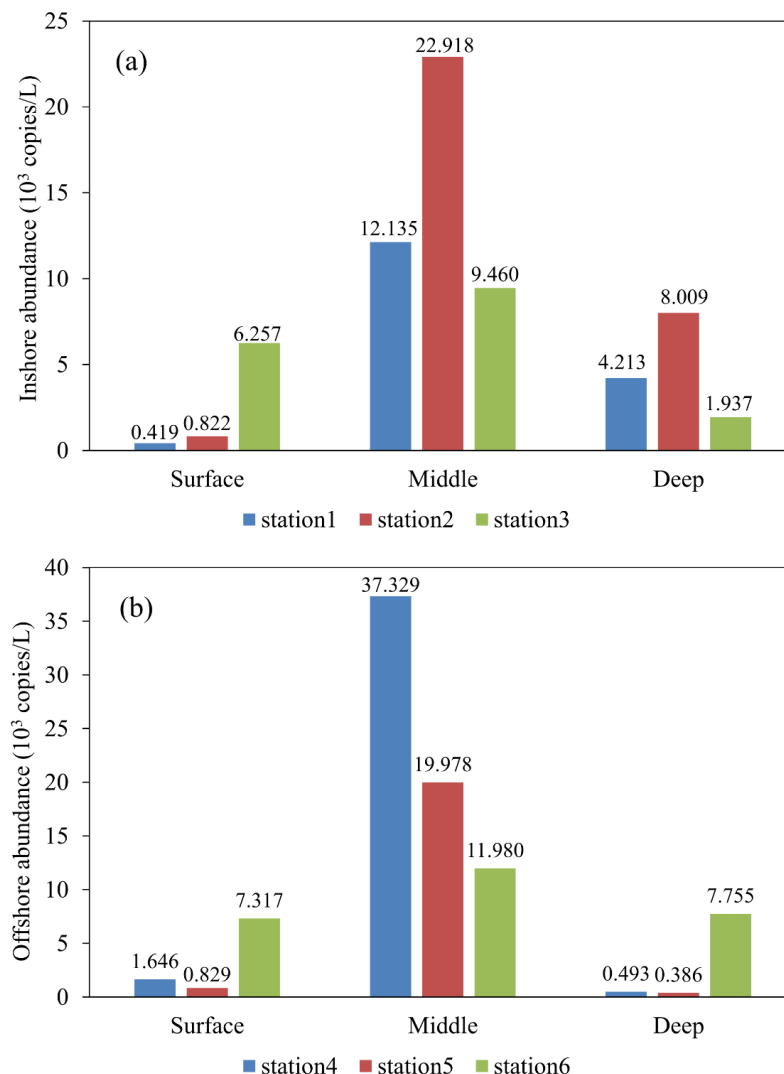


Figure 2. 18S rRNA gene abundance of *Labyrinthulomycetes* protists in the different zones of (a) inshore; and (b) offshore habitats.

3.2. Diversity Patterns of *Labyrinthulomycetes* Protists

In this study, a total of 399,059 LP sequences and 192 LP ASVs were generated from 12 seawater samples. The results showed that the LP richness, evenness, and Shannon diversity did not differ significantly between the inshore and offshore stations (ANOVA, $p > 0.05$) (Figure 3). The ASV richness ranged from 9 to 49, with the highest and lowest values observed in the inshore and offshore bottom waters, respectively. The LP abundance showed significant differences (ANOVA, $p < 0.05$) between the offshore surface and bottom layers, but not between the inshore surface and bottom layers, suggesting greater stability in LP abundance between the inshore surface and bottom waters. Furthermore, the inshore deep water showed higher LP abundance than the offshore deep water (ANOVA, $p < 0.05$). Although the differences were not statistically significant, the inshore deep water had a higher Shannon diversity than the offshore deep water and the inshore surface water had a lower Shannon diversity than the offshore surface water. The lower evenness in the inshore surface water indicated that the LP population in this habitat was dominated by a few

species while many species have a relatively low abundance. Furthermore, the PCoA result showed that the inshore surface water samples (stations 1–3) were clustered (Figure S2), suggesting that the LP community composition in this habitat was less diverse and more similar in species composition, in agreement with the evenness results.

