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Diverse Deep-Sea Fungi from the South China Sea and Their Antimicrobial Activity

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Abstract We investigated the diversity of fungal communities in nine different deep-sea sediment samples of the South China Sea by culture-dependent methods followed by analysis of fungal internal transcribed spacer (ITS) sequences. Although 14 out of 27 identified species were reported in a previous study, 13 species were isolated from sediments of deep-sea environments for the first report. Moreover, these ITS sequences of six isolates shared 84–92 % similarity with their closest matches in GenBank, which suggested that they might be novel phylotypes of genera *Ajellomyces*, *Podosordaria*, *Torula*, and *Xylaria*. The antimicrobial activities of these fungal isolates were explored using a double-layer technique. A relatively high proportion (56 %) of fungal isolates exhibited antimicrobial activity against at least one pathogenic bacterium or fungus among four marine pathogenic microbes (*Micrococcus luteus*, *Pseudoaltermonas piscida*, *Aspergerillus versicolor*, and *A. sydowii*). Out of these antimicrobial fungi, the genera *Arthrinium*, *Aspergillus*, and *Penicillium* exhibited antibacterial and antifungal activities, while genus *Aureobasidium* displayed only antibacterial activity, and genera *Acremonium*, *Cladosporium*, *Geomyces*, and *Phaeosphaeriopsis* displayed only antifungal activity. To our knowledge, this is the first report to investigate the diversity and antimicrobial activity of culturable deep-sea-derived fungi in the South China Sea. These results suggest that diverse deep-sea fungi from the South China Sea are a potential source for antibiotics' discovery and further increase the pool of fungi available for natural bioactive product screening.

Introduction

Drug resistance in microorganisms and overuse of antibiotics are becoming serious concerns around the world. As a result, there is an urgent need to search for effective new antibiotics in the treatment of infectious disease at present [28]. To maximize the antibiotics' diversity available from microorganisms, new sources of microbes are needed [14]. In recent times, deep-sea fungi have received considerable attention as new sources for new antibiotics' discovery [8, 9].

Although the extreme conditions are characterized by the absence of sunlight irradiation, predominantly low temperature, and high hydrostatic pressure in deep-sea environments, it is now well known that diverse fungal community is abundant in deep-sea environments [11]. The isolation of deep-sea fungi was first reported approximately 50 years ago from the Atlantic Ocean at a depth of 4,450 m [17]. Since this first report, an increasing number of fungal species were found in several deep-sea environments, e.g., sediments from the Mariana Trench at 11,500 m depth [21], calcareous sediments [15], the Chagos Trench at a depth of 5,500 m [16], the Central Indian Basin at about 5,000 m depth [3, 20], and deep-sea coral *Lophelia pertusa* [5]. These developments clearly illustrate the increasing attention being paid to fungal abundance and diversity in deep-sea environments. However, little is now known

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about the deep-sea fungal community in the South China Sea. The aim of the present study was to isolate and test antimicrobial activity of culturable fungi from different deep-sea sediment cores at about 2,400–4,000 m depth from the South China Sea.

Materials and Methods

Deep-Sea Sediment Samples

Deep-sea sediment samples at about 2,400–4,000 m depth were collected on the South China Sea Open Cruise in August 2011. Latitudes and longitudes of the collected deep-sea sediment samples A-I are 17°59′742 N and 111°48′092E, 18°01′654 N and 112°30′203E, 18°01′N841 and 114°30′315E, 19°28′581 N and 115°27′751E, 19°00′368 N and 117°58′223E, 18°05′255 N and 118°30′989E, 19°41′569 N and 119°19′896E, 18°44′606 N and 119°44′263E, and 20°22′971 N and 120°00′250E, respectively (Fig. 1). Deep-sea sampling was performed using the Remote Operated Vehicle. The collected sediments were mostly undisturbed and compact. The average length of sediment cores obtained from these locations was about 30 cm. Sub-cores of these sediments were collected from a box corer using an alcohol-sterilized PVC cylinder of 5 cm inner diameter. Subsections of these sediments were cut from the above sediment sub-cores and directly introduced into sterile plastic bags to avoid any aerial contamination [19]. The bags were closed and carried to the microbiology laboratory on board for fungal isolation.

