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Biodiversity and Enzyme Activity of Marine Fungi with 28 New Records from the Tropical Coastal Ecosystems in Vietnam

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ABSTRACT

The coastal marine ecosystems of Vietnam are one of the global biodiversity hotspots, but the biodiversity of marine fungi is not well known. To fill this major gap of knowledge, we assessed the genetic diversity (ITS sequence) of 75 fungal strains isolated from 11 surface coastal marine and deeper waters in Nha Trang Bay and Van Phong Bay using a culture-dependent approach and 5 OTUs (Operational Taxonomic Units) of fungi in three representative sampling sites using next-generation sequencing. The results from both approaches shared similar fungal taxonomy to the most abundant phylum (Ascomycota), genera (*Candida* and *Aspergillus*) and species (*Candida blankii*) but were different at less common taxa. Culturable fungal strains in this study belong to 3 phyla, 5 subdivisions, 7 classes, 12 orders, 17 families, 22 genera and at least 40 species, of which 29 species have been identified and several species are likely novel. Among identified species, 12 and 28 are new records in global and Vietnamese marine areas, respectively. The analysis of enzyme activity and the checklist of trophic mode and guild assignment provided valuable additional biological information and suggested the ecological function of planktonic fungi in the marine food web. This is the largest dataset of marine fungal biodiversity on morphology, phylogeny and enzyme activity in the tropical coastal ecosystems of Vietnam and Southeast Asia. Biogeographic aspects, ecological factors and human impact may structure mycoplankton communities in such aquatic habitats.

1. Introduction

Vietnamese coastal marine ecosystem is one of the most biologically diverse marine ecosystems, overlapping with the Coral Triangle and 4 out of 35 global biodiversity hotspots with unique biota. This region is identified as a high-priority area for marine conservation [1]. However, the marine ecosystems of Vietnam are severely impacted by climate change [2] and anthropogenic pollutions [3–7]. Severe ecological impacts from anthropogenic disturbance on the marine biodiversity of Vietnam have been observed in recent years [3,7,8]. Among marine organisms, fungi play a critical role in biogeochemical cycles [9,10]. Assessing the biodiversity of marine fungi in the coastal environment of Vietnam is the first but critical step to provide a scientific base for conservation as rapid biodiversity loss is observed in this region [11]. Furthermore, it opens the opportunity to explore bioactive compounds from marine fungi that may have great pharmacological and industrial applications [12–16]. However, the biodiversity of marine fungi and their

ecological roles in the coastal environment of Vietnam is largely unknown.

A marine fungus is defined as "any fungus that is recovered repeatedly from marine habitats and: 1) is able to grow and/or sporulate (on a substrate) in marine environments; 2) forms symbiotic relationships with other marine organisms; or 3) is shown to adapt and evolve at the genetic level or be metabolically active in marine environments" [17]. The updated classification of marine fungi has 1,112 species, 472 genera, 129 families and 65 orders. Major phyla include Ascomycota, Basidiomycota, Chytridiomycota (chytrids) and related phyla, Zygomycota, and Blastocladiomycota. The Halosphaeriaceae remains the largest family of marine fungi with 141 species in 59 genera, while the most specious genera are *Candida* (64 species), *Aspergillus* (47 species), and *Penicillium* (39 species) [18]. The members of Zygomycota are now part of two phyla Mucoromycota and Zoopagomycota, and mostly terrestrial in habitat [19]. Marine fungi were retrieved exclusively from the sea surface to the

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⊕ Supplemental data for this article can be accessed here.

depth of kilometers beneath the ocean sediments. Despite their diverse roles in nutrition cycles and food webs, as well as their commensal interaction or pathogenicity to other organisms, little is known about the diversity of this major eukaryotic branch in their marine ecosystems or their ecological functions [9].

The diversity of marine plankton fungi has been studied from a few coastal locations such as Portugal [20], Taiwan [21], India [22], Chile [23,40], Brazil [24], France [25], and China [26,27]. A few notable studies were performed, e.g., molecular analysis across 130 European marine samples [28]. More studies were reported on fungi in the marine environments associated with animals, plants and algae [29–36] and in deepsea waters [37–42]. These molecular studies have retrieved marine fungal sequences belonging to Ascomycota, Basidiomycota, and Chytridiomycota, and some of them were grouped into novel environmental clusters.

In this study, we comprehensively assessed the biodiversity of fungi in the coastal marine habitats in Nha Trang Bay and Van Phong Bay of the Central Vietnam region where is experiencing a rapid increase in anthropogenic disturbances from urbanization, tourism, shipping, and aquaculture development that may seriously affect the marine environment and accelerate biodiversity loss [6,11,43–46]. Importantly we used two different methods that are culture-dependent and culture-independent approaches that may provide complementary results to have a more comprehensive assessment of marine fungal biodiversity. Culturable fungal strains were identified based on ITS sequence, followed by the description of morphology, phylogeny and taxonomy of major fungal classes and orders. To provide valuable additional biology information and mechanistically assess the ecological role of fungi in the marine ecosystems, we analyzed the total relative enzyme activity and checklist of trophic mode and guild assignment.

2. Materials & methods

2.1. Site description and sampling strategy

A total of 11 seawater samples were collected from coastal marine habitats of Nha Trang Bay and Van Phong Bay at Khanh Hoa Province, Vietnam from May to August 2017 (Table 1, Figure 1) using a 2.2-liter sterile sampler (Horizontal Beta Bottle style, Wildco, Yulee, Florida, USA). Water samples were carried in 1.5 liters sterile, screw-capped plastic bottles, stored in ice and transferred immediately to the laboratory after collection. Also, the temperature, pH, and salinity of the water samples were monitored at each sampling site using a multiparameter

Table 1. Details regarding colony forming units (CFUs) of fungal colonies on the SDA medium plates and strains identified with ITS sequence.

Sampling date	Location	Lat (°N)	Long (°E)	Habitat	Depth (m)	pH	Temperature (°C)	Salinity (ppt)	CFU L^{-1}	No. of colonies (strains)		
										Yeast	Filamentous fungi	Total
07/05/2017	Nha Trang Central Beach (NB)	12°14′	109°11′	Coastal marine waters (CMW)	0.03–0.05	7.95	29	1.6	6.0×10^2	6 (5)	2 (2)	8 (7)
07/05/2017	Song Cai River Estuarine (SC)	12°15′	109°11′	CMW	0.03–0.05	8.15	28	3.5	2.0×10^3	6 (2)	7 (5)	13 (7)
20/5/2017	Doc Let Beach (DL)	12°33′	109°13′	CMW	0.03–0.05	7.79	31	3.45	9.3×10^2	10 (5)	2 (2)	12 (7)
24/5/2017	Dam Mon Lagoon (DM)	12°40′	109°24′	CMW	0.03–0.05	8.4	32	3.5	2.4×10^4	10 (7)	3 (3)	13 (10)
24/5/2017	Tu Bong River Estuarine (TB)	12°46′	109°19′	CMW	0.03–0.05	8.38	33.5	3.5	3.1×10^4	7 (5)	4 (4)	11 (9)
24/5/2017	Ran Trao Marine Ecosystem Reserve (RT)	12°37′	109°12′	CMW	0.03–0.05	7.96	32	3.25	1.4×10^4	16 (3)	1 (1)	17 (4)
09/06/2017	Cau Da Port (CD)	12°12′	109°13′	CMW	0.03–0.05	8.01	29.5	3.3	5.4×10^4	19 (4)	3 (2)	22 (6)
10/8/2017	Vung Ngan Aquaculture Zone (VN)	12°11′06″	109°16′25″	CMW	0.03–0.05	8.01	29	3.25	5.7×10^3	13 (2)	5 (4)	18 (6)
10/8/2017	Hon Mun Core Zone (HMC)	12°10′33″	109°18′16″	CMW	0.03–0.05	7.97	29	3.3	1.1×10^5	21 (3)	1 (1)	22 (4)
10/8/2017	Hon Mun Buffer Zone (HMB)	12°10′72″	109°18′56″	CMW	0.03–0.05	7.96	30	3.4	1.8×10^5	7 (2)	4 (4)	11 (6)
01/6/2017	Deep Waters (DW)	12°18′31″	109°31′67″	Deep waters	40–47	7.0	22	3.44	8.8×10^2	0 (0)	16 (9)	16 (9)
Total										115 (38)	48 (37)	163 (75)
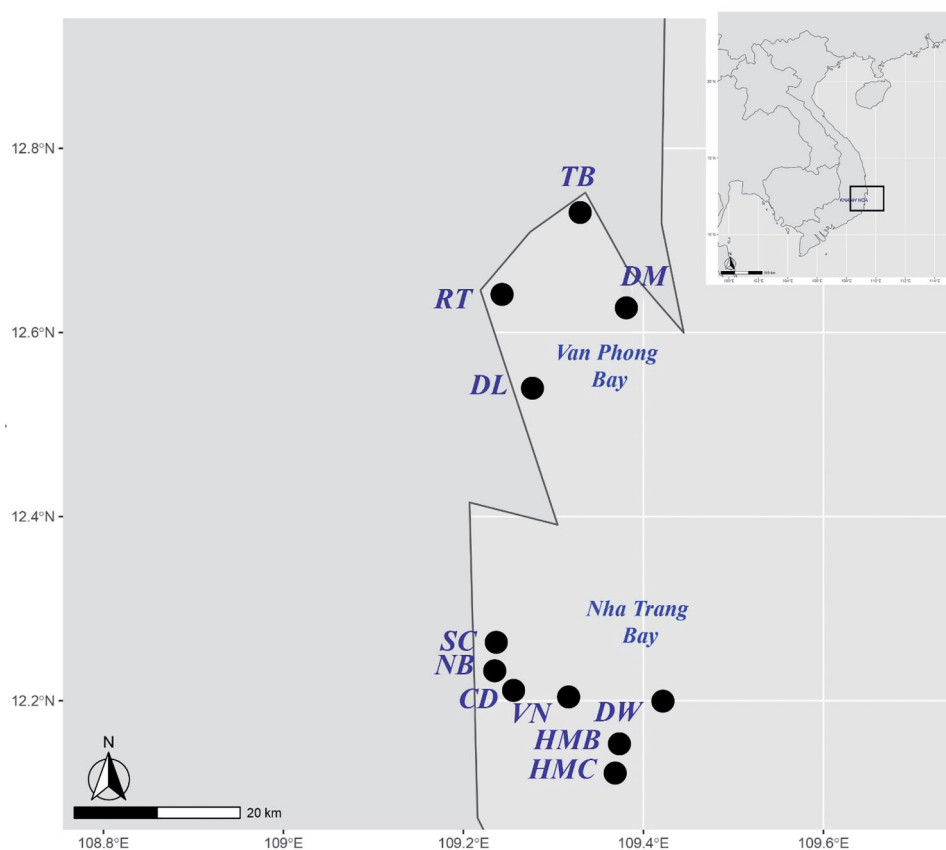
Figure 1. Sampling positions at Nha Trang Bay and Van Phong Bay of Khanh Hoa Province, Vietnam. Detail information on locations was given in the Table 1.

meter. The sample sets were randomly split into two subsamples to employ the two culture-dependent and culture-independent approaches simultaneously.

