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High Fungal Diversity and Abundance Recovered in the Deep-Sea Sediments of the Pacific Ocean

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Abstract Knowledge about the presence and ecological significance of bacteria and archaea in the deep-sea environments has been well recognized, but the eukaryotic microorganisms, such as fungi, have rarely been reported. The present study investigated the composition and abundance of fungal community in the deep-sea sediments of the Pacific Ocean. In this study, a total of 1,947 internal transcribed spacer (ITS) regions of fungal rRNA gene clones were recovered from five sediment samples at the Pacific Ocean (water depths ranging from 5,017 to 6,986 m) using three different PCR primer sets. There were 16, 17, and 15 different operational taxonomic units (OTUs) identified from fungal-universal, Ascomycota-, and Basidiomycota-specific clone libraries, respectively. Majority of the recovered sequences belonged to diverse phylotypes of Ascomycota (25 phylotypes) and Basidiomycota (18 phylotypes). The multiple primer approach totally recovered 27 phylotypes which showed low similarities (≤97 %) with available fungal sequences in the GenBank, suggesting possible new fungal taxa occurring in the deep-sea environments or belonging to taxa not represented in the GenBank. Our results also recovered high fungal LSU rRNA gene copy numbers (3.52×10^6 to 5.23×10^7copies/g wet sediment) from the Pacific Ocean sediment samples, suggesting that the fungi might be involved in important ecological functions in the deep-sea environments.

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Introduction

Marine fungi are a taxonomically diverse group and are ubiquitous on substrata within coastal environments [21]. They serve as major decomposers of organic matters in marine ecosystems [19] but have also been reported as parasites, pathogens, and mutualists with other marine organisms [1, 23, 36, 37]. Deep-sea environments are recognized as an extreme marine habitat, which is characterized by an absence of sunlight, low nutrient input, extreme low temperatures (<4 °C, except near hydrothermal vents), and high hydrostatic pressure (up to 110 MPa) [33]. Burgaud et al. [4] suggested that the deep-sea floor and especially hydrothermal vent environment are under-explored habitats for fungi, which may possess unique fungal communities.

Although the isolation of deep-sea fungi was first reported approximately 50 years ago from the Atlantic Ocean [39], knowledge of "marine fungi" in deep-sea regions of the ocean is still limited. The recognition and importance of fungal organisms in marine ecosystems has increased over the last several decades, and recent studies have focused on fungal diversity in various deep-sea habitats, such as hydrothermal vents [5, 6, 15, 26], seawater and sediments [2, 9, 10, 12, 41–43], oxygen-depleted regions [20], and methane hydrate-bearing sediments [25, 31, 46, 47]. Most fungi isolated from deep-sea environments belong to Phylum Ascomycota, with a few species being affiliated with Basidiomycota [32]. In addition, Chytridiomycota and other basal fungal lineages have also

been recovered from deep-sea environments, which were identified using culture-independent techniques [2, 26, 31, 32].

Earlier studies revealed the diversity of fungi from deep-sea sediments by conventional approach wherein several culture media and isolation techniques were used in conjunction with molecular identification [10, 15, 41]. With the development of culture-independent molecular techniques, the presence of fungi has also been directly reported from deep-sea DNA samples. These techniques involve an initial polymerase chain reaction (PCR) step to amplify genes of interest and a DNA separation technique, such as single-strand conformation polymorphism (SSCP), amplified rRNA intergenic spacer analysis (ARISA), restriction fragment length polymorphism (RFLP), terminal-RFLP, thermal and denaturing gradient gel electrophoresis (TGGE and DGGE), and length heterogeneity PCR (LH-PCR) analysis. However, universal PCR primers for all eukaryotic organisms were employed in most of these studies; hence, our knowledge on the diversity of fungi in the deep-sea environments is fragmentary [11, 28, 27, 29, 46]. Only recently, various fungal-specific PCR primers have been designed and used to report fungal communities in these environments [2, 25, 26, 31, 33]. Bass et al. [2] applied a fungal-specific primer set of the 18S rRNA gene to investigate the fungal diversity in deep-sea samples collected from various oceanic locations (water depths ranging from 1,500 to 4,000 m). Nagano et al. [33] investigated the diversity of fungal communities in deep-sea sediments collected at locations off the Japanese islands in the Pacific Ocean (depths ranging from 1,200 to 10,000 m) using three fungal-specific primer sets of the ITS of rRNA gene. Nagahama et al. [32] used two PCR primer pairs of the SSU rRNA to study the fungi inhabiting the methane cold seeps at Sagami Bay (depths 850–1,200 m). All of these studies revealed a wide occurrence of previously unidentified fungal taxa, which occupy a wide variety of niches in deep-sea environments. In recent years, quantitative PCR (qPCR) has emerged as a promising tool for quantifying microbial communities [14]. qPCR approach has been shown to be highly useful in assessing the abundance of specific phylogenetic groups of microorganisms in soils [22, 24, 44], which is also applied to fungal abundance in some environment samples [3, 14, 26].

Although the use of culture-independent molecular techniques with fungal-specific primers has revealed the presence of diverse fungi, including various unknown species in deep-sea environments, the distribution and diversity of fungal communities within the deep-sea floor sediments are still largely unknown, especially in regions where water depths are greater than 5,000 m. In the present study, we examined the composition and abundance of fungal communities in the deep-sea sediment samples from five locations of the Pacific Ocean (water depths ranging from 5,000 to 7,000 m) using universal fungal and phylum-specific primer sets, targeting the ITS1-5.8S-ITS2-28S rRNA regions, in order to advance our knowledge of fungal communities in deep-sea ecosystems.

