Microb Ecol (2011) 62:644–654
DOI 10.1007/s00248-011-9859-y

INVERTEBRATE MICROBIOLOGY

Phylogenetically Diverse Cultivable Fungal Community and Polyketide Synthase (PKS), Non-ribosomal Peptide Synthase (NRPS) Genes Associated with the South China Sea Sponges

Kang Zhou · Xia Zhang · Fengli Zhang · Zhiyong Li

Received: 27 November 2010 / Accepted: 10 April 2011 / Published online: 26 April 2011

Abstract Compared with sponge-associated bacteria, the phylogenetic diversity of fungi in sponge and the association of sponge fungi remain largely unknown. Meanwhile, no detection of polyketide synthase (PKS) or non-ribosomal peptide synthase (NRPS) genes in sponge-associated fungi has been attempted. In this study, diverse and novel cultivable fungi including 10 genera (*Aspergillus*, *Ascomycete*, *Fusarium*, *Isaria*, *Penicillium*, *Plectosphaerella*, *Pseudonectria*, *Simplicillium*, *Trichoderma*, and *Volutella*) in four orders (*Eurotiales*, *Hypocreales*, *Microascales*, and *Phyllachorales*) of phylum *Ascomycota* were isolated from 10 species marine sponges in the South China Sea. *Eurotiales* and *Hypocreales* fungi were suggested as sponge generalists. The predominant isolates were *Penicillium* and *Aspergillus* in *Eurotiales* followed by *Volutella* in *Hypocreales*. Based on the conserved Beta-ketosynthase of PKS and A domain of NRPS, 15 polyketide synthases, and four non-ribosomal peptides synthesis genes, including non-reducing and reducing PKSs and hybrid PKS–NRPS, were detected in these fungal isolates. A lateral gene transfer event was indicated in the comparison between the phylogenetic diversity of 18S rRNA genes and β-ketoacyl synthase domain sequences. Some fungi, especially those with PKS or NRPS genes, showed antimicrobial activity against *P. fluorescens*, *S. aureus* and *B. subtilis*. It was the first time to investigate PKS and NRPS genes in sponge-associated fungi. Based on the detected antibiotics biosynthesis-related PKS and NRPS genes and antimicrobial activity, the potential ecological role of sponge-associated fungi in the chemical defense for sponge host was suggested. This study extended our knowledge of sponge-associated fungal phylogenetic diversity and their potential roles in the chemical defense.

Introduction

Marine fungi are widely distributed in marine habitats and are suggested to play an important ecological role [14, 33]. But, compared with terrestrial fungi, marine fungi remain the most underexplored group in the marine environment [21, 46], especially marine fungi associated with marine macro-organisms. Sponges (phylum Porifera), which are among the oldest of the multicellular animals (Metazoa), form close associations with a wide variety of microorganisms [11, 42, 43], some of which are thought to be involved in the production of secondary metabolites which contribute to the host's chemical defense and ecological functions [20, 26, 29]. However, in contrast to prokaryotic microbes associated with sponges [11, 16, 17, 22, 42, 43], our knowledge on the occurrence of fungi in sponges is still

Electronic supplementary material The online version of this article (doi:10.1007/s00248-011-9859-y) contains supplementary material, which is available to authorized users.

K. Zhou · X. Zhang · F. Zhang · Z. Li
Marine Biotechnology Laboratory,
State Key Laboratory of Microbial Metabolism,
Shanghai Jiao Tong University,
Shanghai 200240, People's Republic of China

K. Zhou · X. Zhang · F. Zhang · Z. Li (✉)
School of Life Sciences and Biotechnology,
Shanghai Jiao Tong University,
800 Dongchuan Road,
Shanghai 200240, People's Republic of China
e-mail: zyli@sjtu.edu.cn

very scarce due to the lack of direct evidence of fungal mycelia, for example, microscopic, immunological, or fluorescence in situ hybridization detection. Though the investigations on the diversity of sponge-associated fungi have just begun by culture-independent [8] and culture-dependent approaches [3, 7, 13, 21, 23, 31, 46], we still lack a clear picture of fungal diversity in sponges. At present, the knowledge of fungal phylogenetic diversity-associated sponges is mainly focused on Hawaiian sponges [8, 21, 46]; the investigation on the diversity of China Sea sponge-associated fungi is rarely carried out compared with sponges from other sea areas [7, 23].

Many marine-derived fungi have been isolated for natural products investigation [1, 9]; in particular, sponge-associated fungi have been proven to be the most abundant resources of diverse bioactive compounds and novel metabolites [4, 20]. But the ecological function and association of sponge fungi also remain largely unanswered [25, 34]; more detailed evidences for sponge-associated fungal functions need to be provided. Activity-based analysis strategy can be used in the sponge-associated fungal function analysis, but it will be greatly limited sometimes because the culture condition during bioassay may not be suitable for the synthesis of related bioactive metabolites. On the other hand, functional gene-based molecular screening strategy could avoid this disadvantage of bioassay to some extent. Polyketides and non-ribosomal peptides have been immensely concerned over the past decades, and numbers of various novel polyketide and non-ribosomal peptide compounds have been found from marine-derived microbes, most of which show different biological activities and ecological functions [4]. The detection of polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) genes in sponge-associated fungi could suggest their potential roles in the chemical defense process of sponge host. The filtration of PKS and NRPS genes from sponge-associated bacteria has started [12, 16–18, 36, 38, 48, 49], but until now, no detection of PKS and NRPS genes in sponge-associated fungi has been attempted.

