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ORIGINAL ARTICLE

Recovery and Phylogenetic Diversity of Culturable Fungi Associated with Marine Sponges *Clathrina luteoculcitella* and *Holoxea* sp. in the South China Sea

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Abstract Sponge-associated fungi represent an important source of marine natural products, but little is known about the fungal diversity and the relationship of sponge–fungal association, especially no research on the fungal diversity in the South China Sea sponge has been reported. In this study, a total of 111 cultivable fungi strains were isolated from two South China Sea sponges *Clathrina luteoculcitella* and *Holoxea* sp. using eight different media. Thirty-two independent representatives were selected for analysis of phylogenetic diversity according to ARDRA and morphological characteristics. The culturable fungal communities consisted of at least 17 genera within ten taxonomic orders of two phyla (nine orders of the phylum *Ascomycota* and one order of the phylum *Basidiomycota*) including some potential novel marine fungi. Particularly, eight genera of *Apiospora, Botryosphaeria*, *Davidiella*, *Didymocrea*, *Lentomitella*, *Marasmius*, *Pestalotiopsis,* and *Rhizomucor* were isolated from sponge for the first time. Sponge *C. luteoculcitella* has greater culturable fungal diversity than sponge *Holoxea* sp. Five genera of *Aspergillus*, *Davidiella*, *Fusarium*, *Paecilomyces,* and *Penicillium* were isolated from both sponges, while 12 genera of *Apiospora*, *Botryosphaeria*, *Candida*, *Marasmius*, *Cladosporium*, *Didymocrea*, *Hypocrea*, *Lentomitella*, *Nigrospora*, *Pestalotiopsis*, *Rhizomucor,* and *Scopulariopsis* were isolated from sponge *C. luteoculcitella* only. Order *Eurotiales* especially genera *Penicillium*, *Aspergillus*, and order *Hypocreales* represented the dominant culturable fungi in these two South China Sea sponges. *Nigrospora oryzae* strain PF18 isolated from sponge *C. luteoculcitella* showed a strong and broad spectrum antimicrobial activities suggesting the potential for antimicrobial compounds production.

Keywords Marine sponge · *Clathrina luteoculcitella* · *Holoxea* sp · Fungus · Phylogenetic diversity · Antimicrobial activity

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Introduction

Marine fungi have been widely found in marine habitats, such as seawater, sediment, marine animals and plants, and are suggested to play an important ecological role in recycling nutrients, decomposition of dead plant and animal tissues, and certain species of marine fungi are pathogenic to marine plants and animals (Bugni and Ireland 2004; Hyde et al. 2000; Raghukumar 2008). But, the ecological role of marine fungi is rarely understood compared with terrestrial fungi. Though many marine fungi have been isolated from different marine habitats for investigation of natural products (Saleem et al. 2007), marine fungi remain the most underexplored group in the marine environment (Li and Wang 2009; Wang et al. 2008). In some cases, marine fungi form symbiotic relationships with other organisms such as sponges, algae, corals, and calcareous tubes of mollusks, but the function of these associations are rarely known (Hyde et al. 2000; Maldonado et al. 2005; Rot et al. 2006).

The unique physicochemical conditions of the marine environment confer marine fungi with special properties

that could be exploited in biotechnology. For instance, a growing number of compounds (e.g., polyketides and their isoprene hybrids, terpenoides, alkaloids, peptides, cerebroside analogs) with antimicrobial, antiprotozoal, and cytotoxic activities have been isolated from marine fungi (Bhadury et al. 2006; Bugni and Ireland 2004; Saleem et al. 2007). Marine sponges are known to harbor diverse prokaryotic and eukaryotic microbes including archaea, bacteria, cyanobacteria, microalgae, fungi, and probably also protozoa and represent one of the important resources for natural products (Blunt et al. 2009; Osinga et al. 2001). Fungi associated with sponges display diverse biological activities and represent the single most prolific source of marine fungi-derived diverse bioactive compounds accounting for the overall highest number of novel marine metabolites to date (Bhadury et al. 2006; Bugni and Ireland 2004; Proksch et al. 2003b). Many "sponge" products might be produced by microorganisms that are associated with the sponge (König et al. 2005; Piel 2009), thus, the revealed biological diversity of sponge-associated fungi will guard us to study the chemical diversity of metabolites of sponge-associated fungi with the aim to find new natural products and new genes with pharmaceutical and industrial potentials. Investigation on the composition of sponge–fungal communities will lay a basis for the revelation of ecological function of sponge–fungi association. Prokaryotic microbial communities associated with sponges have been well studied by both cultivation-dependent and cultivation-independent approaches (Taylor et al. 2007; Hentschel et al. 2006), whereas eukaryotic microbial communities are rarely investigated. Studies on sponge-associated fungi have typically concentrated on natural product chemistry (Amagata et al. 2006; Bugni and Ireland 2004; Lin et al. 2003; Proksch et al. 2003a; Saleem et al. 2007; Wang et al. 2002), while investigations on biology of sponge-associated fungi have been started recently and are rare (Gao et al. 2008; Baker et al. 2009; Li and Wang 2009; Menezes et al. 2009; Wang et al. 2008). It is estimated that there are several thousands of sponge species in the China Sea (Zhang et al. 2003) but no research on the diversity of fungi associated with South China Sea sponges has been reported.

The aim of this study was to reveal the diversity of cultivable fungal diversity associated with two South China Sea sponges *Clathrina luteoculcitella* and *Holoxea* sp. and find some sponge-derived fungi with pharmaceutical potential. Eight different media were used to recover fungi from sponges and the phylogenetic diversity was revealed based on 18S rRNA gene and internal transcribed spacer (ITS) region sequences and amplified rDNA restriction analysis (ARDRA). Meanwhile, the antimicrobial activity bioassay was carried out to evaluate the bioactive potential of the isolated fungi.