Materials and Methods

Sediment Sampling

The deep-sea sediment samples were collected at five locations (water depths ranging from 5,017 to 6,986 m) in the Pacific Ocean during the cruise of DY115-23 of R/V 'Hai-Yang-Liu-Hao' from June to October 2011, including a sample from the deepest known part of the world's oceans, the Mariana Trench. Details of the collected samples are described in Table 1. All sediment samples were immediately kept at −20 °C on the ship and brought to the laboratory for molecular analysis. Sediment temperature was measured with a portable electronic thermometer, while pH and salinity were determined using the pore water of sediments with pH meter and salinity meter, respectively.

DNA extraction, PCR Amplification, and Clone Library Construction

Total DNA was extracted from 0.5 g of sediment sample (wet weight) using a FastDNA® Spin Kit for Soil (MP Biomedicals, USA) according to the manufacturer's protocol. Extractions were performed in triplicate for each sample. DNA extracts were pooled and then quantified by a NanoDrop1000 spectrophotometer (Thermo Scientific, USA) according to the manufacturer's protocols.

Fungal-universal primer pair ITS1/ITS4 and five taxon phylum-specific primer sets ITS5/ITS4Asco (Ascomycota), ITS1F/ITS4B (Basidiomycota), ITS5/ITS4Chytrid (Chytridiomycota), ITS5/ITS4Oo (Oomycota), and ITS5/ITS4Zygo (Zygomycota) were used for ITS region amplification, respectively (Table 2). Due to the weak band obtained on 1 % agarose gel electrophoresis for the ITS1F/ITS4B primer set (Basidiomycota), a nested PCR approach using ITS1F/ITS4B as the first PCR primer set and ITS1/ITS4B as the second PCR primer set was applied. No products were obtained when using the nested PCR for the phylum of Chytridiomycota, Oomycota, and Zygomycota. PCR reaction was carried out for an initial 5 min at 95 °C, 32 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, and a final extension of 7 min at 72 °C. Triplicate PCR reaction products were pooled together to minimize PCR bias. The amplified products were gel-purified and ligated into PGEM-T easy vectors (Progema, USA), which were transformed into *Escherichia coli* DH5α competent cells (Takara, China) to construct clone libraries. Recombinant transformants were

Table 1 Details and physicochemical characteristics of the sediment samples collected from the Pacific Ocean

Sample name	location	Sampling area	Depth (m)	Salinity (%)	*T* (°C)	pH
CQ	171°20′ E/19°12′ N	Northwest Pacific Ocean	5,017	3.4	3	7.36
CW	170°40′ E/19°50′ N	Northwest Pacific Ocean	5,215	3.4	3	7.40
JK	141°58′ E/11°00′ N	Mariana Trench area	6,986	3.4	3	7.47
W	154°25′ W/10°00′ N	Central Pacific Ocean	5,145	3.4	3	7.48
WS	154°00′ W/10°03′ N	Central Pacific Ocean	5,062	3.4	3	7.53

selected using Luria–Bertani (LB) indicator plates supplemented with ampicillin (100 mg l^{-1}).

Screened clones were subjected to colony PCR using the vector M13F/RV primers. Over 100 positive clones with expected size from each library were subjected to RFLP analysis with restriction enzymes *Afa I* and *Msp I* (Takara, China). The digested products were size-fractioned on a 3 % (*w*/*v*) agarose gel electrophoresis and grouped according to their restriction patterns. The representative clones with different digestion profiles were selected for sequencing by Majorbio (Shanghai, China) using ABI 3730XL sequencer (ABI, USA).

Community Structure Analysis

The ITS gene sequences were edited using DNAstar software package (DNASTAR, USA) to clip the redundant sequences. All sequences from the environmental libraries were checked using Check_Chimera utility (Ribosomal Database Project) to eliminate chimeric sequences [7]. Representative sequences from each clone library and their closest relative matches retrieved from the GenBank were aligned using Clustal W (http://www.clustal.org/). Operational taxonomic units (OTUs) were defined as groups of sequences that differed ≤3 %. The library coverage values were calculated using the formula [1−(*n*/*N*)], in which *n* is the number of OTUs represented only by a single clone and *N* is the total number of clones in the library. Rarefaction curves to the numbers of observed OTUs were calculated within the fungal assemblage for each dataset. Phylogenetic trees were constructed by selecting a representative sequence of each OTU in different samples and the reference sequences retrieved from the GenBank using neighbor-joining method in MEGA software (version 5.01). The relative confidence of the tree topologies was estimated by performing 1,000 bootstrap replicates. Gene sequences reported in this study have been deposited in GenBank under accession numbers KJ194276–KJ194455.

Quantitative PCR Analyses

Primers targeting the nuclear LSU rRNA gene fragment of the fungi (universal primers), Ascomycota, and Basidiomycota were chosen for an abundance analysis by qPCR (Table 2). Standard curves were generated using a tenfold serial dilution

Table 2 Details of the primers used in this study

Target genomic region	Primers	Sequence (5′–3′)	Reference
ITS (fungal-universal)	ITS1	TCCGTAGGTGAACCTGCGG	[48]
	ITS4	CGTTACTRRGGCAATCCCTGTTG	[48]
ITS (Ascomycota-specific)	ITS5	GGAAGTAAAAGTCGTAAACAAGG	[48]
	ITS4Asco	CGTTACTRRGGCAATCCCTGTTG	[34]
ITS (Basidiomycota-specific)	ITS1F	CTTGGTCATTTAGAGGAAGTAA	[48]
	ITS4B	CAGGAGACTTGTACACGGTCCAG	[16]
ITS (Chytridiomycota-specific)	ITS5	GGAAGTAAAAGTCGTAAACAAGG	[48]
	ITS4Chytrid	TTTTCCCGTTTCATTCGCCA	[34]
ITS (Zygomycota-specific)	ITS5	GGAAGTAAAAGTCGTAAACAAGG	[48]
	ITS4Zygo	AAAACGTWTCTTCAAA	[34]
ITS (Oomycota-specific)	ITS5	GGAAGTAAAAGTCGTAAACAAGG	[48]
	ITS4Oo	ATAGACTACAATTCGCC	[34]
Fungal 28S rRNA (universal, for Q-PCR)	NL1F	ATATCAATAAGCGGAGGAAAAG	[35]
	LS2R	ATTCCCAAACAACTCGACTC	[6]
Ascomycota-specific 28S rRNA (for Q-PCR)	LROR	ACCCGCTGAACTTAAGC	[48]
	ITS4Asco	CGTTACTRRGGCAATCCCTGTTG	[34]
Basidiomycota-specific 28S rRNA (for Q-PCR)	NL1F	ATATCAATAAGCGGAGGAAAAG	[35]
	ITS4B	CAGGAGACTTGTACACGGTCCAG	[16]

