Microb Ecol (2012) 64:617–627
DOI 10.1007/s00248-012-0050-x

FUNGAL MICROBIOLOGY

Diversity and Antimicrobial Activity of Culturable Fungi Isolated from Six Species of the South China Sea Gorgonians

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Received: 16 December 2011 / Accepted: 27 March 2012 / Published online: 15 April 2012

Abstract Fungi in gorgonians are now known to cause gorgonian diseases, but little attention has been paid to the nature of fungal communities associated with gorgonians. The diversity of culturable fungi associated with six species of healthy South China Sea gorgonians were investigated using a culture-dependent method followed by analysis of fungal internal transcribed spacer sequences. A total of 121 fungal isolates were recovered and identified using the Basic Local Alignment Search Tool search program. These belonged to 41 fungal species from 20 genera. Of these, 30 species and 12 genera are new reports for gorgonians, and the genera *Aspergillus* and *Penicillium* were the most diverse and common in the six gorgonian species. Comparison of the fungal communities in the six gorgonian species, together with results from previous relevant studies, indicated that different gorgonian species and the same gorgonian species living in different geographic locations had different fungal communities. The gorgonian *Dichotella gemmacea* harbored the most fungal species and isolates, while *Echinogorgia aurantiaca* had the least fungal diversity. Among the six media used for fungal isolation, potato glucose agar yielded the highest isolates (27 isolates), while glucose peptone starch agar had the best recoverability of fungal species (15 species). The antimicrobial activity of the 121 fungal isolates was tested against three marine bacteria and two marine gorgonian pathogenic fungi. A relatively high proportion (38 %) of fungal isolates displayed distinct antibacterial and antifungal activity, suggesting that the gorgonian-associated fungi may aid their hosts in protection against pathogens. This is the first report comparing the diversity of fungal communities among the South China Sea gorgonians. It contributes to our knowledge of gorgonian-associated fungi and further increases the pool of fungi available for natural bioactive product screening.

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Introduction

Coral reefs are widely recognized as one of the most diverse ecosystems, containing large and diverse microbial communities. Schimel et al. [1] reported that complex microbial communities were extremely important in coral ecosystems, with significant influence on these ecosystems. Corals provide a structurally and environmentally complex array of habitats supporting a broad microbial diversity that influences both host physiology and ultimately ecosystem processes [2]. Many studies have indicated that microbial communities occupy a range of niches on corals, from within the surface mucus layer [3, 4] to on and within the coral tissue layers [5, 6]. Furthermore, a variety of studies have suggested that coral-associated microorganisms may be saprophytic or pathogenic or may provide other important functions for corals [7, 8]. Microorganisms found in corals might, for instance, protect the host against pathogens [9, 10] and/or supply nutrients [11].

As part of our ongoing investigation of the diversity and antimicrobial activities of culturable microorganisms associated with South China Sea gorgonians, fungi attracted our attention because the fungal strains found exhibited relatively high diversity and antimicrobial activity. The fungal diversity of corals has been poorly understood [12]. Initially

there were few reports of fungi associated with scleractinian corals. Kendricks et al. [13] and Kohlmeyer et al. [14–17] isolated several endolithic fungi from reef-building corals and hydrozoans in Caribbean and Australian waters. Betis et al. [18] reported unknown fungal hyphae associated with two reef-building corals from the Pacific Ocean. Priess et al. [19] recovered an *Aspergillus*-like endolithic fungus from the reef-building corals in Mayotte and Moorea Islands.

Later reports have increasingly emphasized fungi associated with gorgonians. Some fungi in gorgonians are now known to cause gorgonian diseases. Since Smith et al. [20] initially reported that *Aspergillus sydowii* was the causal agent of the aspergillosis of gorgonians (*Gorgonia ventalina* and *Gorgonia flabellum*) in the Caribbean, a few studies have found that widespread diseases and mortality in Caribbean and West Indian gorgonians were also caused by the fungus *A. sydowii* [21–23]. In addition, a recent disease outbreak of *Aspergillus versicolor*, affecting 250 fallen gorgonians in Southeast Asia indicated that other fungal species were susceptible to gorgonian diseases [24]. However, some of the fungi that have been identified in diseased colonies and implicated in gorgonian diseases have also been found in healthy gorgonians [25]. Furthermore, some gorgonian diseases are believed to be caused by fungal communities and not by single pathogenic fungi [26]. These findings highlight our ignorance of the basic fungal ecology of gorgonians. Investigation of the nature of fungal communities in gorgonians is therefore vital for providing a general understanding of these microbes, their diversity, distribution, and ecology, as well as giving opportunities for further exploitation of novel fungi and bioactive natural products.