Fungal Isolation and Identification

About 1 g of sediment from the middle of each subsection that had not been in contact with the walls of the PVC cylinder was removed with a flame-sterilized spatula and placed in sterile vials for isolation of fungi [16]. Fungal isolation was performed through the modified particle plating technique described by Cathrine and Raghukumar [2]. The media used for isolation were malt extract agar (MEA), Czapek Dox agar (CDA), glucose peptone starch agar (GPSA), and potato glucose agar (PDA) [3]. All the media were used at 1/5 strength to simulate the low nutrient conditions in the deep sea.

To inhibit the growth of bacteria, 0.5 g L^{-1} benzylpenicillin and 0.03 g L^{-1} rose bengal were added to the basic media. The inoculated plates were cultured in the dark at 10 °C for 1–3 months until the morphology of the fungi could be distinguished. Fungal isolates were chosen and transferred onto new corresponding agar plates on the basis of their morphological differences based on visible examination of growth characteristics, mycelia, and diffusible pigments. The resulting plates were incubated at 10 °C for pure culture.

Fungal isolates were identified by combining morphological observations with internal transcribed spacer sequences (ITS). Total genomic DNA was extracted from all selected fungal strains, as described by Lai et al. [7]. From the genomic DNA, nearly full-length ITS sequences were amplified by polymerase chain reaction using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTA TTGATATGC-3′) [26]. Detailed information of fungal ITS gene sequencing and identification was given by Zhang et al. [29].

Phylogenetic and Data Analysis

Nearly full-length ITS sequences of selected fungal isolates were aligned using Clustal W in MEGA 5.0 along with phylogenetic anchor sequences obtained from the GenBank BLASTn matches and rooted with a fungal outgroup [22]. Phylogenetic trees of ITS sequences were created using the

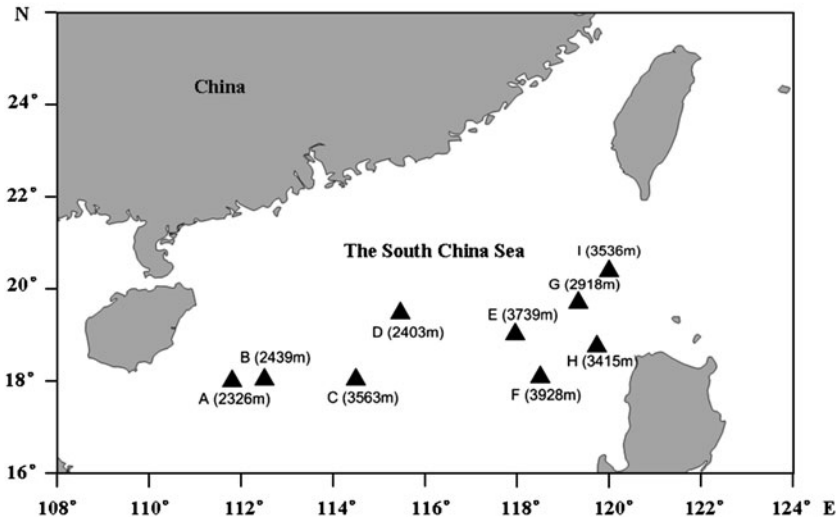

Fig. 1 Map of the South China Sea, location and depth of the sampling sites

neighbor-joining method with bootstrap values >50 % shown, based on 1,000 boot strap iterations [23].

A richness/species abundance coefficient Bray-Curtis was estimated based on the presence/absence matrix of fungi isolated from the nine sediment samples. Bray-Curtis analysis was performed through using SPSS for Window Soft (Version 11.5) [24]. Fungal ITS sequences of the 27 representative isolates were deposited in GenBank under accession numbers JX156347, JX156349–JX156356, JX15 6358–JX156375, and JX156378.

Determination of Antimicrobial Activity

The antimicrobial activities of fungal isolates were determined by a double-layer technique [27]. Selected fungal isolates were grown on PDA medium at 10 °C for 5–14 days depending on the growth rate of various isolates. Two marine pathogenic bacteria (*Micrococcus luteus* (ML) and *Pseudoaltermonas piscida* (PP) and two marine pathogenic fungi (*Aspergillus versicolor* (AV) and *A. sydowii* (AS) were used as the indicator microorganisms for the double-layer assay. Detailed information about the antimicrobial activity test was given by Zhang et al. [29] and the antimicrobial activity was expressed as the diameter of the zone of inhibition (mm) [29, 30].