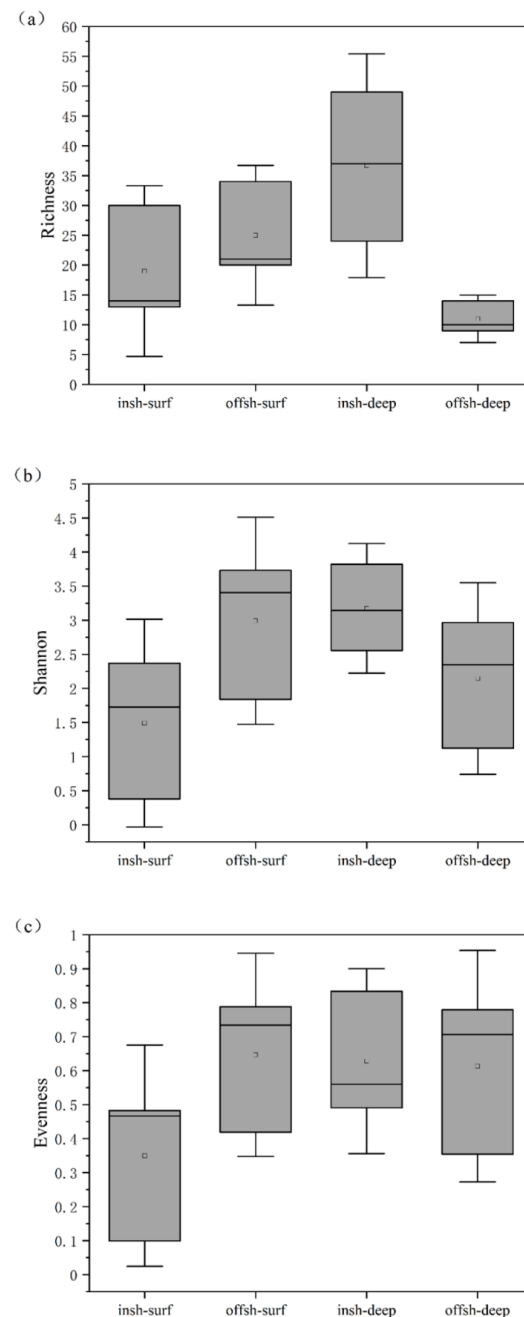


Figure 3. Richness (a); Shannon diversity (b); and evenness (c) for the *Labyrinthulomycetes* communities from different habitats. Labels “insh-surf”, “offsh-surf”, “insh-deep” and “offsh-deep” represent inshore surface water, offshore surface water, inshore deep water, and offshore deep water, respectively. The boxes represent the ranges of the first and third quartiles, the line and small square inside each box represent the median and mean values, and the ends of the whiskers represent the minimum and maximum values.

3.3. Community Structure of *Labyrinthulomycetes* Protists

The distribution of common and unique LP ASVs across the four habitats was analyzed. There were 36, 69, 43, and 17 ASVs that were unique to the inshore surface, inshore deep,

offshore surface, and offshore deep, respectively (Figure S3). Only three ASVs were found in all four habitats, accounting for only 2.09% of the total ASVs, but these three ASVs made up 51.33% of the relative abundance (Table S2). One of the shared ASVs, *Aplanochytrium*, accounted for 77.86% of the relative abundance of shared ASVs. The relative abundance of shared ASVs was higher in the inshore habitat (77.86%) compared to the offshore (43.90%), suggesting that the inshore water column was more stable in terms of species composition.

In this study, we identified three annotated genera: *Aplanochytrium*, *Aurantiochytrium*, and *Ulkenia*, as well as many unclassified taxa within Thraustochytriaceae or Labyrinthulomycetes (Figure 4). These three genera were found only in three stations, S3, S5 and D3, and made up 29.69% of the total sequences of the three genera. *Aplanochytrium* dominated, accounting for 91.02% of the total number of sequences in inshore surface water and 48.45% of all samples. *Aurantiochytrium* and *Ulkenia* accounted for 0.3% and 1.11% of the total sequences, respectively. *Aurantiochytrium* was mostly located in the inshore deep and offshore surface layer, while *Ulkenia* was primarily found in the inshore deep. The distribution of *Aplanochytrium* was inclined towards the surface layer in station D3, whereas the composition of station S5 was more deep-layer oriented. A large portion (50.14%) of LP ASVs were not clearly classified, especially in the deep water.