Our aim was to investigate and compare the diversity and antimicrobial activities of culturable fungi associated with six species of South China Sea gorgonians: *Dichotella gemmacea*, *Echinogorgia aurantiaca*, *Melitodes squamata*, *Muricella flexuosa*, *Subergorgia suberosa*, and *Verrucella umbraculum*. These six gorgonians are unevenly distributed in the South China Sea [27]. Six different isolation media were utilized for fungal isolation. The fungi phylogenetic diversity was analyzed based on internal transcribed spacer (ITS) sequences of the culturable fungi associated with the six gorgonians. In addition, the antimicrobial activities of the fungal isolates were primarily assayed using a double-layer technique with five indicator microorganisms including three marine bacteria and two gorgonian pathogenic fungi.

Materials and Methods

Sample Sites and Sample Collection

Healthy gorgonian samples including *D. gemmacea*, *E. Aurantiaca*, *M. squamata*, *M. flexuosa*, *S. suberosa*, and *V. umbraculum* were collected from the South China Sea, near Sanya City (18°11′ N, 109°25′ E), in August 2010 (Fig. 1). Three to five samples were obtained for each gorgonian species. Gorgonian species were identified by Dr. Hui Huang and Xiu-bao Li (South China Sea Institute of Oceanology, Chinese Academy of Sciences) [27]. Samples were transferred directly to sterile plastic bags without seawater and then sent to the laboratory as soon as possible, maintaining ice-cold conditions to enable fungal isolation.

Fungal Isolation and Comparison

Gorgonian samples (15–20 g) were rinsed three times in sterile seawater to remove transient and loosely attached microorganisms. The washed samples were then cut into 1 cm^3 pieces and thoroughly homogenized using a sterile mortar with the addition of 2 volumes of sterile seawater. A 10-fold dilution was made and 0.1 ml of the resulting solution was plated on agar. The inoculated plates were cultured at 28°C for 1–3 weeks 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 28°C for pure culture.

Six isolation media were used to isolate gorgonian-associated fungi; the compositions of the six media are shown in Table 1. To prohibit the growth of bacteria, 0.5 g L^{-1} benzylpenicillin and 0.03 gL^{-1} rose bengal were added to the basic media.

DNA Preparation, ITS Gene Sequencing, and Identification of Fungi

For DNA extraction, the selected fungal strains were inoculated into 7 ml centrifuge tubes containing 1 ml potato glucose medium and cultured at 28°C with shaking at 150 rpm for 7 days. Total genomic DNA was extracted from all selected strains, as described by Lai et al. [28]. From the genomic DNA, nearly full-length ITS sequences were amplified by polymerase chain reaction using primers ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGAT ATGC-3′). All primers were synthesized by SBS Genetech (China). The polymerase chain reaction mixtures consisted of 12.5 μl Taq premix (TakaRa, China), 0.25 μl (10 μM) of each primer (TakaRa, China), 0.75 μl DMSO, 10.25 μl water, and 1 μl template DNA. After denaturation at 94°C for 6 min, amplification was performed with 30 cycles of 45 s at 94°C, 45 s at 53°C, 2 min at 72°C and a final extension at 72°C for 10 min.

DNA sequencing of the selected fungal strains was carried out by Invitrogen (China). Sequences were corrected