of a plasmid containing 10^2–10^8copies of either one of universal fungal, Ascomycota, and Basidiomycota LSU rRNA gene fragment. Concentrations of the plasmid DNA were measured using a NanoDrop1000 spectrophotometer (Thermo, USA). Three primer sets, NL1F/LS2R, LROR/ITS4Asco, and NL1F/ITS4B, were used to amplify the LSU rRNA gene of total fungi, Ascomycota, and Basidiomycota, respectively (Table 2). The PCR protocols were performed as follows: 95 °C for 10 min, followed by 45 cycles of 20 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C. Negative controls containing no template DNA were subjected to the same qPCR procedure to exclude any possible contamination. qPCR reactions were run in triplicate with the DNA extracted from each sediment sample. The specificity of the qPCR amplification of correct size was determined by the melting curve and checked with 1 % agarose gel electrophoresis. Cycle thresholds were determined by comparison with standard curves constructed using a tenfold serial dilution standard sample. Relative copy numbers among target groups were quantified via Pico Green analysis using the Rotor-Gene 6000 software.

Statistical Analysis

The weighted Jackknife environmental clustering analysis and principal coordinate analysis (PCoA) were conducted using the online software UniFrac (http://bmf2.colorado.edu/unifrac/) to evaluate the community similarity based on the gene sequence data. Comparison of the microbial communities was carried out using genetic distances based on the gene sequence data [30]. Environment cluster trees were projected in MEGA 5.01.

Results

Selectivity of Different Primer Sets

Six fungal-specific primer sets including one fungal-universal primer pair and five phylum-specific primer sets were applied to amplify the fungal ITS regions from the five sediment samples in the Pacific Ocean. PCR products were successfully obtained for all samples using the fungal-universal, Ascomycota- and Basidiomycota-specific primers, while no products were obtained for the primers specific for Oomycota, Zygomycota, and Chytridiomycota. Clone libraries were constructed for a total of 15 amplified PCR fragments (five samples, three primer sets), with 1,947 positive clones selected for restriction enzyme analysis including 766, 600, and 581 clones from the fungal-universal, Ascomycota-, and Basidiomycota-specific ITS clone libraries, respectively (Table 3). A total of 140, 86, and 62 restriction enzyme patterns was respectively obtained from the fungal-universal, Ascomycota-, and Basidiomycota-specific ITS clone libraries, which were subsequently subjected for sequencing. After analysis by BLASTN search in GenBank, six unique RFLP patterns (4.29 %) obtained from the fungal-universal primer set turned out to be of non-fungal origin. These six non-fungal sequences were affiliated with the genera of *Aspidisca* and *Engelmanniella* in the Alveolata [18]. No non-fungal taxa were retrieved from the phylum-specific primer sets.

The remaining 282 fungal sequences resulted in 48 fungal OTUs based on 3 % gene sequence distance cutoff: 16 (OTU1 to OTU16) in fungal-universal, 17 (OTUA1 to OTUA17) in Ascomycota-specific, and 15 (OTUB1 to OTUB15) in Basidiomycota-specific clone libraries (Table 3). The calculated coverage values showed that more than 95 % of the ITS diversity was captured in all clone libraries, which was further confirmed by rarefaction analysis.

Fungal Composition

Sixteen OTUs were obtained from the fungal-universal ITS primers; 12 OTUs were referred to four classes of Ascomycota—Eurotiomycetes, Saccharomycetes, Dothideomycetes, Sordariomycetes, while the other four OTUs (OTU9, OTU12, OTU15, and OTU16) belonged to the classes Agaricomycetes, Exobasidiomycetes, and Tremellomycetes of Basidiomycota (Table 4 and Fig. 1). Twelve Ascomycete OTUs belonged to six genera (*Aspergillus*, *Aureobasidium*, *Candida*, *Exophiala*, *Fusarium*, and *Periconia*) and an uncultured deep-sea fungal group (DSF-group 1), in which OTU3 with a sequence similarity of 77 % to *Fusarium* spp. and OTU5 with a sequence similarity of 99 % to *Candida metapsilosis* were the most prevalent OTUs appearing in all five clone libraries. Eight OTUs were only recovered from single clone libraries, within which five (OTU10–OTU13 and OTU15) were represented by a single clone. Compared with Ascomycota, there was a lower diversity of Basidiomycota recovered. Only four Basidiomycete OTUs were obtained from two samples (JK and WS), which belonged to the genera *Malassezia*, *Cryptococcus*, *Pleurotus*, and *Auricularia*. The first three Basidiomycetous genera were obtained from WS, while *Auricularia* was only recovered from JK. These Basidiomycetous genera accounted for less than 1 % of the total clones in individual clone libraries except *Pleurotus* which formed a major portion (61.7 %) of the clones in sample WS.