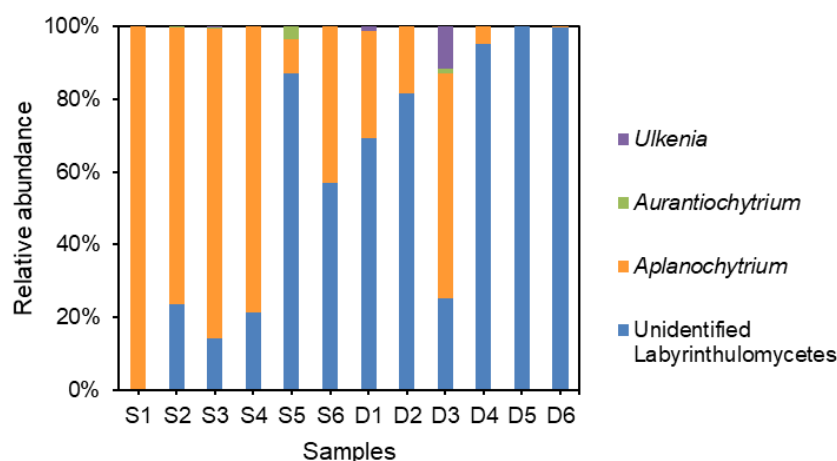


Figure 4. Composition of Labyrinthulomycetes communities (at the genus level) across samples from inshore and offshore habitats. S1–S3: inshore surface; S4–S6: offshore surface; D1–D3: inshore deep; and D4–D6: offshore deep.

The relationship between LP community composition and environmental parameters (depth, temperature, salinity, chlorophyll a, and seven nutrients) was explored using redundancy analysis (RDA) (Figure S4). The results showed that only salinity had a significant (adjusted $p < 0.05$) correlation with LP community composition at the genus level. *Aurantiochytrium* and *Aplanochytrium* were negatively correlated with salinity, whereas *Ulkenia* was positively correlated with salinity. A further correlation test (Spearman's rank correlation test) revealed that *Aplanochytrium* had a negative correlation with depth and salinity ($p < 0.05$), whereas *Aurantiochytrium* was negatively correlated with total nitrogen ($p < 0.05$) and *Ulkenia* was positively correlated with chlorophyll a ($p < 0.01$).

3.4. Distribution Patterns of Dominant Labyrinthulomycetes Protists

To better understand the distribution patterns of LP in different habitats in the Qiongdong Sea, the 40 most abundant LP ASVs were analyzed with a heatmap. The average relative abundance of these ASVs in all samples was greater than 0.25% (93.26% of the total) (Figure 5). The most abundant ASV, ASV1, was present in all 12 samples and accounted for 39.97% of the total sequences and 31.25% of the total ASVs. It belonged to the genus *Aplanochytrium*, which is common in the South China Sea. Ward's hierarchical clustering showed that the Qiongdong Sea ASVs could be divided into four ecotypes (six

clusters), each with different distribution patterns. The first ecotype (clusters 1 and 3) was concentrated in the offshore habitat and belonged to the “offshore type”; the second ecotype (clusters 2 and 6) was distributed evenly across different habitats and belonged to the “relatively uniform type”; the third ecotype (cluster 4) was only found in the inshore bottom and belonged to the “inshore bottom type”; the fourth ecotype (cluster 5) was concentrated in the inshore habitat and belonged to the “inshore type”. Most of the ASVs in cluster 1 belonged to *Aplanochytrium*, and ASVs in cluster 6 accounted for 53.74% of the 40 most abundant ASVs. *Aplanochytrium* was mainly found in clusters 1 and 5, and 77.5% of the 40 most abundant ASVs did not have a clear classification.