Results

Fungal Isolation and Phylogenetic Diversity

A total of nine deep-sea sediment samples of the South China Sea were processed for fungal isolation on four media plates. Totally, 72 fungal isolates were obtained from the nine sediment samples. ITS sequence analysis and observation of morphological characteristics showed that all the isolates had 95–100 % sequence similarities to previously cultured and well-described fungi, with the exception of six unclassified fungal isolates which shared 84–92 % similarity with their closest NCBI relatives. These identified isolates belonged to 27 fungal species of ten families (Table 1). Phylogenetic tree and more detailed subtrees of partial ITS sequences of the 27 fungal representatives are shown in the supplementary materials. Among the ten families, *Davidiellaceae* and *Trichocomaceae* were the most common and diverse fungal families in these sediment samples, which included three genera (*Cladosporium*, *Aspergillus*, and *Penicillium*) and 18 species (Table 1). On the other hand, *Apiosporaceae*, *Dothioraceae*, *Pleosporaceae*, and *Teratosphaeriaceae* were the rarest fungal families with a single isolate, and most of the other families occurred as two or three isolates.

Distribution and Comparison of Fungal Community

The distribution and comparison of fungal species in nine different sediment samples are summarized in Table 1. Among the 27 identified fungal species, most species could only be recovered from one sample, except for *Aspergillus sydowii*, *A. vitricola*, *Cladosporium cladosporioides*, *Engyodontium album,* and *Penicillium brevicompactum* that could be recovered from two or three different samples. Both samples A and D had the most diverse fungal species and isolates with six species and 12 isolates, while samples G, H, and I harbored less fungal species and isolates with two or three species and four or five isolates. Further analysis of Bray-Curtis showed 100 % dissimilarity of the fungal community between each two samples with the exception of 33.3 % dissimilarity between sample H and samples B and C.

Distribution of Fungi with Antimicrobial Activity

All of the 27 fungal representatives were tested against two marine pathogenic bacteria and two marine pathogenic fungi to examine their spectra of antimicrobial activity. The antimicrobial activities of the 27 fungal representatives are shown in Table 2. About 56 % of the 27 representatives showed antimicrobial activity. Among them, six fungal species exhibited distinct antimicrobial activity against more than one pathogenic microorganism, and one species *A. westerdijkiae* displayed relatively strong activity against all the four indicator microorganisms.

Discussion

Although there were a few papers that reported on the fungal diversity in deep-sea environments by conventional culture-dependent methods [3, 5, 15–17, 20, 21], this study is the first report about the phylogenetic survey and antimicrobial activity of culturable deep-sea-derived fungi from the South China Sea. A total of 72 fungal isolates belonging to 27 species were recovered from the nine sediment samples of the South China Sea. And, out of the 27 fungal species, 13 species (Table 1) were first isolated from sediments of deep-sea environments, which might be due to the use of effective isolation media that were successfully used for isolating deep-sea fungi from the Central Indian Basin [3]. Combined with previous studies [3, 5, 15–17, 20, 21], our results indicate that an increasing number of previously unidentified fungal communities widely occur in the deep-sea environments. The deep-sea environments are now recognized as dynamically hosting a wealth of unique organisms. In particular, the discovery of hydrothermal vents, methane cold seeps, and surrounding ecosystems has resulted in completely new concepts for

Table 1 Diversity and distribution of fungi isolated from nine different deep-sea sediment samples of the South China sea