Materials and Methods

Sponge Collection

Marine sponge *C. luteoculcitella* and *Holoxea* sp. were collected nearby Yongxing Island (112°20′E, 16°50′N) in the South China Sea at depth of ca. 20 m. Latex gloves were worn during collection. Sponge was transferred directly to Zip-lock bags containing seawater to prevent contact of sponge tissue with air. The samples were transported to the laboratory and processed immediately for fungal isolation. Alternatively, sponge was stored at −40°C for future use.

Isolation of Sponge-Associated Fungi

To get rid of nonspecific fungal propagules from seawater column on sponge surface and inner cavity, sponge was rinsed three times with sterile artificial seawater (ASW; Li and Liu 2006). The inner tissue (1.0 g) was taken out with a scalpel and then homogenized using a blender containing 10 ml of sterile ASW. The resulting homogenate was diluted with sterile ASW at three dilutions (original, 1:10, 1:100), each dilution was mixed using a vortex mixer for 30 min. For fungal isolation, 100 μl of each dilution was plated onto eight isolating media (Table 1) in triplicate. All the media were prepared with ASW and added with ampicillin and streptomycin (100 mg/ml each). The plates were incubated at 28°C for 1–3 weeks until the morphology of fungi could be distinguished. Single isolate was transferred onto a new Martin agar plates containing streptomycin and incubated at room temperature for pure culture isolation.

Table 1 Composition of media used for sponge-associated fungal isolation

Medium	Composition (L^{-1})[a]
Martin	Glucose 10 g, peptone 5 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 20 g
Sabouraud	Peptone 10 g, maltose 40 g, agar 20 g
Czapek	Sucrose 30 g, $NaNO_3$ 3 g,K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4$ 0.5 g,$FeSO_4$ 0.01 g, agar 20 g
Malt	Malt extract 20 g, agar 20 g
MYPG	Malt extract 3 g, yeast extract 3 g, peptone 5 g, glucose 20 g, NH_4NO_2 5 g, K_2HPO_4 1 g, $MgSO_4$ 0.5 g, $FeCl_3 \cdot 6H_2O$ 0.2 g, agar 20 g
2216	Marine broth 2216 plus 20 g agar
Soya germ	Soya germ 100 g, glucose 50 g, agar 20 g
Gause I	Starch 20 g, KNO_3 1.0 g, K_3HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NaCl 0.5 g, $FeSO_4$ 0.01 g, agar 20 g

[a] All media were prepared with ASW (artificial seawater; Li and Liu 2006)

Genomic DNA Extraction

Morphological traits (e.g., morphology and color of spore and mycelia) were examined to exclude replicates of fungal isolates. Before the extraction of genomic DNA, fungal isolate was cultured in Martin broth at 180 rpm, 28°C for 5–7 days. Fungal DNA was extracted using a modified method based on Li et al (2006) and Van Burik et al. (1998). Mycelium were picked out to a mortar, then 600 μl of CTAB lysis buffer (1% CTAB, 1% Triton X-100, 1% SDS, 1.4 M NaCl, 100 mM Tris, 20 mM EDTA, 2% PVP) was added to grind at 65°C. The mycelial mixture was transferred to a 1.5-ml Eppendorf tube and water-bathed at 65°C for 30 min, then an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. After brief mixing and centrifugation (12,000×*g*, 15 min, 4°C), the aqueous phase was transferred to a new microtube and extracted with chloroform/isoamyl alcohol (24:1). Finally, DNA was precipitated by adding equal volume of isopropanol at −20°C. A DNA pellet was collected by centrifugation (12,000×*g*, 15 min), washed with 75% ethanol, and resuspended in sterile water. RNA was removed by adding 2 μl of RNase A (10 mg/ml) (Invitrogen) at 60°C for 10 min.

PCR Amplification and ARDRA

The resulting genomic DNA was used as template to amplify the fungal 18S rRNA gene fragment (ca.760 bp) using the primers nu-SSU-0817(5′-TTAGCATGGAA TAATRRAATAGGA-3′) and nu-SSU-1536 (5′-ATTG CAATGCYCTATCCCCA-3′; Borneman and Hartin 2000). The PCR reaction mixture contained 2.5 μl of 10× Taq Buffer with $(NH_4)_2SO_4$, and 3.5 μl 25 mM $MgCl_2$ (Fermentas EP0402), 2 μl of 2 mM dNTPs (Fermentas), 2 μl of each primer (10 pmol), 1 μl of fungal DNA, 0.25 μl of Taq DNA polymerase (5 U μl^{-1}, Fermentas), and 14.75 μl of ddH_2O. PCR was carried out as follows: initial denaturation (94°C for 5 min), 35 cycles of denaturation (94°C for 30 s), annealing (56°C for 30 s) and elongation (72°C for 1 min), a final elongation at 72°C for 10 min.

In the case of fungal isolate, for which the PCR amplification of 18S rRNA gene was not successful, primer set ITS1 (5′-TCCGTAGGTGAACCTGCG-3′)/ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) was used to amplify the ITS region (White et al. 1990). PCR was carried out as follows: initial denaturation (94°Cfor 5 min), 35 cycles of denaturation (94°C for 50 s), annealing (55°C for 50 s) and elongation (72°C for 1 min), a final elongation at 72°C for 10 min.

After PCR amplification, restriction enzymes Taq I, Hinf I, and Hae III were used to accomplish ARDRA, PCR products which belonged to different patterns were purified using QIA quick PCR Purification Kit (Qiagen) and directly analyzed.