2.2. Culture-dependent approach

2.2.1. Isolation and identification of marine fungi

Fungi from the seawater samples were isolated within three hours of collection using the membrane filtration technique [26]. Briefly, seawater samples were diluted into concentrations at 10^{-1}, 10^{-2}, and 10^{-3}. Subsequently, diluted water samples (15 ml, triplicate) were filtered through sterile 0.45 μm cellulose esters membrane (MilliporeSigma, Burlington, Massachusetts, USA). These membranes were then placed on solid media plates of Sabouraud Dextrose Agar (SDA) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with antibiotics (0.075% streptomycin sulfate and 0.05% ampicillin) (Thermo Fisher Scientific) to suppress bacterial growth. The plates were incubated at 28 °C for 2–3 days and examined daily for the growth of fungi. Fungal colonies that developed were subcultured onto fresh Potato Dextrose Agar (PDA) (Thermo Fisher Scientific) plate (for filamentous fungi) or Yeast Extract Peptone and Dextrose (YPD) (Thermo Fisher Scientific) plate (for yeast) for pure, single colony isolation and identification. Filamentous fungi were identified based on

macroscopic and microscopic morphological characteristics (Figure S1, Supplementary Material). Strains were named according to the name of the sampling location, the order of strains isolated from each site, and a final symbol distinguishing between yeast (Y) and mold (M) (Table S1, Supplementary Material). For example, the strain VN1Y means that it was sampled at Vung Ngan (VN) as the first (1) strain isolated from this site and it is a yeast (Y). Fungal strains with different morphology have been selected for further analysis and deposited in Microbiological Culture Collection at Nha Trang University (NTU), Vietnam with isolate IDs shown in Table S1 (Supplementary Material).

2.2.2. DNA isolation, PCR and sequencing

For DNA isolation, yeasts were grown in a YPD broth medium and shaken at 170 rpm for 1–3 days at 25–28 °C, whereas a PDA plate was used for growing filamentous fungi for 3–5 days at the same temperature. Yeast cells were harvested by centrifugation and genomic DNA was extracted according to the method of Harju et al. [47]. For mold, mycelia were harvested, lyophilized and crushed in a mortar with pestle using liquid nitrogen to a fine powder. Genomic DNA from mold isolates was extracted using the EZ − 10 Spin Column Plant Genomic DNA Miniprep Kit (Bio Basic, Canada) as

directed by the manufacturer. The extracted DNA samples were stored at $-20\,°C$.

The ITS domain of nuclear ribosomal genes was amplified by polymerase chain reaction (PCR) using the appropriate specific primers to target Ascomycota and Basidiomycota (ITS1-F_KYO2: 5'-TAG AGG AAG TAA AAG TCG TAA-3' [48] and ITS4-R: 5'-TCC TCC GCT TAT TGA TAT GC-3' [49]). The PCR method was performed as described previously [46,50] with some modifications. Briefly, one microliter of DNA ($\sim$25 ng) was added to 50 μl reaction volume containing 1 μl of Taq polymerase (Bioline, Memphis, Tennessee, USA), 10 μl of 5X MyTaq reaction buffer, 35 μl of distilled deionized water and 10 pmoles of each primer. The PCR program of ITS rRNA gene was run for initial denaturation step at $93\,°C$ for 3 min, followed by 35 cycles of 0.5 min at $93\,°C$, 0.5 min at $55\text{-}60\,°C$ and 0.5 min at $72\,°C$, and a final extension at $72\,°C$ for 5 min. The amplified products were separated on a 1% (w v^{-1}) agarose gel stained with ethidium bromide and visualized under a UV transilluminator.

PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany), and then Sanger sequenced at Macrogen (Soul, Korea) in both directions with the same PCR primers as described above using Big Dye terminator in a 3730×l DNA Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). The DNA sequences generated in this study are deposited in GenBank under the accession numbers from MT102787 to MT102861 for the ITS gene.

2.2.3. Sequence alignment and phylogenetic analyses

Partial gene sequences of fungi samples obtained in this study and reference sequences available in GenBank were used for sequence analysis at the National Center for Biotechnology Information (NCBI) using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). Gene sequences were aligned using MUSCLE [51], and regions with gaps were removed using BioEdit [52]. Model selection was used to determine the best fit model with the lowest Bayesian Information Criterion score for Maximum Likelihood, Minimum Evolution, and Neighbor-Joining method [53], which was then used to construct a phylogenetic tree using the MEGA X program [54]. The evolutionary distances were computed by using the Kimura 2-parameter model and Tamura 3-parameter model [55]. The robustness of the tree topology was tested by bootstrap analysis with 1000 re-samplings [56].

2.3. Culture-independent approach

2.3.1. DNA extraction from seawater samples for metabarcoding

As the three seawater samples HMC, DW and CD showed the highest density of marine fungi by culture-dependent approach at Nha Trang Bay, they were selected as 3 representatives for culture-independent studies (Table 1). Seawater samples were filtered through sterile 0.45 μm cellulose ester membranes (MilliporeSigma, Burlington, Massachusetts, USA). DNA samples were extracted directly from filters using the PowerWater DNA Isolation Kit (Qiagen) according to the manufacturer's instructions. All the DNA samples were quantitated and quality checked with the NanoDrop ND-2000C spectrophotometer (ThermoFisher Scientific, Dreieich, Germany). DNA extracts were then stored at $-20\,°C$ for further analysis.

2.3.2. Marine fungi analysis: Sequencing

Metagenome amplicon libraries were generated from DNA extracts and sequenced at Macrogen (Soul, Korea) using the standard protocol as described in the manufacturer's instructions (Illumina, San Diego, California, USA). Briefly, amplification of ITS regions was performed using the sequence-specific primers (ITS1-F_KYO2 and ITS4-R) described above in a two-step dual-indexed PCR approach designed specifically for Illumina instruments, in which the gene-specific sequence was fused to the Illumina sequencing primers. The quantity of DNA was checked by picogreen method with Quant-iT^{TM} PicoGreen^{TM} dsDNA Assay Kit (Introgene, Leiden, Netherlands) using Victor 3 fluorometry. The library quality was assessed on an Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip to verify the size of PCR enriched fragments. For library quantity check, qPCR was used according to the Illumina qPCR Quantification Protocol Guide to achieving the highest quality of data on Illumina sequencing platforms. To generate a standard curve of fluorescence readings and calculate the library sample concentration, Roche's Rapid library standard Quantification solution and calculator were used. At last, the library was sequenced on an Illumina MiSeq2500 platform and 301 bp paired-end reads were generated.

2.3.3. Marine fungi analysis: Bioinformatic analysis

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 (V1.2.11, http://ccb.jhu.edu/software/FLASH/) [57] to significantly improve read assemblies.

To remove noise data and generate OTU clusters, assembled reads were pre-processed and clustered using CD-HIT-OTU (http://weizhongli-lab.org/cd-hit-otu/) [58] according to the User's Guide (http://weizhong-lab.ucsd.edu/cd-hit-otu/wiki/doku.php?id=cd-hit-otu_user_guide). Briefly, a three-step clustering was used for identifying OTUs. The first-step clustering was raw read filtering and trimming. The second step was error-free reads picking. In the last step, OTU clustering was generated at different distance cutoffs (0.03).

The program QIIME [59] with script *assign_taxonomy.py* (http://qiime.org/scripts/assign_taxonomy.html) was used to assign taxonomy of the representative OTUs against the Unite Database (https://unite.ut.ee) [60] using the Blast algorithm (≥80% identity and coverage).

The ITS fragments were extracted from both the Sanger sequencing (traditional) and Illumina sequencing datasets and the sequence similarity-based comparison was performed using the direct matching based on the Blast algorithm (≥95% identity and coverage) at the species level.

The eukaryote ITS rDNA genes Illumina sequencing data are deposited in the NCBI under the BioProject with GenBank accession numbers from MT364507 to MT364634.

2.4. Screening for extracellular enzyme activities

The fungal isolates were screened using a qualitative plate assay for different enzymes (protease, amylase, phytase, cellulase and chitinase) by streaking them on the modified Czapek-Dox agar plates, supplemented with ampicillin (0.05%) and streptomycin sulfate (0.075%) as well as specific substrates. Modified Czapek-Dox agar medium included $NaNO_3$ 2 g L^{-1}, K_2HPO_4 1 g L^{-1}, $MgSO_4$ $7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1}, $FeSO_4$ $7H_2O$ 0.01 g L^{-1}, agar-agar 15 g L^{-1}, at pH 6.5 ± 0.2. Sterile seawater was used at 50% (w w^{-1}).

Protease activity was detected using modified Czapek–Dox agar plates after substitution $NaNO_3$ with 1.0% skim milk [61]. The fungal isolates were grown on these media plates at 28 °C for 5 days. The diameter of the clear zone around the colonies indicated protease activity.

Amylase activity was detected using modified Czapek–Dox agar plates supplemented with 1.0% soluble starch as the sole carbon source [62]. The fungal isolates were grown on these media plates at 28 °C for 5 days. Then all culture plates (duplicates) were flooded with Lugol's iodine solution. The diameter of the clear zone surrounding the colonies indicated amylase activity.

Phytase activity was detected using modified Czapek-Dox agar plates supplemented with sodium phytate (Sigma-Aldrich, St. Louis, Missouri, USA) at the concentration of 10 g L^{-1} [63]. The fungal isolates were grown on these media plates at 28 °C for 3 days. Then, petri dishes were flooded with Lugol's iodine solution. The diameter of the clear zone around the colonies indicated phytase activity.

Cellulase activity was detected using modified Czapek-Dox agar plates supplemented with 0,5% cacboxylmethyl cellulose [64]. The fungal isolates were grown on these media plates at 28 °C for 4 days. Then, petri dishes were flooded with an aqueous solution of 0.1% Congo red and shaken at 80 rpm for 15 min on a rotary shaker. The diameter of the clear zone around the colonies on media plates indicated cellulase activity.

Chitinase activity was detected using modified Czapek–Dox agar plates supplemented with colloidal chitin 10 g L^{-1} [65]. The fungal isolates were grown on these media plates at 28 °C for 7 days. The diameter of the clear zone around the colonies on media plates indicated chitinase activity.

2.5. Fungal functional assignment and checklist

Fungal functional guilds were assigned using an open annotation tool (FUNGuild) [66, online version: http://www.funguild.org/). Only the guild assignment with at least "possible" confidence rankings was accepted. Distribution and disease notes were confirmed and checklists from Fungal Databases of U.S. National Fungus Collections [67] and previous studies published on National Center for Biotechnology Information (https://pubmed.ncbi.nlm.nih.gov/) by 23 March 2021.

2.6. Statistic analysis

The program QIIME [59] with scripts such as *alpha_diversity.py* (http://qiime.org/scripts/alpha_diversity.html) was used to calculate alpha diversity (OTUs, Chao1, Shannon, Simpson) related to fungal OTU richness; *alpha_rarefaction.py* (http://qiime.org/scripts/alpha_rarefaction.html) to produce rarefaction curve graph; and *make_2d_plots.py* (http://qiime.org/scripts/make_2d_plots.html) to make 2 D PCoA Plots.