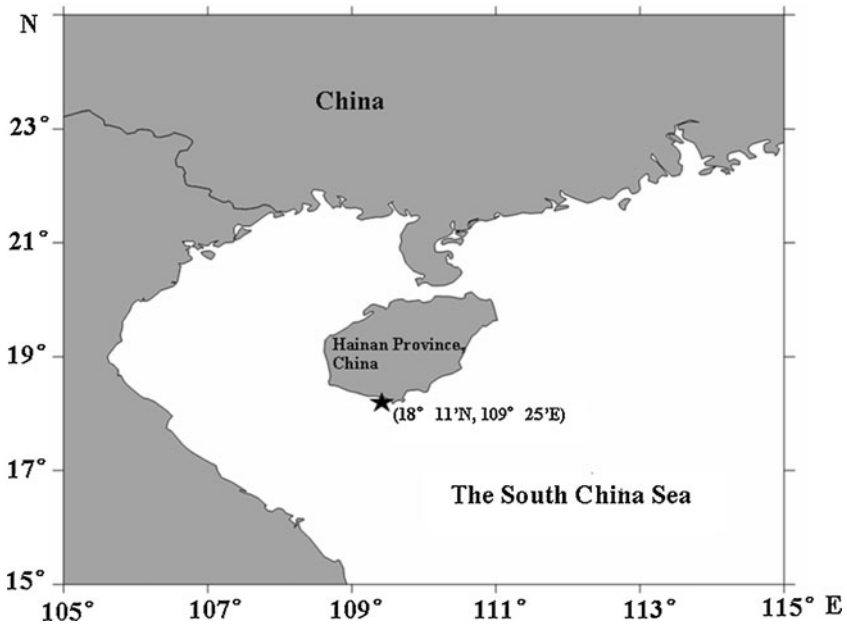

Figure 1 Map of the South China Sea and location of the sampling site

using Sequencher, and the most similar sequences in GenBank were found using Basic Local Alignment Search Tool (BLAST) searches. When the top three matching BLAST hits were from the same species and were ≥95 % similar to the query sequence, this species name was assigned to the selected isolate [29].

Determination of Antimicrobial Activity

The antimicrobial activities of isolated fungi were determined by a double-layer technique [30]. Selected strains were grown on potato glucose agar (PDA) (200 g diced potatoes, 20 g glucose, 20 g agar, and 1 l of seawater) that was allowed to grow for 5–14 days depending upon the growth rate of the various strains. The agar blocks containing the cells were then excised and placed on the assay plates spread with five indicator microorganisms including three marine bacteria (*Micrococcus luteus* UST950701-006, *Pseudoaltermonas piscida* UST010723-006, and *Vibrio alginolyticus* UST981130-062) [31, 32] and two marine gorgonian pathogenic fungi (*A. versicolor* SCSGAF0096 and *A. sydowii* SCSGAF0035) [20, 24]. The indicator bacteria were cultured in nutrient agar (5 g peptone, 3 g yeast extract, 20 g agar, and 1 l of seawater) at 37°C for 18 h, and the indicator fungi were cultured on PDA at 30°C for 60 h. The antimicrobial activity was expressed as the diameter of the growth inhibition zone (in milliliter). Each test was performed three times. All the indicator microorganisms were from the South China Sea Institute of Oceanology, Chinese Academy of Sciences.

Table 1 Compositions and concentrations of the isolation media

Media type	Compositions/g L^{-1}
GPA	Glucose 10, peptone 1, K_2HPO_4 1, $MgSO_4$ 0.25, agar 20
GPSA	Glucose 10, peptone 1, starch 10, K_2HPO_4 1, $MgSO_4$ 1, agar 20
GYMA	Glucose 4, yeast extract 4, malt extract 5, agar 20
GYPA	Glucose 5, yeast extract 1, peptone 5, agar 20
PDA	Potato 200, glucose 20, agar 20
SYA	Starch 10, yeast extract 5, agar 20

All media contained 30 gL^{-1} sea salt adjusted to pH 7.2

Data Analysis

A richness/species abundance coefficient Bray–Curtis was estimated based on presence/absence matrix of fungi isolated from the six gorgonian species [12]. And the Bray–Curtis analysis was performed through using SPSS for Window Soft (Version 11.5).

Nucleotide Sequence Accession Number

Fungal ITS sequences of the 84 representative isolates we obtained were deposited in GenBank under accession numbers JN850975–JN851058.

Results

Blast Searching and Phylogenetic Analyses Based on ITS Sequences

A total of 208 fungal strains were isolated from the six species of gorgonians from the South China Sea, and 127

isolates of these were selected for analysis. The ITS sequences of the 127 isolates were sequenced and compared with those in GenBank. The ITS sequences of 121 strains shared 95–100 % similarity with their closest NCBI relatives, the other six isolates shared 88–94 % similarity with their closest NCBI relatives. Furthermore, BLAST analysis revealed that the 121 fungal isolates belonged to 41 fungal species from 20 genera (Table 2). *Penicillium* was the most common genus with 43 isolates, while *Aspergillus* was the most diverse genus with 14 species. Most of the remaining genera occurred as singletons or doubletons.