Sequencing of Representative Fungi and Phylogenetic Analysis

Sequencing analysis was performed on an ABI 3730 XL (Applied Biosystems) automated sequencer using the nu-SSU-0817/nu-SSU-1536 and ITS1/ITS4 primers. Sequences of fungal 18S rRNA genes and ITS region sequences were compared to those in GenBank database by BLAST algorithm to identify sequences similarity. 18S rDNA and ITS sequences were aligned using Clustal X software, and the phylogenetic tree was generated using the neighbor-joining algorithms in Mega II software.

Bioassay of Antimicrobial Activity of Fungal Isolates

Fungal isolates were cultured in Martin broth at 28°C, 180 rpm for 14 days. Mycelial mixture was centrifuged at 12,000×*g* for 15 min and the mycelial deposit was discarded. 200 μl mycelial solution of each isolate was transferred into one Oxford cup to accomplish antimicrobial test in triplicate using six indicator microorganisms: *Candida albicans*, *Aspergillus niger*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas Fluorescens,* and *Escherichia coli*.

Nucleotide Sequence Accession Number

Fungal 18S rRNA gene sequences obtained in this study were deposited in GenBank under accession numbers FJ941851-FJ941881. Fungal ITS-rDNA sequence was deposited in GenBank under accession number FJ941882.

Results

Phylogenetic Diversity of Culturable Fungi Associated with Sponge *C. luteoculcitella*

A total of 62 fungal isolates were isolated from sponge *C. luteoculcitella*. According to the morphological characteristics and ARDRA, twenty-four representative isolates were selected for sequencing. According to BLAST and phylogenetic analysis based on 18S rRNA gene sequences, among the 24 representative isolates, twenty-two were grouped into the phylum *Ascomycota* including nine taxonomic orders: *Boliniales*, *Capnodiales*, *Eurotiales*, *Hypocreales*, *Microascales*, *Mucorales*, *Pleosporales*, *Saccharomycetales,* and *Xylariales*. Particularly, the PCR amplification of 18S rRNA gene of isolate PF24 was unsuccessful indicating that it was a definitely different one from other isolates. Finally, isolate PF24 was grouped in genus *Botryosphaeria* of order *Pleosporales* based on ITS sequence (Table 2). Only one representative isolate,

Marasmius alliaceus strain PF19 in the phylum *Basidiomycota*, was identified based on 18S rRNA gene sequence (Table 2, Fig. 1).

In total, diverse fungi of 17 genera in ten orders, two phyla: *Apiospora*, *Aspergillus*, *Botryosphaeria*, *Candida*, *Marasmius*, *Cladosporium*, *Davidiella*, *Didymocrea*, *Fusarium*, *Hypocrea*, *Lentomitella*, *Nigrospora*, *Penicillium*, *Pestalotiopsis*, *Paecilomyces*, *Rhizomucor,* and *Scopulariopsis* were isolated from sponge *C. luteoculcitella* as well as one unidentified (isolate PF01). *Eurotiales* fungi dominated the culturable fungi in sponge *C. luteoculcitella* accounting for 33.3% of the 24 representatives fungi.

Phylogenetic Diversity of Culturable Fungi Associated with Sponge *Holoxea* sp

In the case of sponge *Holoxea* sp., eight representative isolates were selected from 49 fungal isolates for sequencing using the same approach as sponge *C. luteoculcitella*. The cultured fungal diversity included five genera of *Aspergillus*, *Davidiella*, *Fusarium*, *Paecilomyces*, *Penicillium,* and one unidentified (isolate TS03) in three orders of *Capnodiales*, *Eurotiales,* and *Hypocreales* in the phylum *Ascomycota* (Fig. 2). As sponge *C. luteoculcitella, Eurotiales* fungi represented the dominant cultivable fungi in

Table 2 Phylogenetic affiliations of cultivable fungi associated with sponges *C. luteoculcitella* and *Holoxea* sp

Isolate ID[a]	Taxon	Accession number	Closest identified relative[b]	Identity (%)	Frequency isolated
PF01	*Eurotiales*	FJ941851	*Ascomycota* sp. (EU887758)	99	8
PF02	*Xylariales*	FJ941852	*Pestalotiopsis guepinii* (EU375526)	99	1
PF03	*Eurotiales*	FJ941872	*Aspergillus* sp. (EU853156)	99	7
PF04	*Hypocreales*	FJ941853	*Hypocrea koningii* (EU722404)	100	2
PF05	*Hypocreales*	FJ941854	*Paecilomyces lilacinus* (FJ461772)	100	7
PF06	*Hypocreales*	FJ941855	*Fusarium* sp. (EU710826)	99	1
PF07	*Eurotiales*	FJ941856	*Aspergillus candidus* (EU883597)	99	6
PF08	*Saccharomycetales*	FJ941870	*Candida parapsilosis* (FJ538165)	99	1
PF09	*Microascales*	FJ941857	*Scopulariopsis brevicaulis* (AY083220)	99	3
PF10	*Eurotiales*	FJ941858	*Aspergillus ochraceus* (AF548065)	94	1
PF11	*Capnodiales*	FJ941859	*Cladosporium* sp. (EU167574)	99	2
PF12	*Pleosporales*	FJ941861	*Didymocrea sadasivanii* (DQ384066)	100	1
PF13	*Microascales*	FJ941862	*Scopulariopsis brevicaulis* (EU263611)	91	2
PF14	*Boliniales*	FJ941873	*Lentomitella cirrhosa* (AY761089)	97	2
PF15	*Eurotiales*	FJ941863	*Penicillium chrysogenum* (AF548087)	99	5
PF16	*Eurotiales*	FJ941860	*Aspergillus* sp. (EU371048)	99	5
PF17	*Xylariales*	FJ941864	*Apiospora montagnei* (AB220230)	99	1
PF18	*Xylariales*	FJ941865	*Nigrospora oryzae* (AB220234)	99	1
PF19	*Agaricales*	FJ941866	*Marasmius alliaceus* (AY787214)	99	1
PF20	*Eurotiales*	FJ941867	*Aspergillus fumigatus* (FJ560718)	99	1
PF21	*Eurotiales*	FJ941868	*Penicillium purpurogenum* (DQ365947)	99	1
PF22	*Mucorales*	FJ941869	*Rhizomucor pusillus* (AF113434)	99	1
PF23	*Capnodiales*	FJ941871	*Davidiella tassiana* (EU343115)	99	1
PF24	*Botryosphaeriales*	FJ941882	*Botryosphaeria rhodina* (EF564147)	99	1
TS01	*Capnodiales*	FJ941874	*Davidiella tassiana* (EU343115)	99	8
TS02	*Hypocreales*	FJ941875	*Paecilomyces lilacinus* (FJ461772)	99	6
TS03	*Eurotiales*	FJ941876	*Ascomycota* sp. (EU887758)	100	5
TS04	*Eurotiales*	FJ941877	*Penicillium chrysogenum* (AF548087)	99	13
TS05	*Eurotiales*	FJ941878	*Aspergillus* sp. (EU853156)	99	8
TS06	*Eurotiales*	FJ941879	*Penicillium pinophilum* (AF245239)	99	4
TS07	*Hypocreales*	FJ941880	*Fusarium* sp. (EU710826)	98	2
TS08	*Eurotiales*	FJ941881	*Aspergillus versicolor* (AF548069)	99	3