Fungal species (The representative isolates' accession numbers in GenBank)	Fungal families	Number of fungal isolates								
		A	B	C	D	E	F	G	H	I
Acremonium implicatum (JX156347)	*Hypocreaceae*							2		
Alternaria tenuissima (JX156349)a	*Pleosporaceae*				1					
Arthrinium phaeospermun (JX156350)a	*Apiosporaceae*	1								
Aspergillus penicillioides (JX156351)	*Trichocomaceae*				2					
A. restrictus (JX156352)	*Trichocomaceae*	3								
A. sydowii (JX156353)	*Trichocomaceae*		1						1	
A. tubingensis (JX156354)a	*Trichocomaceae*								2	
A. unguis (JX156355)a	*Trichocomaceae*					2				
A. versicolor (JX156356)	*Trichocomaceae*	3				1				
A. vitricola (JX156357)	*Trichocomaceae*				3		1			
A. westerdijkiae (JX156359)a	*Trichocomaceae*							1		
Aureobasidium pullulans (JX156360)a	*Dothioraceae*				2					
Catenulostroma protearum (JX156361)a	*Teratosphaeriaceae*	1								
Cladosporium cladosporioides (JX156362)	*Davidiellaceae*		2	2		2				
C. colombiae (JX156363)	*Davidiellaceae*				2					
C. oxysporum (JX156364)	*Davidiellaceae*									3
C. sphaerospermun (JX156365)	*Davidiellaceae*				2			2		
C. uredinicola (JX156366)	*Davidiellaceae*			1					1	
Engyodontium album (JX156368)	Incertae sedis		3			3				
Geomyces vinaceus (JX156369)a	*Myxotrichaceae*									2
Penicillium biourgeianum (JX156370)a	*Trichocomaceae*	3								
P. brevicompactum (JX156371)a	*Trichocomaceae*			2			1			
P. chrysogenum (JX156372)	*Trichocomaceae*						1			
P. commune (JX156373)a	*Trichocomaceae*					1				
P. verruculosum (JX156374)a	*Trichocomaceae*	1								
Phaeosphaeriopsis musae (JX156375)a	*Phaeosphaeriaceae*			3						
Rhodotorula mucilaginosa (JX156378)	*Erythrobasidiaceae*						2			
Total number of fungal isolates		12	6	8	12	9	5	5	4	5

Species marked by a letter (a) are new reports for deep-sea environments

Detailed information of deep-sea sediment samples A-I is described in Fig. 1

considering the energy sources available for sustaining life in deep oceans [11]. In addition, fungi, one of the most extremotolerant and ecologically important groups of microorganisms, have been relatively underexplored in the deep-sea environments [11].

In this study, most of fungal isolates shared 95–100 % similarity with the existing fungal ITS sequences in the NCBI database. However, several isolates, DFFSCS002, 0030, 0031, 0033, 0035, and 0036 (in GenBank under accession numbers JX156348, JX156376, JX156377, JX156379, JX156381, and JX156382, respectively), shared 84–92 % similarity with their closest matches, suggesting their possibility of being novel phylotypes of genera *Ajellomyces*, *Podosordaria*, *Torula*, and *Xylaria*. Nagano et al. found that a majority of deep-sea fungal communities in the Japanese island including the Mariana Trench were novel species by PCR-mediated ITS regions of rRNA gene clone analysis [12]. These results suggest that great fungal diversity is likely to be revealed by combining the culture-dependent methods with culture-independent approaches. Recently, an increasing number of studies have indicated that comparisons between fungal community compositions obtained by culture-dependent and independent methods highlight different fungi, emphasizing the need for complementary approaches to assess the fungal assemblage within unusual environments [10, 18]. Singh et al. revealed a greater diversity of fungal signatures in deep-sea sediments of the Central Indian Basin by combining culture-dependent and culture-independent approaches than earlier reports using a single approach [19].

The other aim of this study was to explore deep-sea-derived fungi from the South China Sea for antibiotics' discovery. The results of antimicrobial activity tests showed that relatively

Table 2 Antimicrobial activity of culturable fungal representative isolates from deep-sea sediments of the South China sea