Distribution and Comparison of Fungal Communities

The distribution and comparison of fungal species associated with the six gorgonian species are summarized in Table 2. Among the 41 identified fungal species, three species *A. sydowii*, *Penicillium citrinum*, and *Penicillium oxalicum* were the most abundant species, which could be isolated from almost all the six gorgonian species. And 20 species (such as *Acremonium polychrorum*, *Myrmecridium schulzeri*, and *Nigrospora oryzae*) were rare and occurred as singletons in the six gorgonian species. The gorgonian *D. gemmacea* harbored the most fungal species and isolates (19 out of the 41 species and 28 out of 121 isolates), followed by *S. suberosa* (13 species and 25 isolates), *V. umbraculum* (12 species and 22 isolates), *M. squamata* (12 species and 18 isolates), *M. flexuosa* (10 species and 11 isolates). And *E. aurantiaca* had the least fungal diversity with 8 species and 17 isolates. The Bray–Curtis analysis (Table 3) showed 53.8–90.5 % dissimilarity of fungal communities between each two gorgonian species, except for 100 % dissimilarity between the gorgonian *V. umbraculum* and the two gorgonians *M. squamata* and *E. aurantiaca*.

Effect of Isolation Media on the Recoverability of Gorgonian-Associated Fungi

Six different fungal isolation media were tested in this study. The results showed that fungi could be recovered on all the six media, but the total number and species of fungal isolates were different in the six media (Table 4). PDA yielded the highest isolates recovery with 27 isolates, while glucose yeast malt agar (GYMA) had the best recoverability of fungal species (15 species). The species *A. sydowii*, *P. citrinum*, and *P. oxalicum* could be isolated on all the six media. *Aspergillus niger*, *Aspergillus tubingensis*, and *Fusarium proliferatum* were only isolated from one medium, PDA and starch yeast agar (SYA), respectively. *Aspergillus fumigatus* was only isolated from GPA and SYA, and *Debaryomyces subglobosus* was only isolated from GPSA and GYPA. The rest of the fungal species were isolated in very small numbers and cannot be concluded to be media-specific.

Distribution of Fungi with Antimicrobial Activity

All of the 121 fungal isolates were tested against three marine bacteria and two marine gorgonian pathogenic fungi to examine their spectrum of antimicrobial activity. Forty-six strains (38 %) displayed antimicrobial activity against at least one bacterium or fungus (Table 5). The numbers of antimicrobial strains isolated from gorgonians *D. gemmacea*, *E. aurantiaca*, *M. squamata*, *M. flexuosa*, *S. suberosa*, and *V. umbraculum* were 11, 11, 7, 6, 5, and 6, respectively. Most antimicrobial strains exhibited moderate or strong activity against the pathogenic fungi *A. versicolor* or *A. sydowii*. A few strains (such as SCSGAF0097 and 0124) displayed relatively high antimicrobial activity against four indicator microorganisms. Two strains (SCSGAF0028 and 0053) exhibited moderate or strong antimicrobial activity against all five indicator microorganisms. Among the bioactive isolates, *Aspergillus* and *Penicillium* isolates had the highest proportions of bioactivity; 33 and 30 %, respectively.

Discussion

Fungal Diversity in Gorgonians

The ecological roles of fungi in marine ecosystems in general and in marine gorgonians in particular are still vague [12, 33]. There are few reports of fungi from gorgonians. The diversity and antimicrobial activity of culturable fungi isolated from six species of healthy gorgonians (*D. gemmacea*, *E. aurantiaca*, *M. squamata*, *M. flexuosa*, *S. suberosa*, and *V. umbraculum*) were here investigated, collected in shallow water of the South China Sea. At least 20 fungal genera and 41 species isolated from the six gorgonians were identified. Of these, 12 genera and 30 species are new reports for marine gorgonians (Table 2). Our results, combined with those of others, including Koh et al. [25], Toledo-Hernandez et al. [12, 29], Wang et al. [34], and Zuluaga-Montero et al. [26], have shown that gorgonians have large and diverse fungal communities. Forty-three fungal genera and 120 species had previously been identified from 16 gorgonian species all over the world, using culture-dependent techniques. Of these 43 fungal genera, *Aspergillus* and *Penicillium* were the most diverse and common in gorgonians. These could be isolated from 12 of 16 healthy gorgonian species [12, 25, 26, 29, 34]. *Aspergillus* and *Penicillium* have also been frequently found in scleractinian corals [19] and other marine invertebrates such as sponges [35, 36]. It appears that these two fungal genera are successful at colonizing different hosts and are ubiquitous in many marine organisms.