[a] Isolates with prefix PF and TS are isolated from *C. luteoculcitella* and *Holoxea* sp., respectively

[b] The closest relatives are identified based on18S-rRNA gene sequences except for PF24 (ITS sequence)

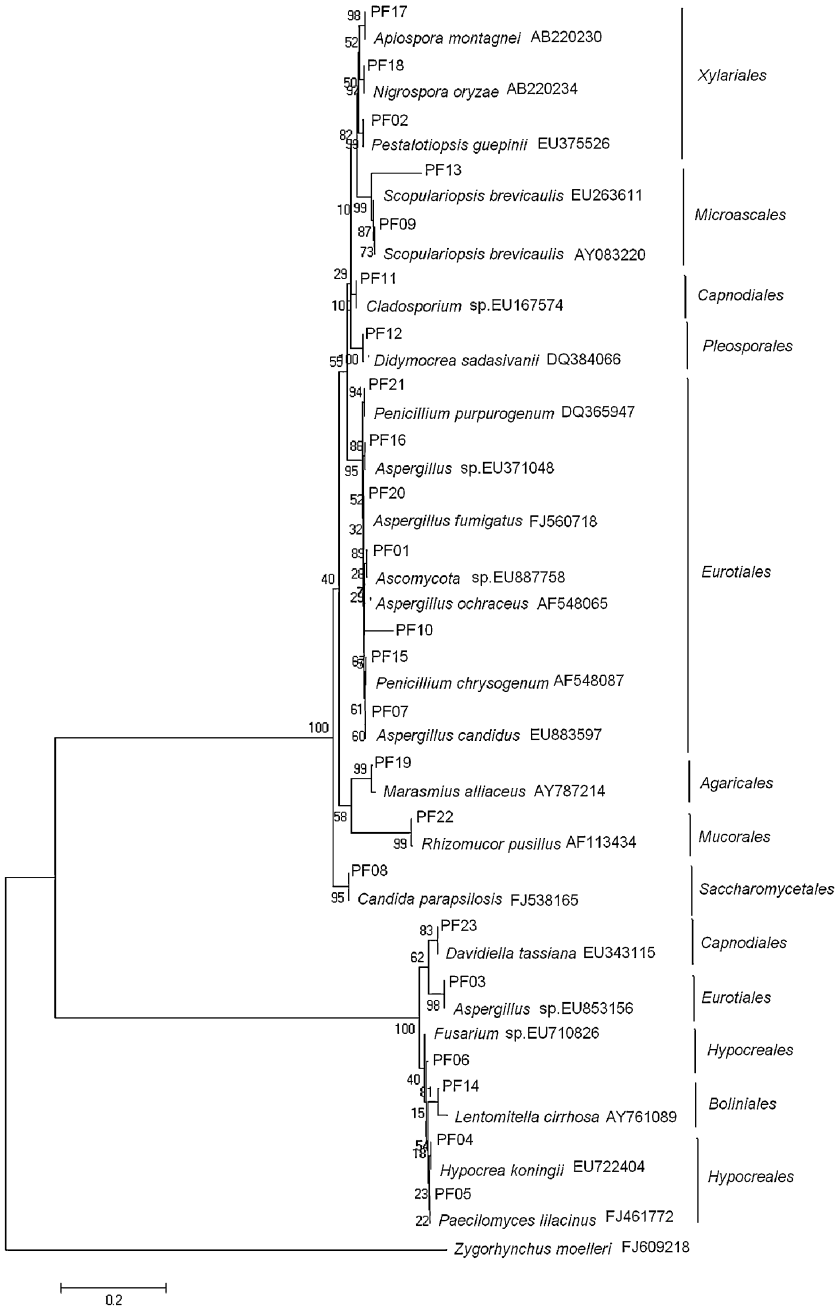

Fig. 1 Neighbor-joining phylogenetic tree of 23 representative isolates from sponge *C. luteoculcitella* based on 18S rRNA gene sequences (ca. 760 bp). The *values at each node* represent the bootstrap values from 1,000 replicates, and the *scale bar* represents 0.2 substitutions per nucleotide. *Zygorhynchus moelleri* FJ609218 was used as outgroup

Holoxea sp. accounting for 62.5% (five representatives) of the total representative fungal isolates (Table 2, Fig. 2).