3. Results

3.1. Fungi density and molecular identification in the coastal ecosystems of Vietnam: Culture-dependent approach

The density of fungi of 11 surfaces coastal marine and deeper waters at Nha Trang Bay and Van

Phong Bay ranged from 10^2 CFU L^{-1} to 10^5 CFU L^{-1} (Table 1). The lowest density of fungi was found in NB (6.0×10^2 CFU L^{-1}) at Nha Trang Bay and DL (9.3×10^2 CFU L^{-1}) at Van Phong Bay (Table 1). The Hon Mun Marine Protected Area of Nha Trang Bay had the highest fungal density, namely 1.8×10^5 CFU L^{-1} in the buffer zone and 1.1×10^5 CFU L^{-1} in the core zone HMC (Table 1), which were two orders of magnitude higher than the fungal density in NB and DL.

Overall, a total of 163 fungal colonies, including 115 yeasts and 48 filamentous fungi, were recovered on SDA medium in all samples with differences in depth, pH, temperature and salinity (Table 1). The Nha Trang central beach NB sample had the lowest number of isolates (8 with 6 yeasts and 2 filamentous fungi), while the deeper water DW sample expressed the highest number of filamentous fungi isolates (16 vs. 1–7 of all other samples) and none of the yeasts (Table 1).

Fungal isolates with morphological differences were further selected for DNA extraction, sequencing and identification based on ITS sequences. Previous studies have shown that some fungal species have at 99% and others at 93% their species-specific sequence similarity [68]. To account for these differences, the UNITE community has clustered all ITS sequences at several sequence similarity thresholds to obtain approximate species-level OTUs referred to as species hypotheses with the 98.5% threshold level given [60]. Moreover, a current systematic study suggested that the optimal identity threshold to discriminate filamentous fungal species is 99.6% for ITS [69]. In the present study, among a total of 75 strains, 38/38 yeasts and 19/37 filamentous fungi were identified into species (Table 2) with the fungal ITS rRNA gene sequences, which showed 99–100% identity with the closest related existing sequences of NCBI database (Table S1, Supplementary Material). All 18 other filamentous fungal strains were identified into genus (Table 2), including 4 sharing 95.6–98.3% identity with one closest related type strain (Group A) and 14 sharing 99–100% identity with at least two closest related type strains (Group B). Group A composes of DW7M (96.6% identity with *Fusicolla acetilerea* IMI 181488 (T)), DL11M (97.2% identity with *Penicillium simplicissimum* CBS 280.39 (T)), DW3M (98.16% identity with *Stagonosporopsis oculi-hominis* CBS 634.92 (T)), and DL12M (98.3% and 95.6% identity with *Phanerochaete chrysosporium* H008 and its type strain IFM 47473) (Table S1, Supplementary Material). Group B includes *Cladosporium colombiae*-like DM11M1 and NB8M; *Aspergillus aflatoxiformans/austwickii*-like TB8M, TB9M, DW1M and DW2M; *A. costaricaensis*-like TB10M; *A. uvarum/japonicus*-like DM11M2 and DM12M; *A. versicolor*-like VN17M, VN18M and HMB11M; *Penicillium cuddlyae*-like DW14M; and *Trichoderma atrobrunneum/lixii*-like SC13M (Table 2). The strains of Group A and some of Group B can be considered as potential novel species but require further studies with other marker genes and morphology (*our unpublished data*). One of these strains, DW14M, has currently been described as a novel species, *Penicillium vietnamense* sp. nov [70], *submitted in parallel to Mycobiology*).

3.2. Fungi diversity in the coastal ecosystems of Vietnam: Culture-independent approach

Three sampling sites HMC, CD, and DW showed a representatively high density of marine fungal community at Nha Trang Bay with differences in ecological characteristics and level of human impact on their habitats. Therefore, these sampling sites were selected for cultivated-independence studies. (1) HMC (Hon Mun Core Zone) is a representative of the core zone of Hon Mun marine protected ecosystems with low human impact, w/ere expressed the highest density of marine fungi at 10^5 CFU L^{-1} from the culture-dependent approach in this study. (2) CD (Cau Da Port), a harbor site with high-frequency human activity, is another worthy representative with the second-highest density of 5.4×10^4 CFU L^{-1}. (3) DW (Deep waters at Nha Trang Bay) had the highest number of filamentous fungal strains isolated and identified, and also a high potential for the presence of uncultivable fungi in a different deeper habitat (lower pH and temperature but higher hydrostatic pressure) compared to surface coastal marine habitats as explained above.

From a total of 749,989 sequences after the assembly, a sum of 402,825 quality-filtered ITS reads were acquired after the expulsion of chimeric and the unique tag (Table S2, Supplementary Material). Subsequent to normalizing all data collections to the smallest sequence read and eliminating all uncommon taxa, the last analyzed data indexes contained 128 microeukaryotes OTUs. The noticed OTU wealth and Shannon variety were utilized straightforwardly for additional investigations. The abundance of total microeukaryotes from various samples was the highest at HMC (108 OTUs), followed by CD (70 OTUs) and lastly, DW (44 OTUs) (Table S2, Supplementary Material).

Among microeukaryote OTUs, fungi were relatively dominated with a total abundance of 13.82% (55 368 fungal read counts/400 639 microeukaryotes read counts) (Table S2, Supplementary Material). However, the richness of fungi was low with a total of 5 fungal OTUs ranging from 1 (DW sample) to 3 (CD and HMC samples) OTUs (Table 3). The majority of fungal OTUs were highly abundant: *Candida_blankii*OTU_2 had a relative abundance of

Table 2. Taxonomy of cultured fungal species found in this study.

No.	Division	Subdivision	Class	Order	Family	Genus	Species	Strain[1]	Relative abundance (%)
1	Ascomycota	Pezizomycotina	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	C. colombiae-like	DM11M1, NB8M	2.67
2							C. cycadicola	DW8M	1.33
3							C. sphaerospermum	VN16M	1.33
4				Dothideales	Saccotheciaceae	Aureobasidium	A. melanogenum	DL9Y	1.33
5				Pleosporales	Didymellaceae	Stagonosporopsis	S. oculi-hominis-like	DW3M	1.33
6					Pleosporaceae	Alternaria	A. alstroemeriae	CD22M	1.33
7			Eurotiomycetes	Chaetothyriales	Cyphellophoraceae	Cyphellophora	C. oxyspora	HMB10M	1.33
8				Eurotiales	Aspergillaceae	Aspergillus	A. aflatoxiformans/austwickii-like	TB8M, TB9M, DW1M, DW2M	5.33
9							A. costaricaensis-like	TB10M	1.33
10							A. uvarum/ japonicus-like	DM11M2, DM12M	2.67
11							A. alabamensis	HMB8M, HMB9M, VN15M	4.00
12							A. versicolor-like	VN17M, VN18M, HMB11M	4.00
13						Penicillium	P. paxilli	NB7M	1.33
14							P. steckii	SC7M	1.33
15							P. citrinum	CD20M	1.33
16							P. oxalicum	TB11M	1.33
17							P. simplicissimum-like	DL11M	1.33
18							P. cuddlyae-like	DW14M	1.33
19			Sordariomycetes	Hypocreales	Cordycipitaceae	Parengyodontium	P. album	DW11M, DW13M	2.67
20						Simplicillium	S. obclavatum	DW15M	1.33
21					Hypocreaceae	Trichoderma	T. lixii	SC9M	1.33
22							T. yunnanense	SC11M	1.33
23							T. atrobrunneum/ lixii-like	SC13M	1.33
24					Nectriaceae	Fusicolla	F. acetilerea-like	DW7M	1.33
25				Trichosphaeriales	Trichosphaeriaceae	Nigrospora	N. sphaerica	HMC22M	1.33
26				Sordariales	Sordariaceae	Gelasinospora	G. saitoi	RT3M	1.33
27		Saccharomycotina	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	Candida	C. blankii	15[2]	20.00
28							C. tropicalis	6[3]	8.00
29							C. akabanensis	TB1Y, TB6Y	2.67
30							C. intermedia	DL6Y, DL8Y	2.67
31						Millerozyma	M. farinosa	NB1Y	1.33
32						Meyerozyma	M. caribbica	TB3Y	1.33
33						Limtongozyma	L. cylindracea	DL7Y	1.33
34					Pichiaceae	Pichia	P. kudriavzevii	TB5Y, DM4Y, VN9Y, DM2Y	4.00
35					Metschnikowiaceae	Kodamaea	K. ohmeri	DM9Y	2.67
36	Basidiomycota	Agaricomycotina	Agaricomycetes	Polyporales	Phanerochaetaceae	Phanerochaete	P. chrysosporium-like	DL12M	1.33
37		Pucciniomycotina	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	Rhodotorula	R. mucilaginosa	NB2Y, CD18Y	2.67
38							R. diobovata	DM5Y	1.33
39						Rhodosporidiobolus	R. fluvialis	CD19Y	1.33
40	Mucoromycota	Mucoromycotina	Mucoromycetes	Mucorales	Mucoraceae	Hyphomucor	H. assamensis	SC10M	1.33
Total	3	5	7	12	17	22	≥ 40	75	100

[1]Strains identified by molecular data in this study. [2]15 *Candida blankii* strains: SC2Y, CD1Y, CD2Y, DM6Y, DM7Y, DM8Y, RT5Y, RT6Y, RT7Y, HMC1Y, HMC2Y, HMC11Y, HMB1Y, HMB7Y, VN1Y; and [3]6 *Candida tropicalis* strains: NB3Y, NB5Y, NB6Y, SC1Y, DL5Y, TB4Y.