Despite the numerous fungal species successfully isolated from different species of gorgonians by culture-dependent

Table 2 Fungi isolated from six gorgonian species as identified by ITS sequences

Fungal species (the representative isolates' accession numbers in GenBank)	Number of fungal isolates					
	DG	EA	MS	MF	SS	VU
Acremonium polychrorum (JN851031)a						1
Aspergillus carneus (JN851024)a			1			
A. flavus (JN850985)	1					
A. fumigatus (JN850983)a	1	1			1	
A. gracilis (JN850991)a	1					
A. insulicola (JN851021)a			2			
A. niger (JN851043)		2				
A. nomius (JN850982)a	1					
A. ochraceopetaliformis (JN850980)	1		1			
A. penicillioides (JN850993)a	1					
A. sclertoiorum (JN850994)a	1			1		1
A. sydowii (JN850979)	3	1	3	1	3	2
A. terreus (JN851009)		1	1	1	1	
A. tubingensis (JN851045)a		1			1	
A. versicolor (JN851010)			1		4	1
Cladosporium cladosporioides (JN850986)	1					
C. sphaerospermum (JN851005)				1		
C. uredinicola (JN851037)a						1
Debaryomyces subglobosus (JN850992)a	1				1	1
Fusarium chlamydosporum (JN851018)a			1			
F. proliferatum (JN850990)a	2					
Gibberella intermedia (JN850990)a						1
Isaria tenuipes (JN851008)a				1		
Lecanicillium fusisporum (JN850976)a	1					
Massarina corticola (JN851057)a				1	1	
Microsphaeropsis arundinis (JN851000)a			3	2		2
Myrmecridium schulzeri (JN851038)a		1				
Myrothecium inundatum (JN851023)a			2			
Nigrospora oryzae (JN851032)a						1
Paecilomyces variotii (JN850996)a	1					
Penicillium citrinum (JN850977)	5	6	1	1	5	1
P. oxalicum (JN850988)	3	4		1	4	8
P. paxilli (JN851050)a					1	
P. radicum (JN851019)a			1			
P. steckii (JN850984)	1					
P. verruculosum (JN851016)a			1			
Peyronellaea glomerata (JN850981)a	1					
Phoma putaminum (JN851036)a						2
Purpureocillium lilacinus (JN850995)a	1				1	
Ramichloridium apiculatum (JN850989)a	1				1	
Tilletiopsis albescens (JN850999)a				1	1	
Total number of fungal isolates	28	17	18	11	25	22

Species marked by a letter (a) are new reports for marine gorgonians

DG Dichotella gemmacea, *EA Echinogorgia aurantiaca*, *MS Melitodes squamata*, *MF Muricella flexuosa*, *SS Subergorgia suberosa*, *VU Verrucella umbraculum*

Table 3 The dissimilarity of fungal community associated with different gorgonian species

	1	2	3	4	5	6	7	8
1	/	89.5 %	90.5 %	90.5 %	66.7 %	89.5 %	100 %	100 %
2	89.5	/	80	80	53.8	100	100	100
3	90.5	80	/	83.3	60	80	100	100
4	90.5	80	83.3	/	86.7	100	100	100
5	66.7	53.8	60	86.7	/	53.8	100	100
6	89.5	100	80	100	53.8	/	100	100
7	100	100	100	100	100	100	/	66.7
8	100	100	100	100	100	100	66.7	/

1–6: gorgonian species from the South China Sea; 7 and 8: gorgonian species from Singapore (Kol et al. 2000)

1 Dichotella gemmacea, *2 Echinogorgia aurantiaca*, *3 Muricella flexuosa*, *4 Melitodes squamata*, *5 Subergorgia suberosa*, *6 Verrucella umbraculum*, *7 D. gemmacea*, *8 Subergorgia suberosa* (gorgonian species)

techniques, greater fungal diversity is likely to be revealed by combining the culture-dependent approach with a culture-independent method (such as direct amplification of DNA from gorgonians). 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 of complementary approaches to assess the fungal assemblage within unusual environments [37–40]. Furthermore, greater fungal diversity would probably be obtained by a rigorous sampling strategy. Toledo-Hernandez et al. reported that when sampling the fungal communities in gorgonians, four points should be considered: sample size, fragment size—use of smaller tissue fragments increases the number and frequency of fungi isolated and minimizes damage to the gorgonians, the method of tissue processing, and tissue collecting influences the results [12].

Comparison of Fungal Communities in Gorgonians

The result of Bray–Curtis analysis (Table 3) displayed that the fungal communities (number and species of fungal isolates) associated with the six different species of South China Sea gorgonians varied significantly. The same result was found in 10 different gorgonian species collected from Raffles Lighthouse in Singapore [24]. Gray et al. [41] reported that abundant and diverse bacterial communities were found in different gorgonian species. These phenomena are probably due to the chances of microbial communities being trapped in gorgonians or the varied morphological structures of the gorgonians.