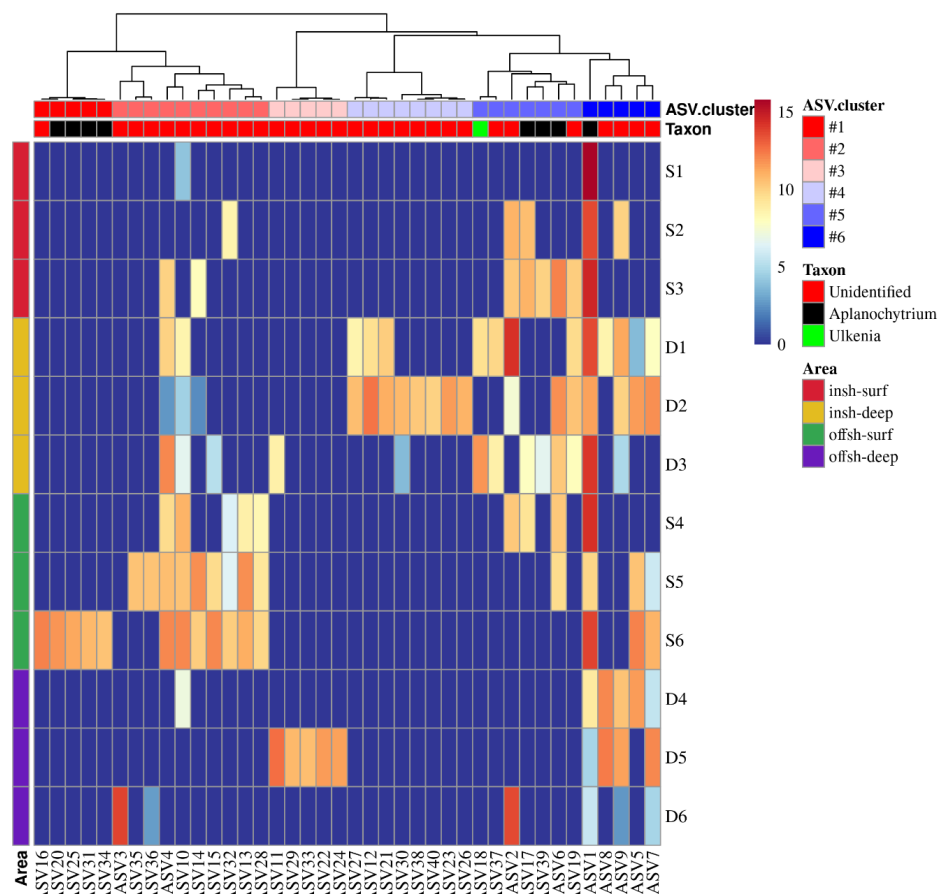


Figure 5. Heatmap illustrating the community structure of the 40 most abundant labyrinthulomycetes ASVs across different zones of inshore and offshore areas. Relative abundances were log2 transformed and scaled, and then clustered by column using Ward’s hierarchical clustering method. The ASVs were classified to the genus level. S: surface water; D: deep water; numbers after “S” or “D” indicate sampling stations.

3.5. Cooccurrence Network of Labyrinthulomycetes and Bacteria

A Spearman correlation-based network was created to investigate the potential interactions between LP and bacteria. The network included LP ASVs (*Aplanochytrium*, *Aurantiochytrium*, *Ulkenia*, p_Laby, o_Laby, and f_Laby) and 124 genera of bacteria with a total sequence count of at least 10 across samples. The data for bacteria were obtained from our previous study [32]. Since the surface and bottom layers showed significant environmental differences ($p < 0.01$ for temperature, salinity, silicate, etc.), two separate ecological networks were created to demonstrate the relationship between LP and bacteria in the surface and bottom habitats.

In the surface habitat network, the relationships between LP and bacteria were visualized with 29 nodes and 30 edges. Fourteen edges showed positive correlations and 16 edges

showed negative correlations (Figure 6a). *Aplanochytrium* and p_Laby played a significant role in the network, with *Aplanochytrium* mostly having negative correlations with bacteria, while p_Laby mostly had positive correlations with bacteria (Table S3). *Aurantiochytrium* was negatively correlated with bacteria, indicating a potential competitive relationship. In the bottom habitat network, with 48 nodes and 61 edges, *Aplanochytrium*, *Aurantiochytrium*, and f_Laby were key members of the network (Figure 6b). *Aplanochytrium* and f_Laby had mostly negative correlations with bacteria, while *Aurantiochytrium* had positive correlations with bacteria (Figure 6b, Table S4).