More diverse OTUs were recovered from the Ascomycota- and Basidiomycota-specific primers. The Ascomycota-specific primer amplified 17 OTUs belonging to five different classes: Sordariomycetes, Eurotiomycetes, Saccharomycetes, Dothideomycetes, and Leotiomycetes (Table 4 and Fig. 1). The number of OTUs obtained from each sample ranged between 4 and 11. Among 17 Ascomycete OTUs, 16 OTUs were referred to 14 named fungal genera (*Fusarium*, *Ramichloridium*, *Chaetomium*, *Cladosporium*,

Table 3 Biodiversity and richness indices of fungal ITS sequences from different clone libraries

Sample	Clone number	RFLP pattern	OTUs	Coverage (%)
Fungi (universal)				
CQ	210	33	8	99.5
CW	161	34	5	100
JK	132	21	7	95.5
W	137	24	6	99.3
WS	126	28	10	97.6
Total	766	140	16[a]	
Ascomycota				
CQ	104	15	6	97.1
CW	102	14	4	100
JK	104	13	4	100
W	149	18	11	96.0
WS	141	26	10	97.9
Total	600	86	17[a]	
Basidiomycota				
CQ	117	13	4	99.1
CW	126	15	7	96.8
JK	112	8	2	100
W	116	13	6	100
WS	110	13	7	99.1
Total	581	62	16[a]	

[a] The total number of OTUs does not equal the sum of OTUs of the individual libraries but reflects the number of unique OTUs for all five individual libraries (obtained with the same primer set) together

Aureobasidium, *Candida*, *Gloeotinia*, *Meyerozyma*, *Lecanicillium*, *Paecilomyces*, *Aspergillus*, *Simplicillium*, *Xylaria*, and *Penicillium*), while the remaining one (OTUA2) was affiliated with an unclassified Pleosporales. OTUA1 showing a sequence similarity of 99 % to *Fusarium solani* and OTUA5 with a sequence similarity of 99 % to *Candida metapsilosis* were the most prevalent OTUs, which occurred in all five clone libraries. Basidiomycota-specific primers recovered 15 OTUs belonging to two different classes: Agaricomycetes and Exobasidiomycetes (Table 4 and Fig. 1). OTUB3 related to *Trichosporon insectorum* with a 98 % sequence similarity was the most prevalent OTU which occurred in all five samples. These 15 Basidiomycete OTUs were affiliated with four known Basidiomycetous genera (*Trichosporon*, *Cryptococcus*, *Pleurotus*, and *Trametes*) and four unclassified or uncultured fungal group (unclassified Polyporales, unclassified Lycoperdaceae, uncultured Trechisporales, and uncultured Thelephoraceae).

Among the 48 fungal OTUs recovered in this study, 11 were unique in the fungal-universal, 13 in the Ascomycota-specific, and 14 in the Basidiomycota-specific ITS clone libraries. Four OTUs occurred in both fungal-universal and Ascomycota-specific ITS clone libraries, while only one OTU was shared in both fungal-universal and Basidiomycota-specific ITS clone libraries (Fig. 2). The number of singletons in libraries constructed with fungal-universal, Ascomycota- and Basidiomycota-specific ITS primer pairs was five, seven, and two, respectively (see table note in Table 4).

Diversity Patterns of Fungal Communities in the Pacific Ocean

A total of 48 OTUs affiliated with eight fungal classes were recovered from five sediment samples in this study. Species richness of fungal taxa differed at different sampling sites: 17, 16, 13, 23, and 27 OTUs were respectively recovered from sites CQ, CW, JK, W, and WS, belonging to 14, 11, 10, 18, and 20 genera, respectively. The highest richness of fungal diversity was recorded at Central Pacific Ocean area (sites W and WS), followed by Northwest Pacific Ocean area (sites CQ and CW), and finally the Mariana Trench area (site JK).

The weighted Jackknife environmental clustering analysis based on ITS sequence diversity of fungal OTUs clearly distinguished three groups of fungal assemblages in the deep-sea sediments of the Pacific Ocean (Fig. 3a). Samples CQ and CW from the Northwest Pacific Ocean areas clustered together and were separated from those collected at sites W and WS in the Central Pacific Ocean areas, while the sample JK was distinct from other Pacific Ocean samples (Fig. 3a). The fungal community at the Mariana Trench area site (JK) showed the highest dissimilarity to all other samples. Similar classification was revealed in the PCoA analysis, and the separation was explained along the first principal coordinate (P1) by 54.41 % of the total community structure variation among all samples (Fig. 3b).

Fungal Abundance in Sediment Samples

Fungal abundance in sediment samples was estimated by qPCR assays targeting the LSU rRNA gene fragment of the total fungi (using universal primers), Ascomycota, and Basidomycota, respectively. For each qPCR assay, more than 50 positive clones were reamplified and sequenced. No non-target sequence was obtained from these three individual qPCR assays. The coefficient of the real-time PCR assays with three primer sets was confirmed by the strong linear inverse relationship between the threshold cycle (C_T) and the log value of gene copy number for both primers sets ($r^2 \geq 0.99$). The PCR amplification efficiencies were 98.9, 94.5, and 91.2 % for the total fungi, Ascomycota, and Basidiomycota, respectively.