Comparing the fungal diversity in these same gorgonian species (*D. gemmacea* and *S. suberosa*) from the South China Sea and Singapore, we found that the fungal species and isolates from gorgonian *D. gemmacea* in the South China Sea (19 fungal species and 28 isolates, Table 2) were significantly higher than that from the same gorgonian species in Singapore (7 fungal species and 17 isolates) [25]; while the fungal species and isolates of gorgonian *S. suberosa* in the South China Sea (13 fungal species and 25 isolates, Table 2) were relatively less than that from the same gorgonian species in Singapore (15 fungal species and 45 isolates) [25]. In addition, none of the fungal species associated with the gorgonians *D. gemmacea* and *S. suberosa* in Singapore [25] could be recovered from these same gorgonian species collected in the South China Sea. Further Bray–Curtis analysis (Table 3) also showed 100 % dissimilarity of fungal communities between these same species (*D. gemmacea* and *S. suberosa*) from the South China Sea and Singapore. These suggest that the same gorgonian species living in different locations harbor significantly variable fungal communities. This is consistent with previous opinions that the fungal consortia of the same sponge species from two distant regions were quite different [37], and the bacterial communities for the same species of three gorgonians collected at different sites were dramatically different [41].

Moreover, the dissimilarity of fungal communities between these same species (*D. gemmacea* and *S. suberosa*) from the South China Sea and Singapore was higher than that between the two gorgonian species (*D. gemmacea* and *S. suberosa*) from the South China Sea and the others gorgonian species from the same location (Table 3). This suggests that the dissimilarity of fungal communities in gorgonians perhaps is more dependent on the surrounding environment (the geographical area) than on the gorgonian species.

Effect of Isolation Media on Fungal Diversity in Gorgonians

The data (Table 4) indicated clearly that different media yielded different numbers and species of fungal isolates. Although most fungal species could be isolated on PDA or GYMA, *A. tubingensis* and *F. proliferatum* were only

Table 4 Differences of fungal isolates using six different media

Fungal species	Number of fungal isolates					
	GPA	GPSA	GYMA	GYPA	PDA	SYA
Acremonium polychrorum			1			
Aspergillus carneus			1			
A. flavus	1					
A. fumigatus	2					1
A. gracilis						1
A. insulicola			1			1
A. niger					2	
A. nomius					1	
A. ochraceopetaliformis		1			1	
A. penicillioides						1
A. sclertoiorum			2			1
A. sydowii	2	2	2	1	3	3
A. terreus		2	1			1
A. tubingensis						2
A. versicolor	1	1	1		3	
Cladosporium cladosporioides	1					
C. sphaerospermum			1			
C. uredinicola			1			
Debaryomyces subglobosus		1		2		
Fusarium chlamydosporum				1		
F. proliferatum						2
Gibberella intermedia					1	
Isaria tenuipes	1					
Lecanicillium fusisporum					1	
Massarina corticola	1		1			
Microsphaeropsis arundinis	1	2	1	3		
Myrmecridium schulzeri						1
Myrothecium inundatum	1	1				
Nigrospora oryzae			1			
Paecilomyces variotii					1	
Penicillium citrinum	3	1	1	3	7	4
P. oxalicum	4	3	4	2	5	2
P. paxilli		1				
P. radicum						1
P. steckii				1		
P. verruculosum		1				
Peyronellaea glomerata		1				
Phoma putaminum	1			1		
Purpureocillium lilacinus		1			1	
Ramichloridium apiculatum			1		1	
Tilletiopsis albescens		1		1		
Total number of fungal isolates/species	19/12	19/14	20/15	15/9	27/12	21/13

recovered on SYA, and *D. subglobosus* was only isolated from GPSA and GYPA. Kol et al. [25] reported that a combination of only two media (PDA and GYA) would be sufficient for isolating fungi from gorgonians. However, a well-designed isolation protocol with multiple isolation media was essential for isolating diverse and abundant fungi from different species of gorgonians in this study.