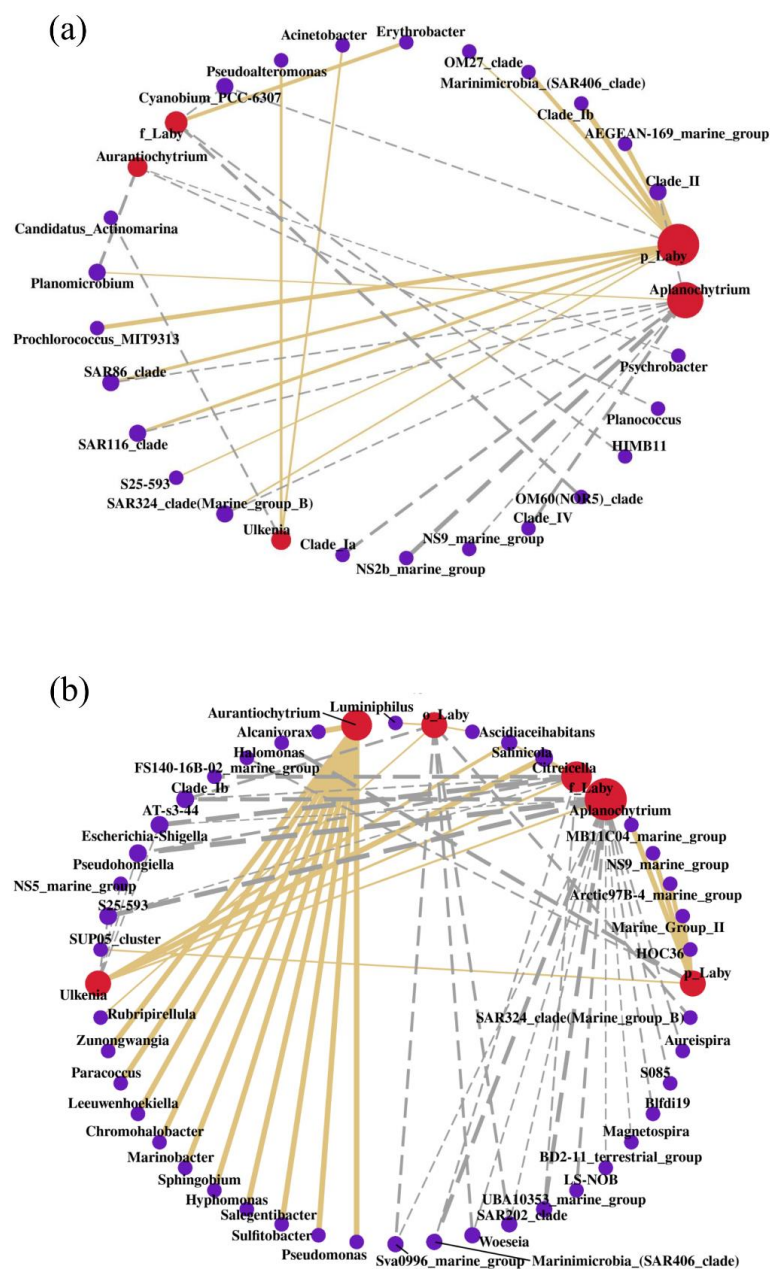


Figure 6. Cooccurrence network of: (a) surface; and (b) bottom Labyrinthulomycetes and bacteria. Red circles represent genera of Labyrinthulomycetes, and purple circles represent genera of bacteria, with larger dots representing a greater number of related objects. The brown solid line represents positive correlation, and the grey dashed line represents negative correlation—the thicker the line, the stronger the correlation surface layer and (b) bottom layer.

The interactions between LP and bacteria in the bottom habitat were more complex than those in the surface layer. *Aurantiochytrium* demonstrated a contrasting correlation pattern with bacteria in different habitats—a negative correlation in surface habitats and a positive correlation in bottom habitats. This suggests that in nutrient-deficient bottom habitats, LP may adapt their survival strategies to coexist with different genera of heterotrophic bacteria, such as *Marinobacter* and *Pseudomonas*, for the degradation and utilization of organic matter or to consume the metabolic waste produced by these bacteria.