Quantitative PCR analysis showed that fungal abundance varied slightly within samples from different sites. The total fungal LSU rRNA gene copy numbers ranged from 3.52×10^6 to 5.23×10^7 copies/g sediment (wet weight), and the highest abundance occurred at sample WS which was five times over

Table 4 Molecular identification of fungal OTUs in this study

OTU no.	Accession number	Top BLAST hit taxon	Class	Sequence similarity (%)
Fungi-universal				
OTU1	KJ194324	*Exophiala xenobiotica* strain: IFM 58546 (AB566310)	Eurotiomycetes	94
OTU2	KJ194325	*Fusarium oxysporum* strain Ppf16 (EF495230)	Sordariomycetes	100
OTU3	KJ194326	*Fusarium equiseti* isolate T34 (FJ459976)	Sordariomycetes	77
OTU4	KJ194327	Uncultured fungus clone G57 (DQ279844)	Saccharomycetes	97
OTU5	KJ194328	*Candida metapsilosis* strain LEMI8448 (JQ585714)	Saccharomycetes	99
OTU6	KJ194329	*Aureobasidium pullulans* culture collection UOA/HCPF:13830 (KC253968)	Dothideomycetes	99
OTU7	KJ194330	*Fusariumsolani* isolate S-0900 (EF152426)	Sordariomycetes	99
OTU8	KJ194331	*Aspergillus sydowii* strain NRRL 250 (AY373868)	Eurotiomycetes	99
OTU9	KJ194332	*Pleurotus pulmonarius* isolate 4203 (AY450349)	Agaricomycetes	99
OTU10[a]	KJ194333	*Fusarium oxysporum* strain H55 (GU566301)	Sordariomycetes	97
OTU11[a]	KJ194334	*Candida metapsilosis* strain LEMI8448 (JQ585714)	Saccharomycetes	95
OTU12[a]	KJ194335	*Auricularia polytricha* cultivar ZH (HM448450)	Agaricomycetes	99
OTU13[a]	KJ194336	*Candida metapsilosis* strain LEMI8448 (JQ585714)	Saccharomycetes	95
OTU14	KJ194337	*Periconia* sp. TMS-2011 voucher SC13d100p7-2 (HQ631028)	Sordariomycetes	83
OTU15[a]	KJ194338	*Malassezia globosa* isolate POL.1.11.IA (KC152884)	Exobasidiomycetes	97
OTU16	KJ194339	*Cryptococcus tephrensis* (DQ000318)	Tremellomycetes	95
Ascomycota-specific				
OTUA1	KJ194276	*Fusarium solani* isolate S-0900 (EU029589)	Sordariomycetes	99
OTUA2	KJ194277	*Pleosporales* sp. 5 TMS-2011 voucher SC15d100p10-3 (HQ631059)	Dothideomycetes	88
OTUA3[a]	KJ194278	*Xylaria angulosa* (AB274815)	Sordariomycetes	94
OTUA4	KJ194279	*Cladosporium* sp.SS-S12 (GU797141)	Dothideomycetes	99
OTUA5	KJ194280	*Candida metapsilosis* strain LEMI8448 (JQ585714)	Saccharomycetes	99
OTUA6	KJ194281	*Aureobasidium* sp. RBF-8B1 (FN665419)	Dothideomycetes	99
OTUA7	KJ194282	*Meyerozyma guilliermondii* strain KAML05 (KC119207)	Saccharomycetes	99
OTUA8[a]	KJ194283	*Gloeotinia temulenta* (DQ235697)	Leotiomycetes	99
OTUA9[a]	KJ194284	*Chaetomium globosum* strain CBS 164.62 (JN209920)	Sordariomycetes	99
OTUA10	KJ194285	*Fusarium equiseti* isolate H02-765S (EU595566)	Sordariomycetes	99
OTUA11	KJ194286	*Cladosporium* sp.CBS 280.49 (EU167574)	Dothideomycetes	99
OTUA12	KJ194287	*Penicillium dipodomyicola* strain ACBF 002-3 (GQ161752)	Eurotiomycetes	99
OTUA13[a]	KJ194288	*Lecanicillium psalliotae* (AB360367)	Sordariomycetes	90
OTUA14[a]	KJ194289	*Paecilomyces lilacinus* strain LTBF 007-1 (GQ229080)	Sordariomycetes	99
OTUA15[a]	KJ194290	*Aspergillus vitricola* isolate NRRL 5125 (EF652046)	Eurotiomycetes	96
OTUA16[a]	KJ194291	*Simplicillium* sp. ID07-F0443 strain: BTCC-F43 (AB378532)	Sordariomycetes	99
OTUA17	KJ194292	*Ramichloridium apiculatum* strain CPC 12310 (GU214687)	Dothideomycetes	96
Basidiomycota-specific				
OTUB1	KJ194409	*Trichosporon dermatis* strain ATCC 20566 (HM802130)	Tremellomycetes	100
OTUB2	KJ194410	*Pleurotus pulmonarius*strain LAU60 (JF736660)	Agaricomycetes	97
OTUB3	KJ194411	*Trichosporon insectorum* strain ATCC 20506 (HM802133)	Tremellomycetes	98
OTUB4	KJ194412	*Polyporales* sp.1 SR-2012 strain 308 (JQ312163)	Agaricomycetes	96
OTUB5	KJ194413	*Pleurotus pulmonarius* isolate 4203 (AY450349)	Agaricomycetes	95
OTUB6[a]	KJ194414	*Lycoperdaceae* sp. TU112130 (JQ657796)	Agaricomycetes	88
OTUB7	KJ194415	*Trametes versicolor* isolate X-02 (KC176306)	Agaricomycetes	91
OTUB8[a]	KJ194416	*Trametes maxima* (AB158315)	Agaricomycetes	93
OTUB9	KJ194417	*Trametes versicolor* isolate X-02 (KC176306)	Agaricomycetes	91
OTUB10	KJ194418	*Trametes versicolor* isolate X-69 (KC176346)	Agaricomycetes	86
OTUB11	KJ194419	Uncultured Trechisporales clone RELIS_K6_C05(JF519274)	Agaricomycetes	79
OTUB12	KJ194420	*Trichosporon insectorum* strain ATCC 20506 (HM802133)	Tremellomycetes	96

Table 4 (continued)