Table 5 Antibacterial and antifungal activities of culturable fungi isolated from the six species of South China Sea gorgonians

Gorgonian species	Fungal isolates	Fungal species	Diameter of the growth inhibition zone (mm)				
			ML	PP	VA	AS	AV
Dichotella gemmacea	SCSGAF0010	*Aspergillus ochraceopetaliformis*					12.5
	SCSGAF0029	*Aspergillus gracilis*				10.0	
	SCSGAF0031	*Aspergillus penicillioides*				14.0	13.0
	SCSGAF0034	*Aspergillus sclertoiorum*		8.1	13.0		12.1
	SCSGAF0032	*Fusarium proliferatum*				17.0	
	SCSGAF0002	*Lecanicillium fusisporum*				10.8	11.2
	SCSGAF0038	*Paecilomyces variotii*					12.5
	SCSGAF0003	*Penicillium citrinum*				18.0	17.6
	SCSGAF0004	*Penicillium citrinum*				15.5	13.0
	SCSGAF0005	*Penicillium citrinum*					15.5
	SCSGAF0036	*Purpureocillium lilacinus*				19.5	14.5
Echinogorgia aurantiaca	SCSGAF0137	*Aspergillus fumigatus*	12.0	19.0			
	SCSGAF0148	*Aspergillus fumigatus*				15.8	16.5
	SCSGAF0145	*Aspergillus niger*				12.4	
	SCSGAF0146	*Aspergillus niger*					9.0
	SCSGAF0162	*Aspergillus terreus*	8.7				
	SCSGAF0179	*Aspergillus terreus*					12.2
	SCSGAF0132	*Penicillium citrinum*					
	SCSGAF0141	*Penicillium citrinum*	13.5			12.7	
	SCSGAF0167	*Penicillium citrinum*				17.6	18.3
	SCSGA 0171	*Penicillium citrinum*				12.8	11.3
	SCSGAF0135	*Myrmecridium schulzeri*	11.9	16.2	17.0		
Melitodes squamata	SCSGAF0071	*Aspergillus flocculosus*	13.0			15.2	12.0
	SCSGAF0097	*Aspergillus ochraceopetaliformis*	15.0	12.1		14.5	14.0
	SCSGAF0080	*Microsphaeropsis arundinis*				13.0	
	SCSGAF0094	*Microsphaeropsis arundinis*				12.5	
	SCSGAF0082	*Penicillium verruculosum*				15.1	15.0
	SCSGAF0091	*Penicillium radicum*		14.1			
	SCSGAF0092	*Penicillium citrinum*				13.5	
Muricella flexuosa	SCSGAF0053	*Aspergillus sclertoiorum*	13.0	21.0	24.7	12.0	12.0
	SCSGAF0059	*Aspergillus terreus*	12.0	14.0			
	SCSGAF0058	*Isaria tenuipes*				10.0	13.0
	SCSGAF0050	*Microsphaeropsis arundinis*				12.1	12.0
	SCSGAF0052	*Penicillium citrinum*	11.2				
	SCSGAF0023	*Penicillium oxalicum*				12.0	
Subergorgia suberosa	SCSGAF0195	*Debaryomyces subglobosus*		9.0			13.0
	SCSGAF0178	*Penicillium citrinum*					11.8
	SCSGAF0192	*Penicillium citrinum*					9.1
	SCSGAF0182	*Purpureocillium lilacinus*				12.5	
	SCSGAF0174	*Tilletiopsis albescens*				17.5	15.0
Verrucella umbraculum	SCSGAF0110	*Acremonium polychrorum*					9.0
	SCSGAF0028	*Gibberella intermedia*	12.0	15.6	10.0	12.1	13.8
	SCSGAF0124	*Aspergillus sclertoiorum*	12.0	25.0	15.0		12.0
	SCSGAF0129	*Cladosporium uredinicola*			14.3		
	SCSGAF0101	*Microsphaeropsis arundinis*			0		12.0
	SCSGAF0111	*Nigrospora oryzae*	12.4		9.1	17.6	16.5

Each test was performed three times. Relatively high antimicrobial activity: zone of inhibition greater than 12 mm

ML Micrococcus luteus, *PP Pseudoaltermonas piscida*, *VV Vibrio alginolyticus* (indicator bacteria), *AV Aspergillus versicolor*, *AS Asperillus sydowii* (indicator fungi)

Potential Antimicrobial Properties of Gorgonian-Associated Fungi

Antimicrobial activities of 121 culturable fungal isolates associated with the six species of South China Sea gorgonians were tested against three marine bacteria and two marine gorgonian pathogenic fungi. After preliminary screening, a relatively high proportion (38 %) of the 121 fungal isolates displayed distinct antibacterial and antifungal activities, suggesting that gorgonian-associated fungi can fend off or develop resistance to certain microbial diseases of gorgonians. This suggestion is supported by the increasing number of findings of new natural products from gorgonian-associated fungi that exhibit antibacterial, antifungal, antifouling, or antimycobacterial activity [42–45]. Gorgonian-associated fungi should be an important source of new, biologically active, natural products.