Comparison of Culturable Fungal Diversity in Two Different Marine Sponges

Table 2 summarizes the representative isolates and their best matches in the NCBI database. Most of the isolates matched their closest relatives with 98% to 100% similarity except for PF13 (91%), PF10 (94%), and PF14 (97%). Three orders *Capnodiales*, *Eurotiales,* and *Hypocreales* including five genera of *Aspergillus*, *Davidiella*, *Fusarium*, *Paecilomyces,* and *Penicillium* were isolated from both sponges, and similarly *Eurotiales* fungi represented the majority of fungal isolates (Table 2, Figs. 1 and 2). Seven orders of *Agaricales*, *Boliniales*, *Microascales*, *Mucorales*, *Pleosporales*, *Saccharomycetales,* and *Xylariales*, including 12 genera of *Apiospora*, *Botryosphaeria*, *Candida*, *Marasmius*, *Cladosporium*, *Didymocrea*, *Hypocrea*, *Lentomitella*, *Nigrospora*, *Pestalotiopsis*, *Rhizomucor,* and *Scopulariopsis*, were found in sponge *C. luteoculcitella* only as well as the only one isolate in the phylum

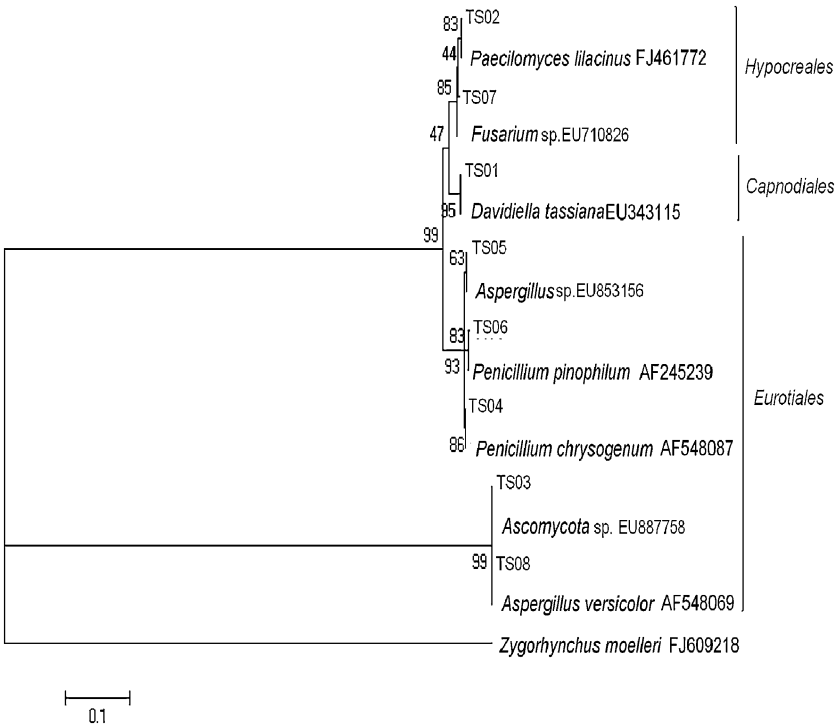

Fig. 2 Neighbor-joining phylogenetic tree of eight representative isolates from sponge *Holoxea* sp. based on 18S rRNA gene sequences (ca. 760 bp). The *values at each node* represent the bootstrap values from 1,000 replicates, and the *scale bar* represents 0.2 substitutions per nucleotide. *Z. moelleri* FJ609218 was used as outgroup

Basidiomycota (Fig. 3). Obviously, the culturable fungal diversity in sponge *C. luteoculcitella* is greater than that in sponge *Holoxea* sp..

Fungal Diversity on Eight Different Isolating Media

Among the eight media tested, Martin and 2216 were proved to be suitable for fungal isolation from sponge *Holoxea* sp., which recovered all the eight representative fungi. In the case of sponge *C. luteoculcitella*, Martin and Gause I were more suitable for isolating diverse fungi (13/18 representatives) than the other six media (Table 3).

Screening of Antimicrobial Activity of Fungi from Sponges *C. luteoculcitella* and *Holoxea* sp

All the 32 representative isolates (24 and eight representatives from *C. luteoculcitella* and *Holoxea* sp., respectively) were screened for the antimicrobial activity against six indicator microorganisms. As a result, *Nigrospora oryzae* strain PF18 from sponge *C. luteoculcitella* showed a strong and broad spectrum antimicrobial activities against *S. aureus, B. subtilis*, *P. fluorescens,* and *E. coli* indicating the potential for antimicrobial compounds production (Fig. 4).

Discussion

Traditionally, morphology-based approach is mainly used for fungal identification. But, many microscopic fungi lack a sexual state, and the majority of fungi associated with sponge are mitosporic or anamorphic (Morrison-Gardiner 2002; Pivkin et al. 2006), so, the identification of sponge-associated fungi is limited in using morphology-based approach. In general, fungi with similar morphology possess a high level of genetic variation, the use of ribosomal DNA sequences has become one of the most useful techniques in fungal identification. Meanwhile, the ITS regions can also be used in fungal taxonomy because they are fairly divergent and vary between species within genus. At present, phylogenetic taxonomy based on 18S rRNA gene or ITS region sequence has changed our understanding of the species concept for different groups of fungi. In this study, among the 32 representative fungal isolates, twenty-seven isolates were classed successfully at genus level based on 18S rRNA gene and ITS region sequences at 98–100% homology with relatives in the NCBI database. Unfortunately, isolates PF01 and TS03,