OTU no.	Accession number	Top BLAST hit taxon	Class	Sequence similarity (%)
OTUB13	KJ194421	*Cryptococcus* sp. HB 1222 (AM160648)	Tremellomycetes	94
OTUB14	KJ194422	*Trichosporon dermatis* strain ATCC 20566 (HM802130)	Tremellomycetes	96
OTUB15	KJ194423	Uncultured Thelephoraceae clone RELIS_K2_E11 (JF519143)	Agaricomycetes	90

[a] Singletons in different clone libraries recovered from this study

the lowest one (sample JK) (Fig. 4). The Ascomycota LSU rRNA gene copy numbers ranged from 2.89×10^6 to 1.58×10^7 copies/g sediment (wet weight), and the highest (sample WS) and the lowest (sample JK) differed by 5.5-fold. The Basidiomycota LSU rRNA gene copy numbers ranged from 5.67×10^4 to 4.22×10^5 copies/g sediment (wet weight), and the highest abundance was found in sample W and differed from the lowest one (sample JK) by 2.5-fold. The ratios of LSU gene abundance for Ascomycota and Basidiomycota to total fungi were 0.82–0.91 and 0.01–0.02, respectively. As a general observation, the highest abundance of total fungi and two dominant fungal taxa occurred in the Central Pacific Ocean areas, followed by Northwest Pacific Ocean area, and Mariana Trench area.

Discussion

The present study revealed 48 fungal OTUs from deep-sea sediments of the Pacific Ocean, including 27 phylotypes, which showed a low similarity (<97 % sequence similarity) with known fungal sequences in the GenBank database. The fungal community was dominated by Ascomycota, followed by Basidiomycota, which was consistent with previous studies on fungal diversity of deep-sea environments [32]. No sequences of Chytridiomycota and the LKM11 clade (a clone group mainly retrieved from oxygen-depleted aquatic environments including deep-sea environments) were detected in sediment samples in this study, suggesting low abundance at the sampling sites examined.

All five different classes of the Ascomycota recovered from the deep-sea environments in previous studies were identified in the present study. The most frequently detected Ascomycete class was Sordariomycetes, followed by Dothideomycetes, Saccharomycetes, Eurotiomycetes, and Leotiomycetes. The Leotiomycetes class has rarely been reported from the deep-sea environments except in the sediments of the Pacific Ocean [31, 33]. The most prevalent Ascomycetous genera recovered in this study were *Candida*, *Fusarium* and uncultured fungal group DSF-group 1. *Candida* occurred in all sediment samples in this study, showing a good agreement with previous deep-sea reports that it is one of the most common genera in deep-sea environments [2, 25, 31, 33, 41–43, 45]. *Fusarium* was represented by a number of OTUs and two of them were affiliated with *Fusarium oxysporum*, which was known as a denitrifying fungus in a previous study [20]. Sequences of the DSF-group 1 formed a major cluster in the Ascomycota with *Metschnikowia*/*Candida* within the Saccharomycotina as its closest described taxa. This group was described by Nagano et al. [33] with fungal sequences from the deep-sea sediments collected at several locations off the Japanese islands based on ITS and LSU rRNA clone library analysis. Analysis using SSU rRNA sequences also recovered this group from other deep-sea regions [2, 32] and oxygen-depleted environments [46]. Apart from *Fusarium*, other sordariomycetous genera including *Periconia*, *Xylaria*, *Lecanicillium*, *Chaetomium*, *Simplicillium*, and *Paecilomyces* were also discovered in this study, challenging the traditional view that the diversity of Sordariomycete phylotypes was low in the deep-sea environments [32].

Only three classes within the phylum Basidiomycota were recovered in this study, including Agaricomycetes, Tremellomycetes, and Exobasidiomycetes. Agaricomycete fungi in marine environments were mostly reported from mangroves [21, 32]. Although molecular studies have shown the ubiquitous presence of Agaricomycete phylotypes in deep-sea environments [2, 26, 31, 32, 40], only one culture (*Fomitopsis* sp.) has been isolated from a deep-sea hydrothermal seafloor mat [8]. A diverse range of Agaricomycete phylotypes retrieved in this study indicated that deep-sea sediments may harbor diverse Agaricomycete fungi, and improved culturing methods are needed to culture them. The Tremellomycete OTUs in this study were affiliated with two genera, *Crytococcus* and *Trichosporon*, which have been widely detected in cold environments such as deep-sea and Polar Regions [2, 13, 25, 32]. The only OTU belonging to Exobasidiomycetes was related to the genus *Malassezia*, which was ubiquitously recovered in the deep-sea environments based on molecular methods. *Malassezia* fungi have been known as the causative agents of skin diseases in marine mammals [31]. As the members of this genus have also been detected in terrestrial soil nematodes [38], it was speculated in some reports that *Malassezia* spp. were associated with small