Table 3. Phylogenetic affiliation and taxonomy of the fungal OTUs obtained with ITS primers based on cultivated-independence studies.

| GenBank Accession	Final taxon	Phylum	Function	Closest relative	GenBank Accession	Identity (Coverage) %	Originally reported habitat	Abundance count				Abundance (%)			
								CD	HMC	DW	Average	CD	HMC	DW	All
MT364509	*Candida_blankii* OTU_2	Ascomycota	Pathotroph: Human, animal and plant Pathogen	*C.blankii* isolate IIC1M.1	MF940143	100 (100)	Sands of Copacabana's beach, Rio de Janeiro, Brazil	2467	51,651	0	18039.33	95.95	97.99	0	97.74
				C. blankii CD1Y, CD2Y, HMC1Y, HMC2Y and HMC11Y	MT102799 MT102800 MT102807 MT102808 MT102809	99.65–100 (95)	CD and HMC samples in this study (direct matching)								
MT364534	*Aspergillus_versicolor* OTU_29	Ascomycota	Saprotroph: Aliphatic and aromatic hydrocarbon degradation	*Aspergillus versicolor* HT-M36	KJ527011	100 (100)	Fen-Daqu fermentation starter (China)	43	782	0	275.00	1.67	1.48	0	1.49
MT364570	*Epicoccum_sorghinum* OTU_65	Ascomycota	Possible plant pathogen	*Epicoccum sorghinum* HM19B	MT125854	100 (100)	Dark Leaf Spot Diseased Rice	0	250	0	83.33	0.00	0.47	0	0.45
MT364606	Chytridiomycota OTU_101	Chytridiomycota	Unknown	Uncultured fungus clone S12T_70	KU163812	100 (94)	Sea water in China	0	0	87	29.00	0.00	0.00	100	0.16
MT364609	Fungal OTU_104	Unclassified Fungi	Possible phytoplankton pathogen	Uncultured fungus isolate DGGE gel band F-SW39	KC491368	90.3 (100)	Sea water in South Florida, Crab Cove, USA	61	27	0	29.33	2.37	0.05	0	0.16
Total								2571	52,710	87	18,456	100	100	100	100

total fungi at 95.95% and 97.99% to CD and HMC samples, respectively, while the DW sample had 100% unclassified ChytridiomycotaOTU_101 (Table 3). The other frequently detected OTUs were Aspergillus_versicolorOTU_29 (1.67% at CD and 1.48% at HMC), Epicoccum_sorghinumOTU_65 (0.47% at HMC), and FungalOTU_104 (2.37% at CD and 0.05% at HMC).

3.3. Comparison of culture-dependent and culture-independent approaches

The results of the fungi diversity and community composition of the 3 surfaces coastal marine and deeper waters (CD, HMC, DW) at Nha Trang Bay using culture-dependent and culture-independent approaches were similar to the most abundant phylum, genus and species but different at less common taxa. In particular, the phylum Ascomycota was found to be most common at 3 samples HMC, CD and DW with the average relative abundance of 89.50% and 99.68% from culture-dependent and culture-independent approaches, respectively (Figure 2). At the genus level, only *Candida* and *Aspergillus* were detected in both approaches (Tables 2 and 3). The results from both approaches showed that members of ascomycetes and these two genera were the most dominant in the mycoplankton community of Nha Trang Bay. Compared to the culture-dependent approach, the culture-independent approach additionally identified members of Chytridiomycota (ChytridiomycotaOTU_101) in the DW sample, other ascomycetes (Epicoccum_sorghinumOTU_65) in the HMC sample, and unclassified uncultured fungi (FungalOTU_104), where the sequence similarity was around 90% with the closest relative, in the CD and HMC samples.

At the species level, *Candida blankii* was detected as the most abundant species in surface coastal marine habitats CD and HMC and absent in the deeper water DW in both approaches. In particular, the *Candida blankii* strains identified from the culture-dependent approach included 33.3% strains found in the CD sample, 75% strains in the HMC sample and none in the DW sample (Table 2), whereas the culture-independent approach measured the abundance of this species at 95.95%, 97.99% and 0.00% in the fungal community of samples CD, HMC and DW, respectively (Table 3). Interestingly, direct matching of the ITS sequences of fungi detected from these two approaches showed that *Candida_blankii*OTU_2 in the CD and HMC samples from the culture-independent approach shared 99.65–100% identity (coverage 95%) to all 5 *Candida blankii* strains isolated from these two
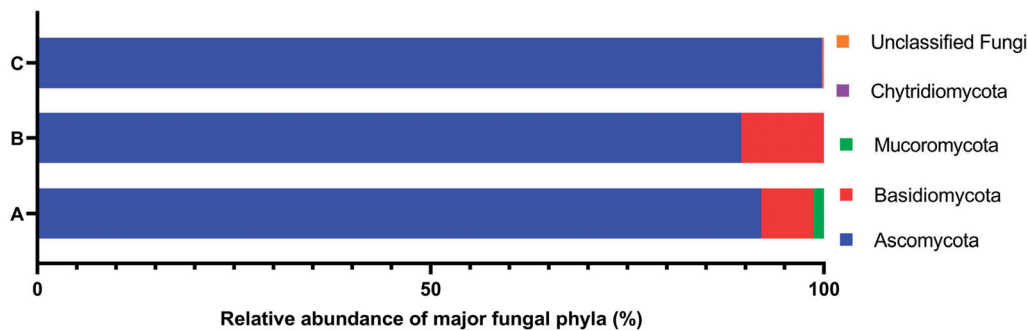
Figure 2. The percentage abundance of major phyla of fungi isolates from all 11 samples using culture-dependent approach (A), fungi isolates from 3 samples HMC, CD and DW using culture-dependent approach (B), and fungal OTUs from 3 samples HMC, CD and DW using culture-independent approach (C).

samples by the culture-dependent approach (Table 3), confirming the exact results from both methods.

### 3.4. Morphology, phylogeny and taxonomy of major fungal classes and orders

Regarding taxonomy, fungal taxa were identified using both morphology and molecular techniques. Identified species are listed in Table 2, morphology is presented in Supplementary Material (Figure S1), phylogenetic relationships in Figures 3–7 and checklist with additional information are provided in the Supplementary Material (Table S3). These results showed that the culturable fungal strains in this study belonged to 3 phyla, 5 subdivisions, 7 classes, 12 orders, 17 families, 22 genera and at least 40 taxa to species level, of which 29 species were identified. *Hyphomucor assamensis* (1.33%) was the only one identified as a member of the phylum Mucoromycota (formerly Zygomycota), whereas Ascomycota and Basidiomycota made up for 91.92% and 6.65% of total fungal strains, respectively (Figure 2). Of the ascomycetes, strains belonged to 18 genera, including *Penicillium* and *Aspergillus* as the most diverse genera in species number (5-6 species each), followed by *Candida* (4 species), *Cladosporium* (3 species), *Trichoderma* (3 species), and other genera with one species each such as *Aureobasidium*, *Stagonosporopsis*, *Alternaria*, *Cyphellophora*, *Parengyodontium*, *Simplicillium*, *Fusicolla*, *Nigrospora*, *Gelasinospora*, *Millerozyma*, *Meyerozyma*, *Pichia*, and *Kodamaea*. Strains of Basidiomycota were affiliated with *Rhodotorula* (2 species), *Rhodosporidiobolus*, and *Phanerochaete* (Table 2).

Importantly, 12 out of 29 identified fungal species were first reported with a marine origin (Table S3, Supplementary Material). Moreover, 17 identified species have not been reported in a neighboring sea area as China (Table S3, Supplementary Material). Only *Candida tropicalis* was found from oil-polluted sediments collected in coastal zones of Vietnam [71]; and thus other 28 identified species detected in this research were new records in a marine ecosystem of Vietnam (Table S3, Supplementary Material).

Interestingly, *Candida* was the most abundant genus with 25 strains isolated in all 10 surface coastal marine habitats. *Candida blankii* was the most abundant species with 15 strains originating from all surface coastal marine habitats, except 2 beaches DL and NB. The deeper waters DW generally shared a common marine fungal community composed of major genera *Aspergillus*, *Penicillium* and *Cladosporium* with surface coastal marine habitats. However, DW did not have *Candida* or any basidiomycetes. The presence of *Parengyodontium*, *Simplicillium*, *Fusicolla*, and *Stagonosporopsis* in DW was also different from fungal communities in the surface marine habitats (Table 2; Table S2, Supplementary Material).

3.5. Enzyme activity of marine fungal strains

We investigated extracellular enzyme activity including protease, amylase, phytase, cellulase and chitinase of all 75 fungal strains obtained from a culture-dependent approach. The results are shown in Table S4 (Supplementary Material). The total relative extracellular enzyme activity (AU) of fungal strains from each sampling site was calculated, in which 1 AU was defined as an enzyme activity unit with hydrolysis zone diameter D–d = 10–15 mm, 2 AU for D–d = 15–20 mm and 3 AU for D–d > 20 mm. As a result, the total relative enzyme activity of each sample ranged from 0 AU in RT to 63 AU in DW (Figure 8). Based on total relative enzyme activity, the samples were divided into 5 groups. Interestingly, each group displayed a specific habitat or ecological niche. Group A (>60 AU) was for deep waters, Group B (23–33 AU) for major aquaculture regions, Group C (20 AU) as a buffer region with some aquaculture and some tourism activity, Group D (12–16 AU) for major tourism regions (beach bathing, anchoring, fishing, and tourism boat running), and Group E (0–5 AU) for marine protected area cores with very limited human activity (Figure 8).
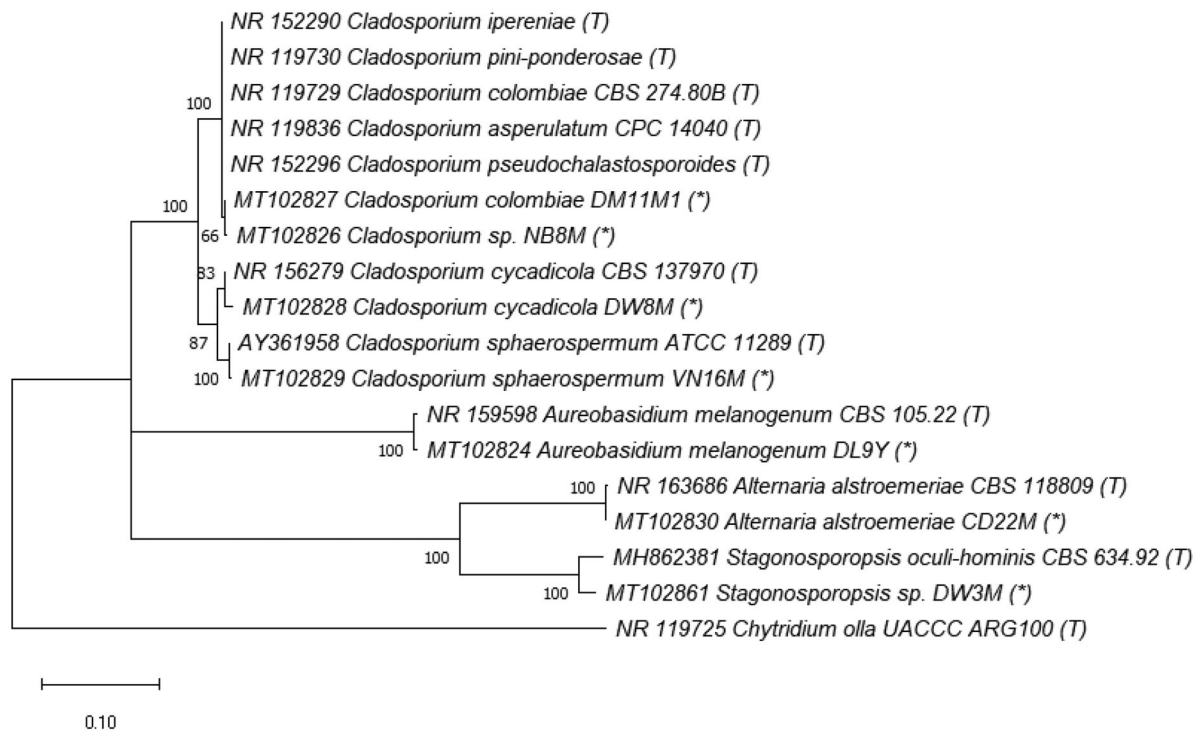
Figure 3. Phylogenetic tree of Orders Capnodiales, Dothideales and Pleosporales (class Dothideomycetes, Phylum Ascomycota) based on ITS rRNA genes. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model [54]. The tree with the highest log likelihood −2876.51) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 34.52% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 18 nucleotide sequences, including ones in this study (*), from type strains (T) and *Chytridium olla* UACCC ARG100 (T) used as an outgroup.