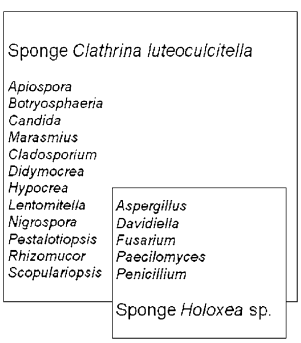

Fig. 3 Comparison of culturable fungal diversity of two different marine sponges *S. aureus, B. subtilis, P. fluorescens, E. coli*

Table 3 The cultured sponge-associated fungi on eight media

Medium	Genus of fungus	
	C. luteoculcitella	*Holoxea* sp.
Martin	*Aspergillus*, *Pestalotiopsis*, *Hypocrea*, *Paecilomyces*, *Scopulariopsis*, *Didymocrea*, *Lentomitella*, *Davidiella*, *Botryosphaeria*	*Aspergillus*, *Paecilomyces*, *Penicillium*, *Davidiella*
Sabouraud	*Pestalotiopsis*, *Marasmius*	*Aspergillus*
Czapek	*Aspergillus*, *Apiospora*, *Nigrospora*	*Aspergillus*, *Davidiella*
Malt	*Candida*, *Rhizomucor*	*Penicillium*
MYPG	*Aspergillus, Marasmius*	*Penicillium*
2216	*Aspergillus*, *Cladosporium*, *Penicillium*	*Aspergillus*, *Penicillium*, *Fusarium*
Soya germ infusion	*Penicillium*, *Aspergillus*	*Aspergillus*, *Davidiella*
Gause I	*Paecilomyces*, *Fusarium*, *Scopulariopsis*, *Aspergillus*, *Penicillium, Cladosporium*	*Aspergillus*, *Hypocrea*

which matched a marine filamentous fungi *Ascomycota* sp. in the phylum *Ascomycota* from deep-sea hydrothermal vents with similarity of 99% and 100%, respectively, were unsuccessfully identified. In addition, isolates PF13, PF10, and PF14 showed low similarity to their closest relatives (91%, 94%, and 97%, respectively). Gao et al. (2008) found the existence of previously undescribed taxa of sponge-inhabiting fungi. These results above indicated the presence of potential novel marine fungi in sponge.

Using culture-independent molecular method, Gao et al. (2008) observed the existence of abundant fungi in the phyla *Ascomycota* and *Basidiomycota* including uncultured fungi in Hawaiian marine sponges *Suberites zeteki* and *Mycale armata*. Though *Basidiomycota* fungi are abundant in sponges *S. zeteki* and *M. armata*, few of them have been successfully isolated (Li and Wang 2009; Wang et al. 2008), which suggests that *Basidiomycota* fungi are difficult to be cultured in vitro. For example, in this study, only one representative isolate PF19 in the phylum *Basidiomycota* was cultured. On the other hand, according to this study and other researches (Baker et al. 2009; Höller et al. 2000; Li and Wang 2009; Pivkin et al. 2006; Wang et al. 2008), *Ascomycota* fungi are relatively easy to cultivate in vitro. *Mycosphaerellales*, *Dothideales*, *Diaporthales*, *Phyllachorales,* and *Trichosphaeriales* isolated from Hawaiian sponges by Li and Wang (2009) and Wang et al. (2008) were not found in these two South China Sea sponges. *Agricomycotina*, *Polysporales*, *Calosphaeriales*, *Chaetothyriales,* and *Helotiales* isolated from sponge *Haliclona simulans* have not been isolated from other sponges (Baker et al. 2009). Though the two different South China Sea sponges were collected in the same location, orders *Agaricales*, *Boliniales*, *Microascales*, *Mucorales*, *Pleosporales*, *Saccharomycetales,* and *Xylariales* were isolated from sponge *C. luteoculcitella* only. These results suggested that the recovery of fungal diversity depended on the species of sponge. Meanwhile, media and culture conditions also affect the revealed fungal diversity. Thus, novel isolation strategy needs to be developed and optimized to get much more diverse fungi from different sponges.

Order *Eurotiales* especially genera *Penicillium*, *Aspergillus*, and order *Hypocreales* represented the dominant cultured fungi in these two South China Sea sponges, which is in agreement with that of sponge *H. simulans* in Gurraig Sound Kilkiet Bay, Galway, Ireland (Baker et al. 2009) and Hawaiian sponges *Gelliodes fibrosa*, *Haliclona caerulea, and M. armata* (Li and Wang 2009; Wang et al. 2008) and other

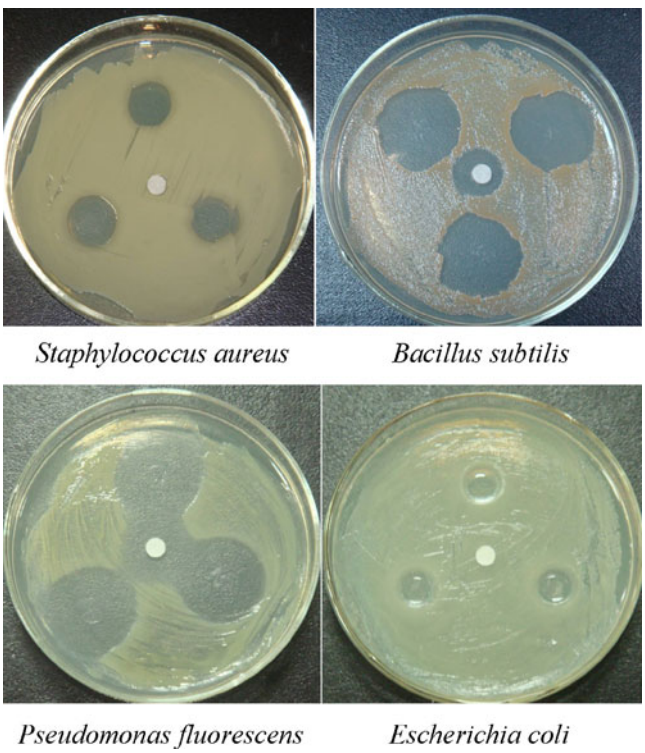

Fig. 4 Antimicrobial activities of *Nigrospora oryzae* strain PF18 (positive control, Amp, 10 mg/ml, 3 μl)

marine sponges (Höller et al. 2000; Menezes et al. 2009; Pivkin et al. 2006). These results indicated that *Eurotiales* and *Hypocreales* fungi, which are present in different sponges, maybe sponge-generalist fungi. Though different fungi were isolated from different sponges in this study, the existence of sponge-specialist fungi was difficult to be suggested because of the lack of direct evidence.