4. Discussion

In this study, the highest abundance of LP was found in the chlorophyll maximum layer, suggesting a somewhat close connection between LP and algal-fixed organic nutrients. Previous studies in offshore Bohai Sea and open Hawaiian waters have also demonstrated the positive correlation between LP and chlorophyll [3,5], indicating their role in secondary production using organic nutrients from algae. Salinity has a significant impact on microbial communities, with a strong correlation between salinity and the planktonic bacterial community (NMDS or MDS, $p < 0.001$, $r > 0.7$) [33,34]. Changes in salinity have been shown to result in the rapid disappearance of freshwater phytoplankton and zooplankton [35,36]. Our previous research in the South China Sea showed that LP communities also change with salinity gradients [37]. Although salinity has a significant effect on LP, particulate and dissolved organic matter content are usually considered the key factors affecting LP abundance and distribution in estuarine and offshore waters [3,15,18,38], and should be considered in future studies.

The dominant genus found in our study was *Aplanochytrium*, accounting for a significant portion of the total ASVs and sequences. Previous studies have shown that *Aplanochytrium* is a prevalent genus in various oceans, including the South China Sea [39], Bohai Sea [3], and Hawaiian waters [5] suggesting it may play a crucial role in regulating ocean primary and secondary production. On the other hand, *Aurantiochytrium*, though not outstanding in cellulase secretion capacity [11], may still play a role in bottom habitats in breaking down plant-derived organic matter in cooperation with other decomposers like fungi and bacteria. While research on the potential ecotypes of LP is limited, a study in the East Indian Ocean showed four main ecotypes with distinctive vertical distribution patterns [20]. In the South China Sea, LP was also classified into multiple ecotypes based on their horizontal position and vertical depth, showing their potential importance in the biological pump through multiple ecotypes in the pelagic [39]. However, further investigation and verification of LP ecotypes are needed.

The interactions of LP with other organisms have been the subject of various studies. In the seawaters of North Carolina, USA, some LP phylotypes have been found to have time-lag correlations or co-occurrences with bacterial, algal, and fungal phylotypes [40]. In Hiroshima Bay, Japan, a massive increase in LP was observed after a raphidophyte outbreak, accompanied by a temporary increase in two unique viruses, indicating a close interaction between LP and phytoplankton and viruses [41]. Of particular note is the cooperative–competitive relationship between LP and heterotrophic bacteria. Despite sharing similar survival strategies, there have been no reports of LP antagonizing bacteria. Instead, LP were found to be positively correlated with bacterial abundance and transparent extracellular polymeric particles (TEPs) with high bacterial extracellular polysaccharide fractions [15,22], indicating that some LP may prefer TEPs derived from bacteria or tend to reside on particulate matter inhabited and utilized by bacteria. Some researchers propose that LP may adopt a “left-over scavenger” survival strategy [42], which allows them to more thoroughly decompose the organic matter remaining after bacterial decomposition.

5. Conclusions

This study provides new insights into the LP community in the coastal zone of Hainan Island in the South China Sea. The highest abundance of LP was found in the chlorophyll maximum layer, and salinity played an important role in the composition of the

LP community. The dominance of *Aplanochytrium* and the different synergistic competitive relationships between *Aurantiochytrium* and bacteria in different habitats were also highlighted. Further studies are needed to better understand the extensive and diverse interactions between LP and other organisms, as well as their potential ecological niche and functional differentiation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w15040738/s1>, Figure S1: Abundance of Labyrinthulomycetes protists at different depths in the inshore and offshore habitats. The data represent the mean $\pm$ S.E of three stations; Figure S2: Principal coordinate analysis (PCoA) based on Bray-Curtis similarities among the communities of Labyrinthulomycetes. Labels “offsh-surf”, “insh-surf”, “offsh-deep” and “insh-deep” represent offshore surface water, inshore surface water, offshore deep water and inshore deep water, respectively; Figure S3: Venn diagram showing the unique and common ASV numbers of different habitats. Labels “insh-deep”, “insh-surf”, “offsh-surf” and “offsh-deep” represent inshore deep water, inshore surface water, offshore surface water, and offshore deep water, respectively; Figure S4: Redundancy analysis (RDA) based on the level of the genus Labyrinthulomycetes. In simple effects and condition effects, only salinity was significantly associated. Table S1: Spearman’s rank correlation coefficients and *p* values between the abundance of LP and environmental factors; Table S2: Taxonomy of shared ASVs and their relative abundance; Table S3: Properties of the interaction network between Labyrinthulomycetes and Bacteria in the surface seawater; Table S4: Properties of the interaction network between Labyrinthulomycetes and Bacteria in the bottom seawater.

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