Fungal isolates	Fungal species	Antimicrobial activity (zone of inhibition/mm)			
		ML	PP	AS	AV
DFFSCS001	*Acremonium implicatum*	–	–	8.90 ± 1.05	9.60 ± 0.36
DFFSCS003	*Alternaria tenuissima*	–	–	–	–
DFFSCS004	*Arthrinium phaeospermun*	–	19.97 ± 1.25	–	12.50 ± 1.61
DFFSCS005	*Aspergillus penicillioides*	11.60 ± 1.73	–	–	–
DFFSCS006	*A. restrictus*	–	–	–	–
DFFSCS007	*A. sydowii*	–	–	–	–
DFFSCS008	*A. tubingensis*	–	17.00 ± 1.20	–	10.10 ± 0.85
DFFSCS009	*A. unguis*	–	21.70 ± 1.18	–	–
DFFSCS010	*A. versicolor*	–	–	–	–
DFFSCS011	*A. vitricola*	–	–	9.23 ± 1.02	–
DFFSCS013	*A. westerdijkiae*	10.60 ± 0.66	22.10 ± 1.05	17.16 ± 0.86	18.30 ± 0.52
DFFSCS014	*Aureobasidium pullulans*	12.33 ± 1.63	13.07 ± 1.10	–	–
DFFSCS015	*Catenulostroma protearum*	–	–	–	–
DFFSCS016	*Cladosporium cladosporioides*	–	–	8.87 ± 0.15	–
DFFSCS017	*C. colombiae*	–	–	10.23 ± 1.67	–
DFFSCS018	*C. oxysporum*	–	–	–	–
DFFSCS019	*C. sphaerospermun*	–	–	16.43 ± 1.11	–
DFFSCS020	*C. uredinicola*	–	–	–	–
DFFSCS021	*Engyodontium album*	–	–	–	–
DFFSCS022	*Geomyces vinaceus*	–	–	9.80 ± 0.26	–
DFFSCS023	*Penicillium biourgeianum*	–	–	–	–
DFFSCS024	*P. brevicompactum*	–	–	–	–
DFFSCS025	*P. chrysogenum*	–	–	–	–
DFFSCS026	*P. commune*	–	22.10 ± 1.05	19.21 ± 0.87	–
DFFSCS027	*P. verruculosum*	–	–	9.20 ± 1.34	10.30 ± 0.36
DFFSCS028	*Phaeosphaeriopsis musae*	–	–	–	9.03 ± 1.07
DFFSCS032	*Rhodotorula mucilaginosa*	–	–	–	–

Each test was performed three times. Strong activity: zone of inhibition greater than 15 mm; Moderate activity: zone of inhibition ranged between 10 and 15 mm; Weak activity: zone of inhibition less than 10 mm; —: traces or no antagonistic effects were observed

Indicator bacteria: *ML Micrococcus luteus*, *PP Pseudoaltermonas piscida*

Indicator fungi: *AV Aspergillus versicolor*, *AS Aspergillus sydowii*

high (56 %) deep-sea fungal isolates belonging to eight genera displayed distinct antibacterial and/or antifungal activity, suggesting that these fungal isolates are potential sources for antibiotics' discovery and further increasing the pool of fungi available for natural bioactive product screening.

Genera *Aspergillus* and *Penicillium* are rich sources of chemically diverse natural products with a broad range of biological activities [6]. In this study, we also found that some isolates belonging to genera *Aspergillus* (such as isolates DFFSCS008 and DFFSCS013) and *Penicillium* (such as isolate DFFSCS026) exhibited relatively high antibacterial and antifungal activity. Genus *Arthrinium* was often recovered from marine sponges [4, 25] and could produce antifungal sesquiterpenoids [13]. In here, the fungal isolate DFFSCS004 belonging to the *Arthrinium* genus displays relatively high antibacterial activity. A previous study reported that fungus *Aureobasidium pullulans* could produce pullulan with significant antagonistic activity against several fungal indicators [1]. In here, the fungal isolate *A.pullulans* DFFSCS014 only displayed antibacterial activity. In this study, genus *Cladosporium* showed antifungal activity; however, in our previous study, genus *Cladosporium* isolated from the South China Sea gorgonians displayed relatively high antibacterial activity [29]. The fungal genera *Acremonium*, *Geomyces*, and *Phaeosphaeriopsis* were found to exhibit relatively high antifungal activity for the first time. These results suggest that deep-sea fungi may evolve metabolic abilities enabling them to produce a variety of bioactive compounds in order to better adapt to different ecosystems.

In summary, the diverse deep-sea-derived fungi from the South China Sea are a versatile reservoir of metabolites

with various antimicrobial activities and can be of potential use to modern medicine, industry, and agriculture. Works of characterizing bioactive metabolites from the potent fungal strains are underway.

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