Based on this study and other reports (Li and Wang 2009; Morrison-Gardiner 2002), some of the sponge-derived fungi were related to common genera in terrestrial habitats, for example *Aspergillus*, *Cladosporium*, *Fusarium,* and *Penicillium*, indicating that these isolates maybe facultative fungi of terrestrial origin. But, many novel natural products that are not found in terrestrial strains have been found in these sponge-derived fungi, suggesting these fungi have adapted to the special environment or marine animal/plant habitats and have their uniqueness of metabolic activities (Bugni and Ireland 2004; Li and Wang 2009). So, it is possible that these fungi in sponges are not simply resulting from terrestrial fungal spores that are trapped in sponge tissues from seawater column during the filter-feeding process. Meanwhile, all the sponge-derived fungi were able to sporulate on media made from artificial seawater, suggesting that these fungi could at least be classified as "marine fungi". About 800 species of obligate marine fungi, which mostly belong to ascomycetes, anamorphs, and a few basidiomycetes, have been reported so far (Hyde et al. 2000). Though *Ascomycota* sp. strain PF01 from sponge *C. luteoculcitella* and *Ascomycota* sp. strain TS03 from *Holoxea* sp. were closely affiliated with fungi in deep-sea hot springs with 99% and 100% homology, respectively, no direct evidence suggested the presence of obligate marine fungi in these sponges.

Fungi associated with sponges are suggested to play ecological roles such as nutrient transfer and chemical defense in sponges (Bugni and Ireland 2004). A previous study found that marine sponges required a mixed diet of bacteria, microalgae, and fungi to fulfill their metabolic needs (Duckworth and Pomponi 2005). In this study, *N. oryzae* strain PF18 showed strong and broad spectrum activities against *S. aureus*, *B. subtilis*, *P. fluorescens,* and *E. coli*, suggesting it may play a role in the antimicrobial defense for sponge host. Indeed, many sponge-derived fungi (e.g., *Eurotiales*, *Hypocreales,* and *Pleosporales*) show obvious antimicrobial activity (Baker et al. 2009), and are able to produce novel antimicrobial compounds (Amagata et al. 2006; Lin et al. 2003; Saleem et al. 2007; Wang et al. 2002). Meanwhile, the majority of relatives (>45%) in this study have enzymic activities or can produce bioactive natural products, indicating the possible biological roles of fungi in the association of sponge–fungi.

According to a few reports (Proksch et al. 2003a, b; Maldonado et al. 2005; Rot et al. 2006), a close relationship between fungus and sponge including symbiosis has been suggested, for instance, several mycelia fungi isolated from sponges yielded natural products identical or related to those formerly attributed to their hosts (Proksch et al. 2003a, b). Particularly, Maldonado et al. (2005) reported the symbiotic relationship between yeast and sponge, where yeast was found to be maternally transmitted from the soma through the oocytes to the fertilized eggs, and Rot et al. (2006) found the horizontal gene transfer of a mitochondrial intron from a fungus to sponge suggesting a symbiotic relationship between sponge and fungi. However, the evidence for the fungal symbiotic relationship with sponge and the fungal function is lacking. In addition, several isolates (e.g., *Pestalotiopsis*) in this study have close affiliations with fungal pathogens of plants and animals, but their pathogenicity towards sponge hosts remains to be confirmed.

Marine microbial natural products have increased tremendously since 2000 (Blunt et al. 2009), particularly, sponge-derived fungi have been proved to be important sources for new bioactive metabolites (Bugni and Ireland 2004; Proksch et al. 2003a). However, marine fungi are still one of the most understudied marine ecological groups, especially our knowledge concerning the diversity and function of fungi associated with sponge is still very limited. In this study, diverse culturable fungi of at least 17 genera within ten orders of two phyla *Ascomycota* and *Basidiomycota*, including some potential novel marine fungi, were revealed in sponges *C. luteoculcitella* and *Holoxea* sp., especially eight genera fungi of *Apiospora, Botryosphaeria*, *Davidiella*, *Didymocrea*, *Lentomitella*, *Marasmius*, *Pestalotiopsis*, and *Rhizomucor* were isolated from sponge for the first time, which extended our knowledge of marine sponge-associated fungal diversity greatly. Future research focused on fungi associated with sponges will help our understanding of the nature of sponge–fungal association, and lead to improve the recovery efficiency of unexplored fungal species with biotechnological potential such as production of novel secondary metabolites.