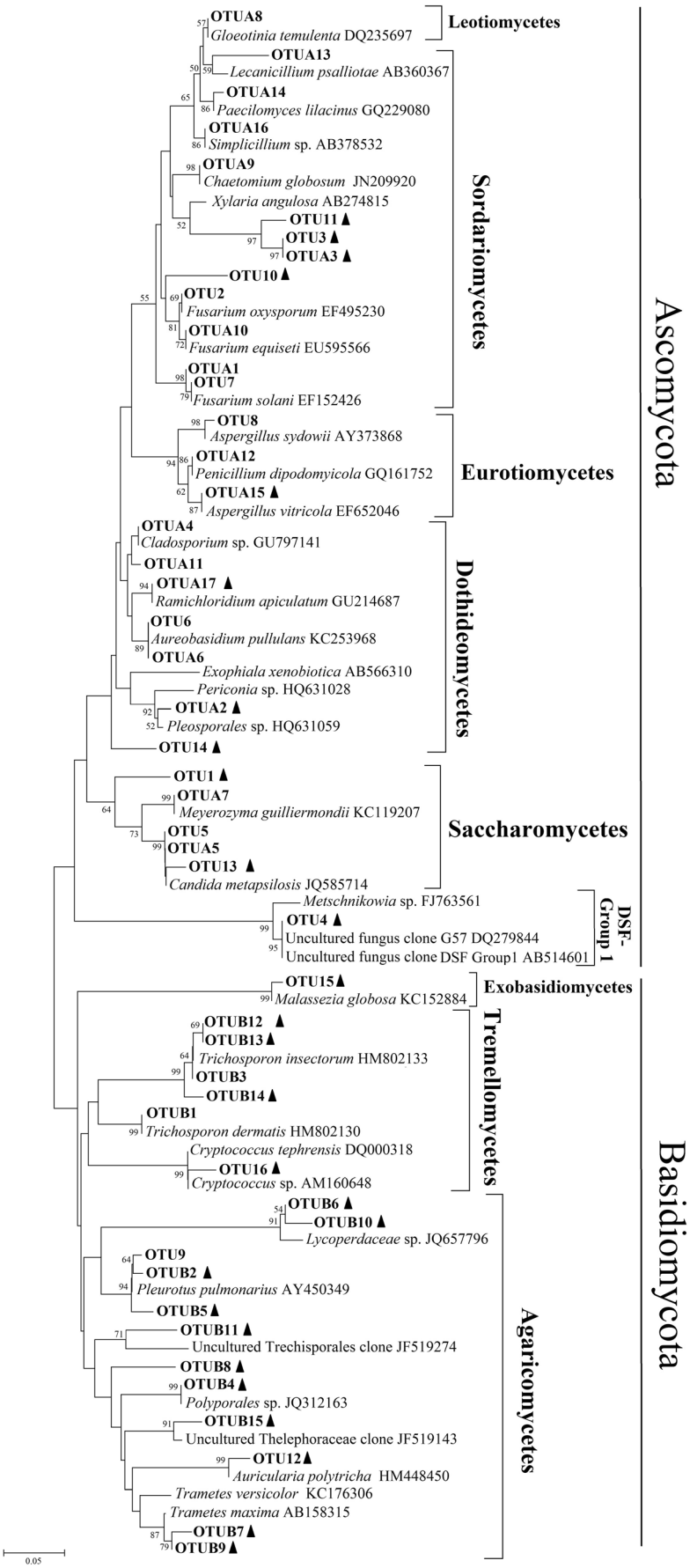


Fig. 1 Neighbor-joining phylogenetic tree for the fungal OTUs constructed based on ITS gene sequences obtained from three fungal primers sets. OTU 1-16 were recovered from fungal-universal primer set ITS1/4, OTU A1-17 were recovered from Ascomycota-specific primer set ITS5/4Asco, and OTU B1-15 were recovered from Basidiomycota-specific primer set ITS1F/4B. *Numbers below* branches indicate bootstrap values (>50 %) from 1,000 replicates. Novel sequences which showed low similarity (≤97 %) with available fungal sequences in the GenBank are marked with a *triangle*

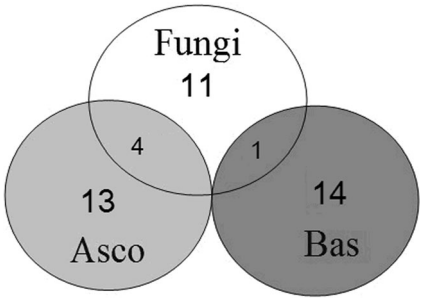


Fig. 2 Venn diagram showing the number of OTUs that were detected simultaneously by two of the primer sets and the number of OTUs amplified exclusively with a single primer set

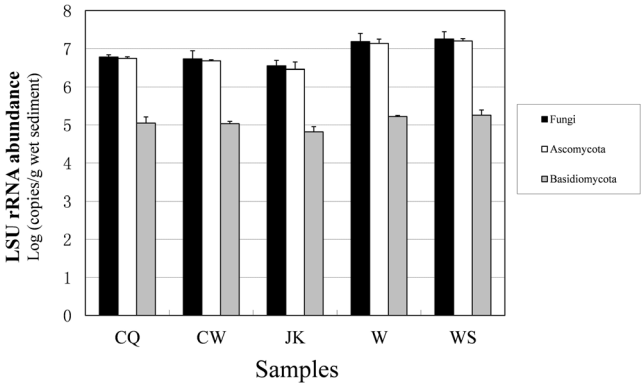
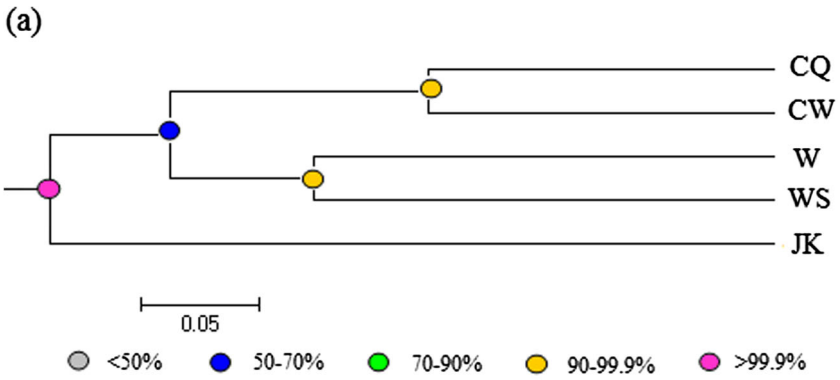
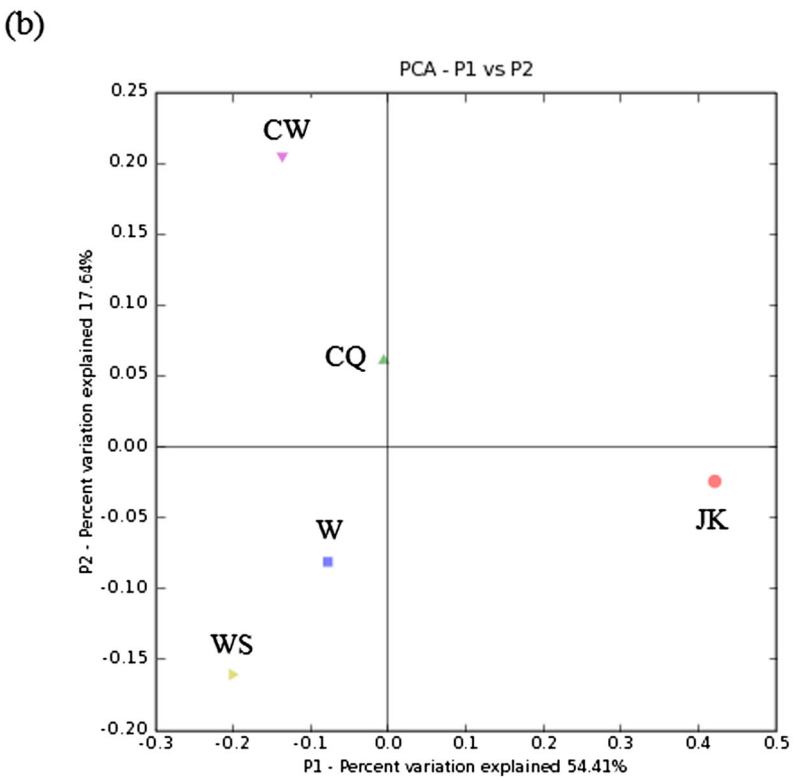