3.6. A checklist of trophic mode and guild analyses of marine fungal strains and OTUs

A checklist of trophic mode and guild analyses of all fungal strains and OTUs identified from the culture-dependent and culture-independent approach in this study, with their possible or confirmed functions, are presented in Table S3, Supplementary Material. The results suggest that marine fungi living in Nha Trang Bay and Van Phong Bay can exist in numerous life modes, such as saprotrophs, endophytes, symbionts, or pathogens to humans, animals, and plants. For example, *Candida* yeasts, which were most frequently detected, can form symbiotic relationships with plants and can be a pathogen to humans and animals. *Aspergillus versicolor*-like group can be saprotrophs with aliphatic and aromatic hydrocarbon degradation activity.

4. Discussion

4.1. Fungi biodiversity, community composition and enzyme activity in the coastal marine ecosystems in Vietnam

This is the first study comprehensively exploring the fungal diversity using culture-dependent and culture-independent approaches of the coastal marine habitats of Nha Trang Bay and Van Phong Bay, Khanh Hoa province in Vietnam. These habitats are experiencing rapid anthropogenic disturbances from urbanization, tourism, shipping and aquaculture expansion [6,43–45,72,73] which are predicted to seriously affect marine habitats. We have retrieved fungal ITS sequences belonging to Ascomycota, Basidiomycota, and some species/genera of Mucoromycota, Chytridiomycota and unclassified and/or uncultured fungi specimens. At the species level, 12, 17 and 28 out of 29 identified fungal species have been not reported in a marine environment, a sea area of China and a sea area of Vietnam, respectively. Therefore, these new records contribute to discovering fungal diversity in the global, regional and national coastal marine habitats.

The present study shared 3 common fungal phyla (Ascomycota, Basidiomycota and Mucoromycota) and 8 common fungal genera (*Aspergillus, Trichoderma, Pleosporales* (currently *Stagonosporopsis* and *Alternaria*), *Aureobasidium, Candida, Rhodotorula* and *Rhodosporidiobolus* (*Rhodosporidium*)) to culturable fungi composition
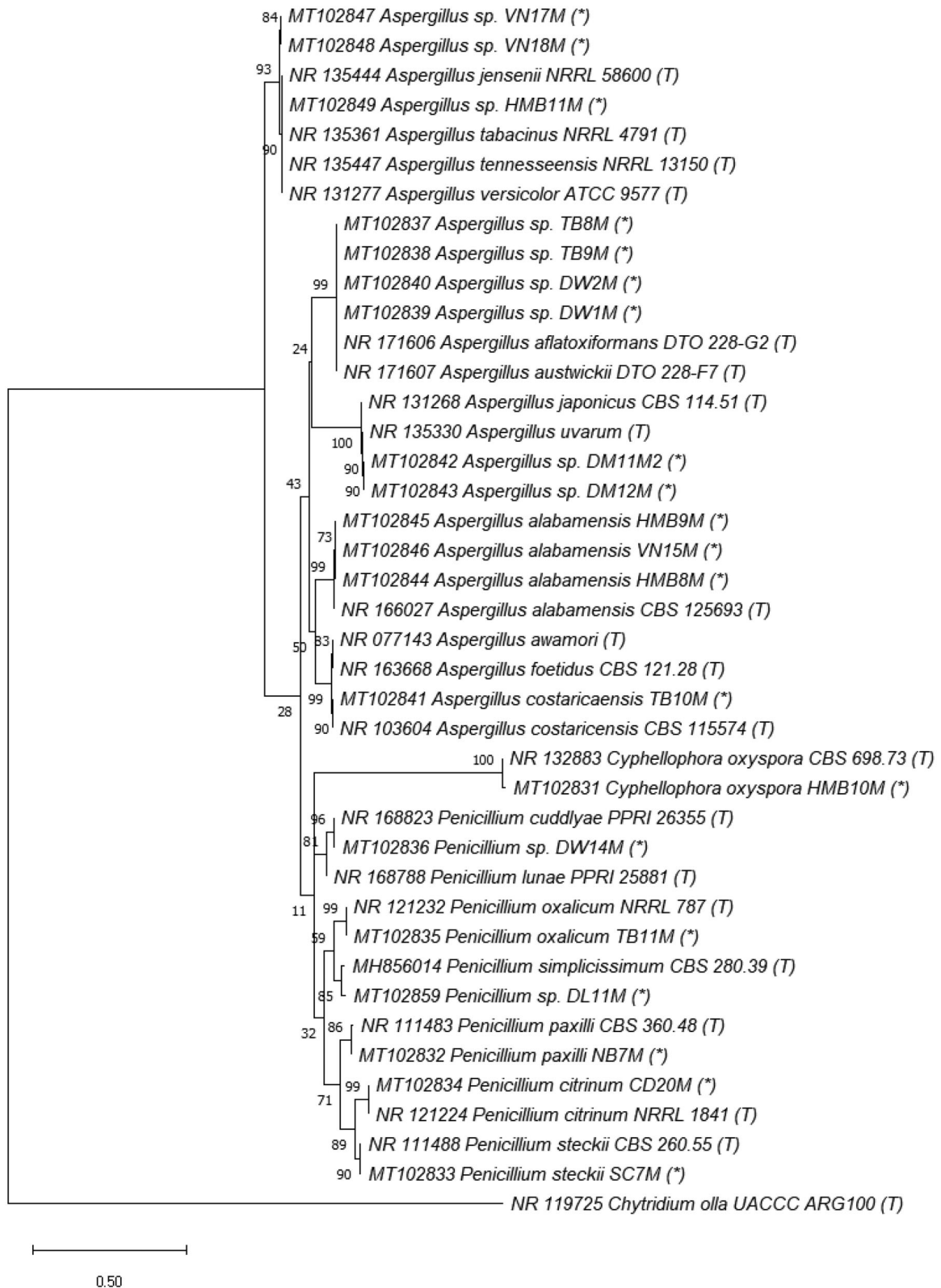
Figure 4. Phylogenetic tree of Orders Chaetothyriales and Eurotiales (Class Eurotiomycetes, Phylum Ascomycota) based on ITS rRNA genes. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model [55]. The tree with the highest log likelihood (−3375.53) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3870)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 41 nucleotide sequences, including ones in this study (*), from type strains (T) and *Chytridium olla* UACCC ARG100 (T) used as an outgroup.
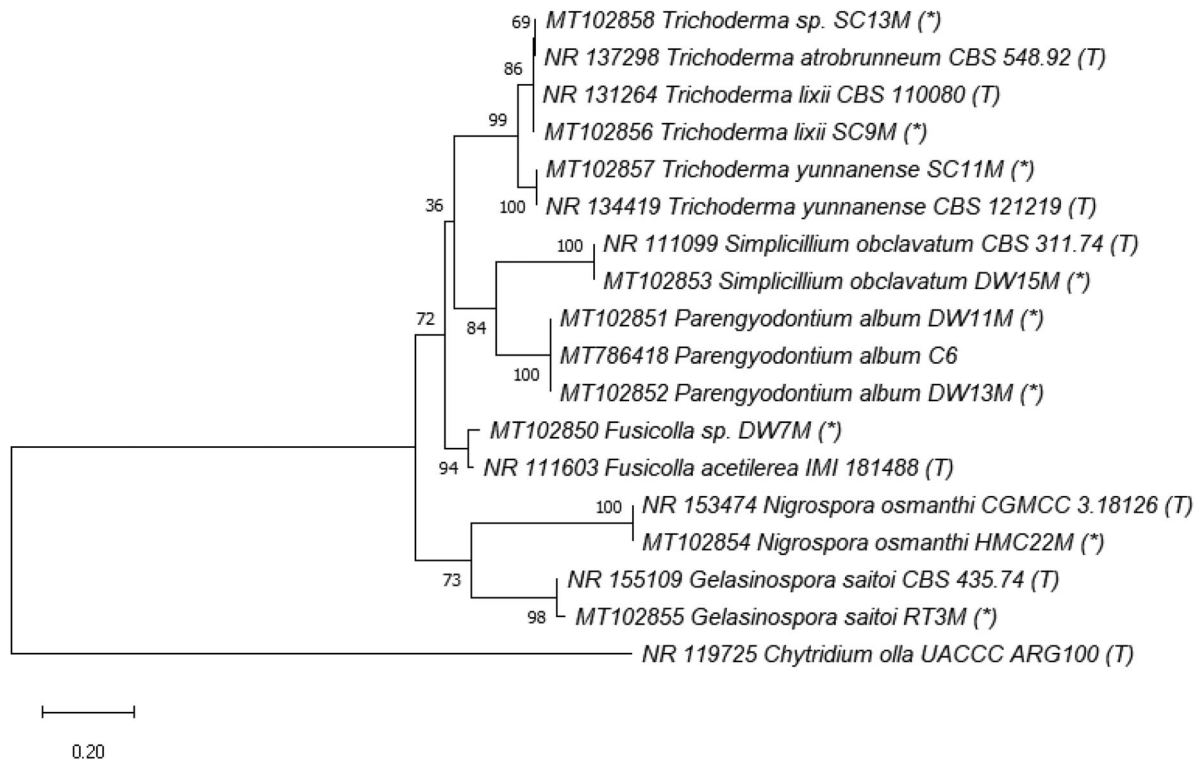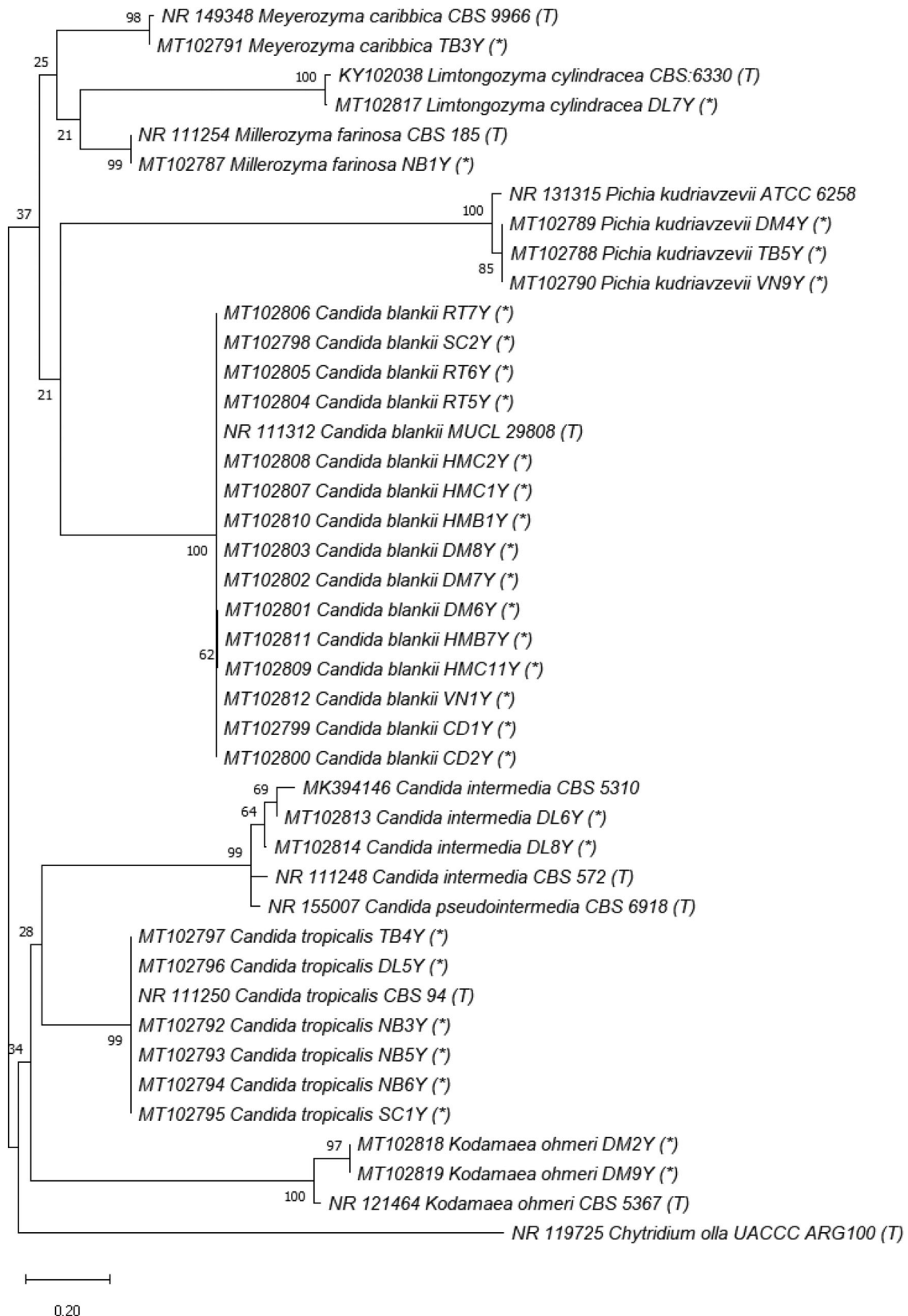
Figure 5. Phylogenetic tree of Orders Hypocreales, Trichosphaeriales and Sordariales (Class Sordariomycetes, Phylum Ascomycota) based on ITS rRNA genes. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model [54]. The tree with the highest log likelihood (−3297.12) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3655)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 18 nucleotide sequences, including ones in this study (*), from type strains (T) and *Chytridium olla* UACCC ARG100 (T) used as an outgroup.