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References

Amagata T, Morinaka BI, Amagata A, Tenney K, Valeriote FA, Lobkovsky E, Clardy J, Crews P (2006) A chemical study of cyclic depsipeptides produced by a sponge-derived fungus. J Nat Prod 69:1560–1565

Baker PW, KennedyJ DADW, Marchesi JR (2009) Phylogenetic diversity and antimicrobial activities of fungi associated with *Haliclona simulans* isolated from Irish coastal waters. Mar Biotechnol 11:540–547

Bhadury P, Mohammad BT, Wright PC (2006) The current status of natural products from marine fungi and their potential as antiinfective agents. J Ind Microbiol Biotech 33:325–337

Blunt JW, Copp BR, Hu WP, Munro MHG, Northcote PT, Prinsep MR (2009) Marine natural products. Nat Prod Rep 26:170–244

Borneman J, Hartin RJ (2000) PCR primers that amplify fungal rRNA genes from environmental samples. Appl Environ Microbiol 66:4356–4360

Bugni TS, Ireland CM (2004) Marine-derived fungi: a chemically and biologically diverse group of microorganisms. Nat Prod Rep 21:143–163

Duckworth AR, Pomponi SA (2005) Relative importance of bacteria, microalgae and yeast for growth of the sponge Haliclondria melanadocia (De Laubenfels, 1936): a laboratory study. J Exp Mar Biol Ecol 323:151–159

Gao Z, Li B, Zheng C, Wang G (2008) Molecular detection of fungal communities in the Hawaiian marine sponges *Suberites zeteki* and *Mycale armata*. Appl Environ Microbiol 74:6091–6101

Hentschel U, Usher KM, Taylor MW (2006) Marine sponges as microbial fermenters. FEMS Microbiol Ecol 55:167–177

Höller U, Wright AD, Mathee GF, Koonig GM, Draeger S, Aust H-J, Schulz B (2000) Fungi from marine sponges: diversity, biological activity and secondary metabolites. Mycol Res 104:1354–1356

Hyde KD, Sarma VV, Jones EBG (2000) Morphology and taxonomy of higher marine fungi. In: Hyde KD, Pointing SB (eds). Marine mycology: a practical approach. Fungal Divers Res Ser 1 1:172–204

König GM, Kehraus S, Seibert SF, Abdel-Lateff A, Müller D (2005) Natural products from marine organisms and their associated microbes. Chembiochem 6:1–10

Li ZY, Liu Y (2006) Marine sponge *Craniella austrialiensis*-associated bacterial diversity revelation based on 16S rDNA library and biologically active actinomycetes screening, phylogenetic analysis. Lett Appl Microbiol 43:410–416

Li Q, Wang G (2009) Diversity of fungal isolates from three Hawaiian marine sponges. Microbiol Res 164:233–241

Li Y, Jiang JZ, Liang N (2006) A rapid method for preparation of filamentous fungi chromosome DNA. J Biol 23(6):52–53

Lin W, Brauers G, Ebel R, Wray V, Berg A, Sudarsono PP (2003) Novel chromone derivatives from fungus *Aspergillus versicolor* isolated from the marine sponge *Xestospongia exigua*. J Nat Prod 66:57–61

Maldonado M, Cortadellas N, Trillas MI, Ruetzler K (2005) Endosymbiotic yeast maternally transmitted in a marine sponge. Biol Bull 209:94–106

Menezes CBA, Bonugli-Santos RC, Miqueletto PB, Passarini MRZ, Silva CHD, Justo MR, Leal RR, Fantinatti-Garboggini F, Oliveira VM, Berlinck RGS, Sette LD (2009) Microbial diversity associated with algae, ascidians and sponges from the north coast of Sao Paulo state, Brazil. Microbiol Res 165:466–482

Morrison-Gardiner S (2002) Dominant fungi from Australian coral reefs. Fungal Divers 9:105–121

Osinga R, Armstrong E, Burgess JG, Hoffmann F, Reitner J, Schumann-Kindel G (2001) Sponge-microbe associations and their importance for sponge bioprocess engineering. Hydrobiologia 461:55–62

Piel J (2009) Metabolites from symbiotic bacteria. Nat Prod Rep 26:338–362

Pivkin MV, Aleshko SA, Krasokhin VB, Khudyakova YV (2006) Fungal assemblages associated with sponges of the southern coast of Sakhalin Island. Russ J Mar Boil 32:249–254

Proksch P, Ebel R, Edrada RA, Schupp P, Lin WH, Sudarsono WV, Steube K (2003a) Detection of pharmacologically active natural products using ecology. Selected examples from indopacific marine invertebrates and sponge-derived fungi. Pure Appl Chem 75:343–352

Proksch P, Edrada-Ebel R-A, Ebel R (2003b) Drugs from the sea: opportunities and obstacles. Mar Drugs 1:5–17

Raghukumar C (2008) Marine fungal biotechnology: an ecological perspective. Fungal Divers 31:19–35

Rot C, Goldfarb I, Ilan M, Huchon D (2006) Putative cross-kingdom horizontal gene transfer in sponge (Porifera) mitochondria. BMC Evol Biol 6:71

Saleem M, Ali SM, Hussain S, Jabbar A, Ashraf M, Lee YS (2007) Marine natural products of fungal origin. Nat Prod Rep 24:1142–1152

Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol Mol Biol R 71:295–347

Van Burik JAH, Schreckhise RW, White TC, Bowden RA, Myerson D (1998) Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Med Mycol 36:299–303

Wang C, Wang B, Brauers G, Guan H, Proksch P, Ebel R (2002) Microsphaerones A and B, two novel ç-pyrone derivatives from the sponge-derived fungus *Microsphaeropsis* sp. J Nat Prod 65:772–775

Wang G, Li Q, Zhu P (2008) Phylogenetic diversity of culturable fungi associated with the Hawaiian sponges *Suberites zeteki* and *Gelliodes fibrosa*. Antonie Leeuwenhoek 93:163–174

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and application. Academic, San Diego, pp 315–322

Zhang W, Xue S, Zhao Q, Zhang X, Li J, Jin M, Yu X, Yuan Q (2003) Biopotentials of marine sponges from China oceans: past and future. Biomol Eng 20:413–419