Fig. 4 Abundance of the fungal, Ascomycete, and Basidiomycete LSU rRNA gene copies in the deep-sea sediments of the Pacific Ocean. *Error bars* represent standard deviations of triplicate analyses

Fig. 3 Weighted Jackknife environmental clustering (**a**) and PCoA (**b**) analyses for fungal ITS sequences in this study





marine invertebrates inhabiting in deep-sea sediments, such as nematodes or polychaetes [31, 32].

Basidiomycota was only successfully detected in two out of five sediment samples and was much less diverse than Ascomycota in each clone library when using a fungal-universal primer set. Similar results were also observed in previous reports using fungal-specific SSU and ITS primers to investigate fungal diversity in marine habitats [2, 26, 33, 41]. This may be due to the high percentage of Ascomycota in deep-sea fungal community, which the fungal-universal primers preferentially amplified. Our qPCR results supported this speculation and showed that Ascomycota was about two orders of magnitude more abundant than Basidomycota in deep-sea sediment samples. A nested PCR approach with the Basidiomycota-specific primer set successfully amplified basidomycete sequences from all sediment samples and a high ratio of novel phylotypes (13/15) was observed in the Basidiomycota clone libraries, suggesting that there are diverse Basidomycota in the deep-sea floor and it is necessary to use improved culture-independent methods for enhanced detection of fungi in deep-sea environment.

In this study, 2.08 % of sequences were of non-fungal origin. These non-fungal sequences were only recovered from the fungal-universal primers, indicating that taxon-specific primers had a higher specificity to fungi compared with fungal-universal primer set. Moreover, the Ascomycota- and Basidiomycota-specific primer sets recovered more diverse fungal genera (15 and 8, respectively) than the fungal-universal primers (eight Ascomycetous and four Basidiomycetous genera), demonstrating that different fungal primer sets may detect different subsets of the fungal community, and the taxon-specific primer sets would be helpful to amplify the less abundant groups of fungi. Therefore, using multiple primer sets, especially the taxon-specific primers, may be essential to investigate the true fungal diversity in deep-sea environments.

Although information is lacking on the abundance of fungi in the deep-sea floor so far, the only report by Le Calvez et al. [26] showed that the fungal SSU rRNA gene copy numbers ranged from 1.91×10^5 to 1.35×10^7 copies/μg genomic DNA in hydrothermal vent ecosystems. Our study recovered a relatively high abundance of fungi (3.52×10^6–5.23×10^7 LSU rRNA gene copies/g wet weight sediment) in the deep-sea sediments of the Pacific Ocean, suggesting that fungi might be involved in important ecological functions in the deep-sea environments.

Mariana Trench, the deepest part of the ocean, is a unique environment on earth. With a depth of almost 11,000 m, a temperature of 2.5 °C, and a pressure of around 111.79 MPa, a comprehensive study of its microecology is technically difficult [17]. Nagano et al. [33] collected a sample from the Mariana Trench, but they failed to get any fungal DNA sequences from it, possibly because the sample predominantly comprised rough sand. This study successfully detected fungi from the sample collected at Mariana Trench area, although the diversity of fungi was low in comparison with that in other sampling sites of the Pacific Ocean. Community similarity analyses also suggested that the fungal community structure at the sample of Mariana Trench was distinct from that at samples from other locations of the Pacific Ocean. The effects of depth, temperature, and hydrostatic pressure on fungal communities may explain these differences, but these need to be studied further.

In conclusion, this study suggests a high diversity and abundance of fungi in deep-sea sediments of the Pacific Ocean, including a sample from the deepest ocean depth, the Mariana Trench. We discovered a high number of unknown fungal species, which accounted for 56.25 % of the total phylotypes, indicating that deep-sea environments may harbor a diverse taxonomically unknown fungal community than we expected. Future investigation should look into the ecological roles of fungi in deep-sea sediment using fluorescent in situ hybridization (FISH) and macro transcriptome approaches. This eventually will improve our understanding of fungal communities responding to extreme conditions, how they interact with other components of the ocean ecosystems, and the ecological role of deep-sea-derived fungi.

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