in the coastal waters of Southern China [26]. Similarly, a culture-independent study on the diversity of planktonic fungi in the coastal waters of the Bohai Sea in China detected more than 60% of unclassified fungi, the dominance of Ascomycota (20.82%), Basidiomycota (9.72%) and Chytridiomycota (2.64%), and episodic low dominance of Cryptomycota (0.64%) and Mucoromycota (0.18%) [27]. Our results showed a super-dominance of Ascomycota (99.68%), followed by Chytridiomycota (0.16%) and unclassified fungi (0.16%). While diverse taxa with 283 genera within Ascomycota were discovered in the Bohai Sea [27], only 5 fungal OTUs were found at Nha Trang Bay in our study. Several previous studies have suggested that mycoplankton can use dissolved organic carbon [74,75], thus, fungal diversity can be attributed to the lower carbon from autochthonous primary production and terrestrially-derived production in the coastal region at Nha Trang Bay than that in the Bohai Sea [23,40,27].

Molecular diversity analysis of marine fungi across 130 European environmental samples also shows the widespread of *Candida* yeasts and two major genera of Basidiomycota yeasts including *Rhodotorula* and *Rhodosporidium* [28], which are similar to our findings. These results suggest that a yeast lifestyle of Dikarya (Ascomycota and Basidiomycota) was the highly dominant fungi in marine ecosystems and adapt well to these environments [37,76]. Especially, many yeast morphotypes detected are closely related to opportunistic animal pathogens, as shown in Table S3, Supplementary Material, suggesting that they may be parasites or opportunistic pathogens of marine animals. We do not exclude the possibility that some filamentous fungi may not be fully recovered from water filtration and/or DNA/RNA extraction [28]. For example, in the present study, Pezizomycotina, or filamentous ascomycetes, were recovered at very low abundance with an exception to *Aspergillus* which appeared at higher abundance in both cultivation and molecular approaches (Tables 2 and 3).

Importantly, our results showed that fungi diversity and community composition of the surface

Figure 6. Phylogenetic tree of Order Saccharomycetales (Class Sacharomycetes, Phylum Ascomycota) based on ITS rRNA genes. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model [54]. The tree with the highest log likelihood (−3834.69) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5029)). This analysis involved 42 nucleotide sequences, including ones in this study (*), from type strains (T) and *Chytridium olla* UACCC ARG100 (T) used as an outgroup.
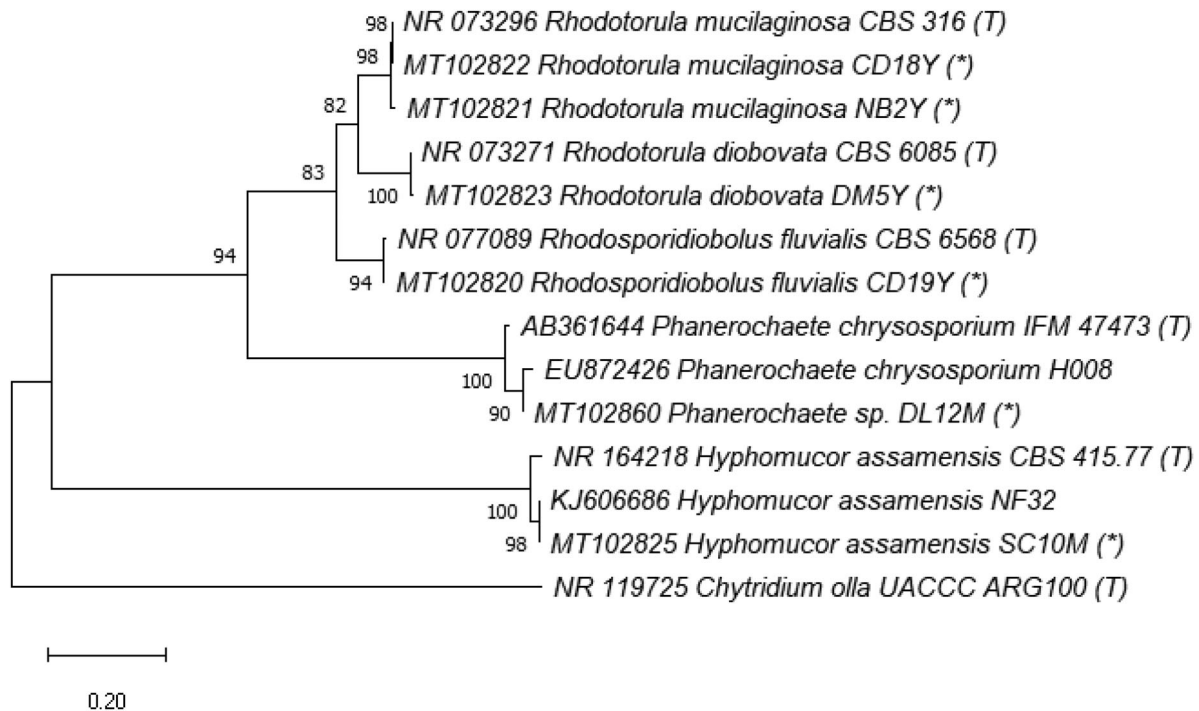
Figure 7. Phylogenetic tree of Order Polyporales (Class Agaricomycetes, Phylum Basidiomycota), Order Sporidiobolales (Class Microbotryomycetes, Phylum Basidiomycota) and Order Mucorales (Class Mucoromycetes, Phylum Mucoromycota) based on ITS rRNA genes. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model [54]. The tree with the highest log likelihood (–3580.61) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories ($+G$, parameter = 0.8620)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 14 nucleotide sequences, including ones in this study (*), from related type strains (T) and *Chytridium olla* UACCC ARG100 (T) used as an outgroup.
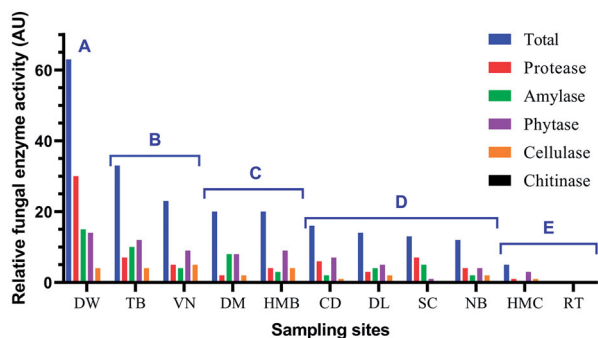
Figure 8. Relative extracellular enzyme activity (AU) of all fungal strains from each sample at Nha Trang Bay and Van Phong Bay, Khanh Hoa province, Vietnam. For each strain, 1 AU was defined as an enzyme activity unit with hydrolysis zone diameter D–d = 10–15 mm, 2 AU for D–d = 15-20 mm and 3 AU for D–d > 20 mm. Based on total relative enzyme activity shown, the samples were divided into 5 groups: Group A (>60 AU), Group B (23–33 AU), Group C (20 AU), Group D (12–16 AU), and Group E (0–5 AU).

coastal marine and deeper waters differed across sampling sites, possibly reflecting the different impact levels from human activities and ecological factors in the marine environment in Khanh Hoa, Vietnam. For example, the lowest density of fungi (10^2 CFU L^{-1}) was found in the samples NB and DL where both locations are beaches with an extremely high level of tourism activity. As expected, the highest density of fungi (10^5 CFU L^{-1}) was found in the Hon Mun Marine Protected Area of Nha Trang Bay where the human impact on this area is minimal. These findings suggest that human activity can negatively impact the biodiversity of marine organisms, including fungi, and such marine protected areas established are likely essential to the life of many marine fungi. For example, some sunscreen lotions and personal-care products contain benzophenone-3, which are highly toxic to corals [77] and the negative effect of this substance on marine fungi is in question. Moreover, the deep water DW had a relatively low density of fungi (8.8×10^2 CFU L^{-1}) but had the highest number of filamentous fungi isolates (16 vs. 1–7 of all other samples) and none of the yeasts were successfully cultivated, which indicated the highest diversity of culturable filamentous fungi and maybe suggested uncultivable fungi existing in such a unique deep habitat that differs from surface sea habitats with lower temperature and higher hydrostatic pressure [10].