We found that some isolates belonging to genera such as *Aspergillus* (SCSGAF0053, 0071, 0097 and 0124), *Gibberella* (SCSGAF0028), and *Nigrospora* (SCSGAF0111) exhibited relatively high antibacterial and antifungal activities. While the genera *Cladosporium* (SCSGAF0129) and *Myrmecridium* (SCSGAF0135) displayed only relatively high antibacterial activity, and the genera *Fusarium* (SCSGAF0032), *Isaria* (SCSGAF0058), *Lecanicillium* (SCSGAF0002), *Microsphaeropsis* (SCSGAF0050, 0080, 0094, and 0101), *Purpureocillium* (SCSGAF0036 and 0182), and *Tilletiopsis* (SCSGAF0174) displayed only relatively high antifungal activity (Table 5). Members of the genus *Aspergillus* are known to be major contributors to bioactive metabolites of fungal origin [30, 46]. Studies have indicated that gorgonian-associated *Aspergillus* species can produce many bioactive compounds. Aspergilone A, a benzylazaphilone with an unprecedented carbon skeleton from the gorgonian-derived fungus *Aspergillus* sp., for example, not only exhibited in vitro selective cytotoxicity but also showed potent antifouling activity [42]. Three of five sesquiterpenoids from another gorgonian-derived *Aspergillus sp*. exhibited antibacterial activity [45]. Members of the genus *Penicillium* are known for production of antibacterial compounds [40]. However, few *Penicillium* strains in our study exhibited antibacterial activity, and most of the bioactive *Penicillium* strains (SCSGAF0003, 0082 and 0167) displayed relatively high activity against the indicator fungi. In order to better adapt to different ecosystems, marine gorgonian-associated fungi may evolve metabolic abilities enabling them to produce a variety of bioactive compounds [40].

Conclusions

We investigated the diversity of fungal species in six gorgonian species from the South China Sea, using culture-dependent methods together with analysis of ITS sequences. All six species of gorgonians contained abundant and diverse fungal species, thus expanding our knowledge of the fungal communities associated with the gorgonians. Comparable analysis indicated that different gorgonian species had significantly variable fungal communities. To the best of our knowledge, this is the first report comparing the diversity of fungal species among the South China Sea gorgonian species. Further comparable analysis indicated that these same gorgonian species (*D. gemmacea* and *S. suberosa*) living in the South China Sea and Singapore harbor significantly variable fungal communities. And the dissimilarity of fungal communities between these same species (*D. gemmacea* and *S. suberosa*) from the South China Sea and Singapore was higher than that between the two gorgonian species (*D. gemmacea* and *S. suberosa*) from the South China Sea and the others gorgonian species from the same location. This suggests that the dissimilarity of fungal communities in gorgonians perhaps is more dependent on the surrounding environment (the geographical area) than on the gorgonian species. The antimicrobial activities of the fungal isolates were tested against three marine bacteria and two marine gorgonian pathogenic fungi. A relatively high proportion (38 %) of fungal isolates exhibited distinct antibacterial and antifungal activities. And about 20 strains belonged to 12 fungal genera displayed relatively high antibacterial or antifungal activities. These suggest that gorgonian-associated fungi may aid their hosts in protection against pathogens. Our study contributes to knowledge of gorgonian-associated fungi and further increases the pool of fungi available for natural bioactive product screening.

Acknowledgments The authors are grateful to the National Basic Research Program of China (grant 2010CB833803), the National Natural Science Foundation of China and Research Grants Council of Hong Kong (NSFC/RGC) Program (grant 40931160435), the CAS/SAFEA International Partnership Program for Creative Research Teams (grant KZCX2-YW-T001), the Knowledge Innovation Program of Chinese Academy of Science (grant KSCX2-EW-G-12B), the National Science Foundation of China (grant 40976090), National Science and Technology Pillar Program of China (grant 2011BAE06B04-03), and Guangdong Natural Science Foundation of China (grant S2011040000144) for financial support. The authors are grateful to Dr. Hui Huang and Xiu-Bao Li (South China Sea Institute of Oceanology, Chinese Academy of Sciences) for their kindness in identifying the gorgonian samples.

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