The impact of human and ecological factors on the biodiversity of marine fungi at Nha Trang Bay and Van Phong Bay was also displayed *via* the total

relative enzyme activity of fungi isolates in each sampling site. As shown in Figure 8, five groups of similarly ecological sampling areas were given, which well explained the correlation between human impact and dissolved organic matter concentration in marine ecosystems. Group A of deep waters at Nha Trang Bay expressed the highest total relative enzyme activity and also the highest activity of protease, amylase and phytase. This can involve a high diversity of fungal isolates and also the availability of organic matters of carbon, nitrogen and phosphorous, in which carbon sources can be sunk from the surface euphotic zone. Group B with the second-highest total enzyme activity and the highest cellulase activity typically represented for environments enriched with cellulose and other organic substrates in major aquaculture regions at Nha Trang Bay and Van Phong Bay. Group C displayed a buffer region with some aquaculture activity and some tourism activity, thus an average number in total relative enzyme activity was recorded. In Group D, tourism activities such as swimming, anchoring, fishing and operating by tourism boats can affect the mycoplankton and phytoplankton diversity and sink organic matters to a lower abundance, thus can lower the enzyme activity of fungi. Finally, Group E for marine protected area cores with very limited human activity displayed a very low or no enzyme activity of fungal isolates, which suggested other enzyme activity or unknown ecological functions of fungal communities living in these ecosystems. Although further direct evidence about the impact of human and ecological factors on fungal biodiversity at Nha Trang Bay and Van Phong Bay is necessary, the results from the present study provide first and valuable clues about their effects.

4.2. Comparison of culture-dependent and culture-independent approaches

The total community of marine fungi, especially uncultured fungi, in the sea areas of Vietnam is not known. We fill this voids by focusing on the planktonic fungi at 3 sampling locations of Nha Trang Bay, Khanh Hoa province, Vietnam using both culture-dependent and culture-independent approaches. Our findings revealed that *Candida blankii* yeasts are the most dominant fungi species at Nha Trang Bay and this result was consistent regardless of the use of the two methods. However, the abundance of other taxa depended on which method was used. In the culture-dependent method, at least 40 species belonging to 22 genera within 3 phyla Ascomycota, Basidiomycota and Mucoromycota were found (Table 2), while in the culture-independent method,

only 5 fungal OTUs were uncovered, in which 3 identified OTUs belonged to 3 different genera within Ascomycota and 2 unidentified OTUs within Chytridiomycota and unclassified environmental clusters (Table 3).

Even though isolated directly from the sea water samples, some fungi were not able to grow on culture media. For example, fungal isolates were obtained on all five different media (MEA, SDA, CMA, PDA and CMA) during isolation from deep-sea sediments [42], whereas only two (MEA and SDA) of these five media were found to recover marine fungi from the coastal waters [26]. In the later case, SDA was found to be a better medium than MEA for the isolation of marine fungi, although there was no statistical significance [26]. In the present study, 75 strains of 3 major phyla Ascomycota, Basidiomycota and Mucoromycota were isolated on SDA, but none of the isolates from the phylum Chytridiomycota were recovered on this medium as found from our metabarcoding analysis. Although chytrids (Chytridiomycota), or "early-diverging lineages" of fungi, have been found to dominate nearshore and sediment samples [28,78], our results suggest that the diversity of chytrids is highly likely based on environmental sequencing data [79].

However, there are several constraints to the culture-independent approach. In the present study, ascomycetes were found to dominate from culture-dependent and culture-independent approaches but for the latter method, it is difficult to detect members of Basidiomycota and Mucoromycota that are present on the SDA medium. The reason for this is that the relative abundance of microbial species in a natural habitat is rarely equal. DNA may not be recovered from all genotypes and the results of next-generation sequencing, which are mainly based on analysis of ITS regions for fungi [80], can be biased toward the most abundant organisms at the time of sampling [81]. Moreover, the ITS rDNA region primers were designed using genomic regions from mainly terrestrial representatives which were biased toward terrestrial Dikarya (Basidiomycota and Ascomycota), resulting in poor representation of marine fungi taxa [9].

The sequencing-based on the fungal ITS rDNA region even expressed both advantages and disadvantages. In the present study, it can coamplify other microeukaryotes and these eukaryotes are reported that they often dominate marine environmental metabarcoding sequence data, resulting in limited representation by marine fungi [9]. Fortunately, ascomycetes were still found to dominate other eukaryotes in the HMC sample, as shown in Figure 2 in this study.

Another disadvantage of the culture-independent approach is that the correspondence of OTU with species can be inconsistent. OTUs are defined dependent on the identity, typically with 97% [82]. However, some species have genes that are 97% identity, which will bring about merged OTUs containing various species. Additionally, a solitary species may have paralogs that are 97% identity, making the species be part across at least two species. Some or even many identified clusters might be wrong because of the artifacts, including reading errors and chimeras [82]. For a long time, sequencing of household and other genes has been utilized exclusively [83], but then a polyphasic approach to taxonomy based on morphological, ecophysiological and molecular analyses appeared to be more effective to discover species [84]. Currently, all-important phenotypic features can be used for classification in agreement with molecular features, e.g., a case of study on *Aspergillus* and *Penicillium* [85]. In the present study, to increase the reliability of species identification compared to the use of ITS1-F and ITS4 primers, DNA barcoding targeting the entire ITS region using ITS1-F_KYO2 and ITS4 primers was designed. This was based on the previous evidence that ITS1-F_KYO2 and ITS4 matched around 99% within the ascomycetes, basidiomycetes, or "non-Dikarya" group [48]. Although the primers ITS1-F_KYO2 and ITS4-R were used to improve coverage across diverse taxonomic groups of fungi compared to previously existing primers [48], these primers may not amplify ITS regions from all marine fungi in our study.

Finally, the culture-independent approach could not distinguish metabolically inactive fungi from true marine fungi as cultivated on media [9]. Thus, this can make it difficult to compare the abundance from both methods and requires additional lines of evidence to capture and characterize marine fungi cells living in their ecosystems. Therefore, the combined use of culture-dependent and culture-independent approaches in the present study may provide a better characterization of marine fungi composition than that obtained when using each approach independently. Most importantly, the direct matching of the ITS sequences of fungi detected from these two approaches has confirmed that molecular analyses are in agreement with phenotype features of fungal isolates.

4.3. Comparison of surface coastal marine and deeper (transition layer) waters

We found consistent evidence that *Aspergillus*, *Penicillium* and *Cladosporium* are dominant genera in both surfaces coastal marine and deeper waters (a transition layer rather than real deepsea) at Nha Trang Bay, Vietnam. Further, we also identified a number of unique and self-contained niches for fungi in such marine habitats. Coastal regions are characterized by eutrophication from terrestrial run-off and high primary production [86]. In contrast, the deepsea layers have the salient features of low temperatures, high hydrostatic pressure, absence of light, and low biological diversity [10]. This leads to higher availability of organic matter as detritus in the coastal marine habitats compared to deepsea regions. Thus, in the coastal waters, prokaryotic heterotrophs are known to play a massive role as primary degraders, followed by eukaryotic heterotrophs, including micro-sized animals and marine fungi [87].

Compared to the coastal marine habitats, the deepsea sediments expressed a very low species richness [41,88], and the majority of the fungi belonged to Ascomycota and Basidiomycota with a large representation of yeasts belonging to both the phyla [37,40]. Molecular studies have revealed the presence of the additional major fungal phyla Chytridiomycota and Zygomycota [10]. In the present study, culturable fungi from deeper waters DW at a depth of 40–47 m, even though not really deepsea, had a relatively low density at 10^2 CFU L^{-1} compared to higher densities ranging from 10^2 to 10^5 CFU L^{-1} of samples in the surface sea habitats, confirming a low fungal richness in the deep habitats as known from previous studies on deepsea. However, DW displayed the dominant number of filamentous fungi isolates and none of the yeasts. It had been previously suggested that yeast forms dominate fungal diversity in the deep-water column and marine sediment environments [37,40]. Therefore, a dominant representation of filamentous fungi in DW suggested that DW could be a "transition layer" to sink and likely reduce fungal filaments from the surface sea to deepsea layers. Moreover, the results showed a wide diversity of fungal phenotypes in different marine fungal communities and were consistent with their molecular diversity analysis [76].

4.4. The first record of Candida blankii *yeast as a dominant fungi species in marine habitats*

Although *Candida* yeast has been found as one of the most widespread fungi species in marine habitats, especially in a big-sized molecular analysis across 130 European marine samples [28], *Candida blankii* has been detected only in a few locations in Japan, Sweden, New Zealand and Canada (https://eol.org/pages/6770416). This study presents the first record of *Candida blankii* as a dominant species in

a marine habitat in Vietnam. This yeast species was detected on SDA medium with 15 isolates of total of 75 fungi isolates (20%) originated from all surface coastal marine habitats at Nha Trang Bay and Van Phong Bay with exceptions from 2 beaches DL and NB only. Similarly, next-generation sequencing method showed its relative abundance to total fungi at 95.95% and 97.99% in CD and HMC samples at Nha Trang Bay, respectively. Moreover, 10 isolates of other *Candida* species were found on SDA medium, which belonged to *C. tropicalis, C. akabanensis* and *C. intermedia*. Collectively, a third of culturable fungi isolates in marine areas of Khanh Hoa province, Vietnam was identified as *Candida* yeasts. Notably, none of *Candida* yeasts were recorded in the deep water DW in both culture-dependent and culture-independent approaches.

Candida is a heterogeneous genus of budding yeast (Saccharomycotina) in the family Saccharomycetaceae and is estimated to constitute 25% of all yeast species. It comprises primarily species related to plants, spoiling vegetation, and plant-feeding insects [89]. The level of *Candida* species can utilize plant materials such as xylose, cellobiose, aliphatic hydrocarbons and starch is reported as 71%, 57%, 29% and 27%, respectively, while 85% of species require vitamins delivered mostly in plant biomass. However, 65% of *Candida* species cannot grow at 37 °C or higher temperatures, therefore just generally a couple of species can infect humans and animals [89].

C. blankii is a rare and uncommon yeast but can form symbiotic relationships with plants [90] or be considered as an opportunistic human pathogen [91]. For example, it was shown as the most common species colonizing flower nectaries in India and making *Azadirachta indica* flower more, although this relationship is not fully understood yet [90]. Regarding human infections, *C. blankii* was first described from the blood of a mink in 1968 [92] and later described from clinical specimens, including the bloodstream of a fungaemia patient [93,94]. Although a few cases of *C. blankii* have been found, other rare and new species of fungi related to human infection have been frequently described in recent years [95,96]. *Candida* species are the third most common cause of sepsis in a recent prevalence survey of adults and children hospitalized in the United States [97]. The outbreaks of fungal sepsis due to emerging and rare *Candida* species, particularly multidrug-resistant *Candida auris* have received great attention from the medical community [95,98]. Currently, a report has emphasized the emergence of *C. blankii* with reduced susceptibility to one or more antifungal agents in nosocomial fungaemia [96].

Moreover, *C. blankii* can grow at elevated temperatures and high salt concentrations, and its genome analysis suggested a strongly altered 1,3-beta-D-glucan synthase protein sequence, which may help it occur in hydrocarbon-rich environments [96,99]. This yeast is studied extensively for use in xylose fermentation and the degradation of hemicellulose hydrolycates. These facts explain why it has appropriate attributes to be a colonizer in surface coastal marine habitats with high temperature and salinity as monitored at the sampling areas in the present study. This was also consistent with evidence that no *Candida* yeasts were detected in the deep water DW with a temperature of 22 °C and higher hydrostatic pressure. In fact, other *Candida* species are also those impacted when grown under marine conditions. For example, a marine yeast *Candida tropicalis* BH-6 isolated from mangrove sludge had the optimum temperature for growth at 37 °C, the initial pH value at 5.0, and the wide NaCl concentrations of 0–6% [100]. *Candida oceani* expressed the optimal growth at 3% sea salt [101] and *Candida viswanathii* displayed filamentous morphology under elevated hydrostatic pressure [38].

In Vietnam, the yeasts *C. viswanathii* and *C. tropicalis* were found from oil-polluted sediments collected in coastal zones with transformation activity of branched aromatic hydrocarbons in biofilm [71,102], while *C. tropicalis* was detected as the most common *Candida* species from blood cultures in two hospitals between May 2013 and May 2015 [103]. Moreover, *C. tropicalis, Diutina rugosa* and *Pichia kudriavzevii* were detected in traditional alcoholic fermentation in Vietnam [104]. In the present study, the dominance of 15 *C. blankii* isolates from all sampled marine habitats with an exception at 2 beaches of Nha Trang Bay and Van Phong Bay, where human contact to seawater occur most frequently and directly, suggests that these isolates are unlikely human pathogens or from a clinical source. It is more likely that *C. blankii* plays a primary role as a hydrocarbon transformer in marine ecosystems as discussed above. A current study has shown that urban and tourism activities are typical pollution sources of polycyclic aromatic hydrocarbons and other organic contaminants in the coastal areas in Spain [105]. The question is that whether the increasing activities from urbanization, tourism, shipping and aquaculture expansion at Nha Trang Bay and Van Phong Bay in Vietnam [7,43,44,73] can give us a clue on the dominance of *C. blankii* species or not. Therefore, the biological nature and ecological role of this species in association with changing environmental conditions in this region should be further studied, including hydrocarbon

transformation activity, human pathogenicity, and originality in terms of marine or clinical sources, or a transition from terrestrial to marine habitats.

4.5. Potential ecological role of mycoplankton in the marine food web

In spite of the fact that the diversity and ecological function of marine fungi have generally been unexplored, some ongoing cultivation and metabarcoding studies of microorganisms in the coastal marine environments and relationship with macroeukaryotes have indicated that fungi are a dominant kingdom and play a significant part in the marine nutrition cycles [9,10]. Marine fungi, especially filamentous fungi, enable to break down organic matters, basically carbohydrates and polyphenols of plant and algae. The plant and algae biomass is the most renewable energy feedstock on the Earth and induces the biosynthesis of microbial extracellular enzymes to catalyze their cleavage into shorter sugars [14,29]. In this manner, marine fungi have generally been found in the ocean, especially connected with sediment, seawater, submerged plant, and algae. Fungi are hypothesized to contribute to phytoplankton population dynamics, biological carbon pump and change in marine sediments [9]. They can act as predators, pathogens and parasites, and can be discovered living in a symbiotic relationship with plants, algae, animals and other organisms [106]. Regardless of the different roles of marine fungi, their ecological functions are still open questions and unsolved issues.

Based on the roles of fungi in the marine carbon cycle as described by [9], the checklist of trophic mode and guild analyses (Table S3, Supplementary Material), and total relative enzyme activity (Figure 8), we hypothesized a contribution of plankton fungi in 3 locations of the study (HMC, CD, DW) at Nha Trang Bay as follows. Many saprotrophic fungi (mainly *Candida*, *Aspergillus*, *Penicillium* and *Cladosporium*) can use particulate and dissolved organic carbon from phytoplankton, which is then fed by zooplanktons in the marine food web. Some other fungi can be pathogens, endophyte, or epiphyte to human, animal, plant and algae.

Identifying the role of marine saprotrophic fungi in ecological cycles remains a key challenge as it does not simply detect their presence but also explores their activity. This can be addressed partly through the activity of extracellular enzymes they produce. Fungal strains isolated from different substrates in marine environments are producers of hydrolytic and/or oxidative enzymes such as alginate lyase, amylase, cellulase, chitinase, glucosidase, inulinase, keratinase, ligninase, lipase, nuclease, phytase,

protease, and xylanase [107]. In the present study, all fungal isolates were investigated with major extracellular enzymes, including protease, amylase, cellulase, phytase and chitinase. The activity of fungal isolates confirmed the roles of saprotrophic mycoplankton in the biogeochemical cycles of carbon, nitrogen and phosphorous. In particular, protease plays an important role in the biogeochemical cycle of nitrogen, whereas amylase and cellulase display a similar function in the biogeochemical cycle of carbon. Phytase, which catalyzes the release of phosphate from phytate (myco-inositol-hexaki-phosphate), plays an essential role in the biogeochemical cycle of phosphorous. Besides, as chitin is a component of the cell walls of fungi and exoskeletal elements of some animals (mollusks and arthropods), some fungi possess degradative chitinases related to their role as detritivores and also to their potential as arthropod pathogens. In our study, none of the chitinase activity was found in all fungal isolates suggested that mycoplankton do not seem to relate to the degradation of chitin.

Our study also provides strong evidence of phytoplankton-derived organic matter consumption by marine saprotrophic mycoplankton. Fungal isolates from this study with amylase and cellulase activity, as shown in Figure 8, suggesting their potential to degrade phytoplankton polysaccharides. Moreover, species of *Cladosporium*, which were found in surface marine coastal and deeper waters in the present study, are considered as active saprotrophs in coastal waters and directly assimilate phytoplankton organic carbon. This is because *Cladosporium* is found to secrete the extracellular enzyme glucan 1,3-β-glucosidase [108]. This enzyme can digest phytoplankton-derived organic matter and its abundance, in particular, correlates with increased abundances of specific diatom species and in the deep chlorophyll maxima regions of the oceans where phytoplankton biomass can be the highest. These results indicate some marine mycoplankton have a saprotrophic functional role in processing phytoplankton polysaccharides.

Although not directly linked to enzyme activity, the dominant presence of some groups of filamentous fungi such as *Cladosporium* and *Aspergillus* are among those known to participate in aliphatic hydrocarbon degradation, while the genera *Penicillium* and *Aspergillus* are among those known to take part in the degradation of aromatic hydrocarbons [109–111]. Fungi have a relatively high tolerance to hydrocarbons [109] and are likely primary degraders of high-molecular-weight hydrocarbons *via* secreted extracellular enzymes and work synergistically with other microorganisms in the oil degradation [112]. Similarly, the yeasts *Candida*

viswanathii and *C. tropicalis* isolated from oil-polluted sediments in Vietnam expressed the transformation activity of branched aromatic hydrocarbons [71,102].

Therefore, the dominance of *Candida*, *Aspergillus, Penicillium* and *Cladosporium* in the present study suggested the contribution of marine saprotrophic ascomycetes fungi to the degradation of oil and other hydrocarbons as organic matter from phytoplankton. The primary function of fungi, including mycoplankton is to degrade detrital organic matter from phytoplankton, which acts as primary producers and provides food for consumers at higher trophic levels. By interacting with microbial communities, mycoplankton productively converts particulate organic matter to dissolved organic matter as part of the biogeochemical cycles. Along with heterotrophic bacteria, mycoplankton play significant functions in the biogeochemical cycles of carbon, nitrogen, phosphorous, and other nutrient fluxes in marine ecosystems. It has been demonstrated that there are higher densities of mycoplankton close to the surface and in shallow waters, proposing their connection to the rise of organic matter. This keeps on connecting with crowded phytoplankton communities at the surface, inferring that mycoplankton is firmly identified with the utilization of organic matter in the euphotic zone [9].

The functional roles of marine mycoplankton are poorly understood, resulting in a lack of understanding of their ecology. Due to the presence of bacterioplankton in the same niche, mycoplankton in the interactions with phytoplankton to transfer particulate organic carbon in marine food webs, therefore, maybe need to be further characterized. Our findings and previous studies suggest that marine fungi can contribute to the action of the biological carbon pump, which transfers the organic matter produced by phytoplankton *via* photosynthesis from the surface euphotic zone to sink as "marine snow" through the epipelagic, mesopelagic and bathypelagic zones to the deep ocean [113]. The significance of fungal biomass in the marine ecosystem, therefore, requires further analysis, especially in terms of the current view that prokaryotes are the principal contributors of heterotrophic microbial biomass in the ocean [114].

5. Conclusions

Fungi diversity and community composition of the surface coastal marine and deeper waters of Khanh Hoa province in Vietnam were uncovered using both culture-dependent and culture-independent approaches. Using a culture-dependent approach, the fungal community of 11 surfaces coastal marine

and deeper waters at Nha Trang Bay and Van Phong Bay accounted for from 10^2 CFU L^{-1} to 10^5 CFU L^{-1} with a total of 75 strains, including 38 yeasts and 37 filamentous fungi successfully isolated and identified based on ITS sequences into species or genus. They belong to 3 phyla, 5 subdivisions, 7 classes, 12 orders, 17 families, 22 genera and at least 40 species. Among them, 29 species have been identified and many potential novel species need to be further studied; 12 and 28 species detected in this research are new records in a global marine source and a sea area of Vietnam, respectively. Using cultivated-independence studies on three representative samples HMC, CD and DW at Nha Trang Bay, the results showed that the marine fungal community displayed a dominance of Ascomycota and a lower abundance of some members of Chytridiomycota and unclassified uncultured fungus, but the richness of fungi was low with 5 fungal OTUs only. The results from both approaches shared similar fungal taxonomy to the most abundant phylum (Ascomycota), genera (*Candida* and *Aspergillus*) and species (*Candida blankii*) but were different at less common taxa. This is the first record of *C. blankii* as a dominant species in a marine area in Vietnam.

Major extracellular enzymes including protease, amylase, cellulase, phytase and chitinase of all fungi isolates were investigated. It suggested the roles of mycoplankton in the biogeochemical cycles of carbon, nitrogen, and phosphorous. Chitinase activity was not found in any mycoplankton. Based on the total relative enzyme activity, five groups of similarly ecological sampling areas were created, which was well explained the correlation to human impact and dissolved organic matter concentration in marine ecosystems. The checklist of marine fungal isolates and OTUs identified from our research indicated that they may be mostly saprotrophs (mainly *Candida*, *Aspergillus, Penicillium* and *Cladosporium*), commensals, or pathogens of human, marine animals, plants and algae.

Disclosure statement

No potential conflict of interest was reported by the authors.

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