In this study, with the aim to know more about the sponge-associated fungal phylogenetic diversity and find evidence for the potential roles of sponge-associated fungi in the host's chemical defense, the culturable fungal phylogenetic diversity of 10 species sponges in the South China Sea was investigated. PKS and NRPS genes were detected in the obtained fungal isolates based on the conserved Beta-ketosynthase of PKS and A domain of NRPS; meanwhile, the bioassay of antimicrobial activity was carried out. According to the detected PKS and NRPS genes and antimicrobial activity, the potential ecological role of sponge-associated fungi in the chemical defense for sponge host was discussed.

Materials and Methods

Sponge and Isolation of Sponge-Associated Fungi

Ten species marine sponges: *Acanthella* sp. (X3), *Aplysinopsis* sp. (X4), *Haliclona oculata* (X5), *Phyllospongia foliascens* (X6), *Pericharax* sp. (X8), *Agelas dispar* (X9), *Rhizaxinella* sp. (X10), *Agelas clathrodes* (X11), *Mycale fibrexilis* (X12), *Pericharax heteroraphis* (X13) were collected opportunistically nearby Yongxing Island (112°20′ E, 16°50′ N) in the South China Sea at depth of ca. 20 m. 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. Sponges were identified by Professor Jinhe Li at Institute of Oceanology, Chinese Academy of Sciences mainly according to the morphology of spicule types.

Sponge was rinsed three times with sterile artificial seawater (ASW) [22] to get rid of nonspecific fungal propagules from seawater column on sponge surface and in inner cavity. Sponge inner issue was cut into pieces (1–3 cm^3) with a sterile scalpel and immersed in sterile calcium-and-magnesium-free ASW for 10 min. Then, the small pieces were homogenized by a high-speed pulse-type homogenizer machine. The homogenate was diluted with sterile ASW at three dilutions (1:1, 1:10, 1:100). One hundred microliters of each dilution was plated onto six different media (Supplement, Table 1) in triplicate and incubated at 20°C for 3–15 days in parallel with 25°C and 30°C. All the media were prepared with ASW and adjusted to pH 7.4–7.6. Medium containing glucose was autoclaved at 115°C for 20 min; others were autoclaved at 121°C for 20 min. Thirty micrograms per liter streptomycin and ampicillin were added in media to inhibit bacterial growth.

Genomic DNA Extraction and PCR Amplification of 18S rRNA Gene

After cultivation in Martins media at 180 rpm, 28°C for 5–7 days, the fungi were centrifuged at 5,000×*g* for 5 min and ground using a mortar containing 600 μL CTAB lysis buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris, 20 mM EDTA, 1% PVP) at 65°C. The mycelial mixture was transferred into a 1.5-mL Eppendorf tube and heated to 65°C for 30 min, extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and washed with chloroform/isoamyl alcohol (24:1). After centrifugation at 10,000×*g* for 5 min, the supernatant was transferred to a new microtube and precipitated by adding equal volume of isopropanol at −20°C for 1 h. Finally, the DNA pellets were collected by centrifugation (12,000×*g*, 15 min), washed with 75% ethanol twice and re-suspended in 40 μL TE Buffer

(10 mM Tris, 1 mM EDTA, pH 8.0). RNA was removed by adding 2 μl of RNase A (10 mg/ml; Invitrogen) at 60°C for 10 min.

The resulting genomic DNA was used as templates to amplify the fungal 18S rRNA gene fragment using primers nu-SSU-0817 (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1536 (TTGCAATGCYCTATCCCCA) [5]. The PCR mixture (50 μL) contained 1 μg DNA template, 5 μL 10×Taq buffer (Fermentas), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 μM of each primer, and 1.25 U Taq DNA Polymerase. The PCR program was carried out with 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 30 s, and final elongation at 72°C for 10 min. PCR products were purified using Cycle-pure Kit (Omega).

Restriction Fragment Length Polymorphism and Phylogenetic Diversity Analyses

PCR products were digested for 2 to 3 h with restriction endonucleases HifI, HeaIII, and TaqI at 37°C, 37°C, 65°C, respectively. After analysis by electrophoresis on a 1.5% (*w*/*v*) agarose gel, different genotypes were picked. PCR products of representative strains of different genotypes were sequenced on an ABI 3730 XL (Applied Biosystems) automated sequencer using primer set of nu-SSU-0817/nu-ssu-1536 in Shanghai Invitrogen Company.

Fungal 18S rRNA gene sequences of ca. 700–800 bp obtained in this study were aligned on the SILVA database (http://www.arb-silva.de/aligner/) to identify sequence similarity [32]. Checking the maximum identity and habitat through the NCBI database, the most appropriate relative sequences were selected and imported into MEGA 4.0 [40]. In order to reflect the genetic distances between different fungal strains intuitively, a rooted phylogenetic tree was constructed using neighbor-joining method combined with bootstrap analysis setting with 1,000 replications.

Bioassay of Fungal Antimicrobial Activity

The fungal isolates were cultivated at 28°C, 180 rpm for 15 days using Martins medium. After centrifugation at 10,000×*g* for 5 min, 300 μl of the supernatant was added into sterile oxford cup placed on the LB agar medium coated with the indicator bacteria *Escherichia coli*, *Pseudomonas fluorescens*, *Staphlococcus aureus*, and *Bacillus subtilis*. One milligram per milliliter ampicillin was added on a filter paper in the center of the Petri dish as positive control. The similar test was performed with *Aspergillus nigers*, *Candida albicans*, and the positive control was 1 mg/ml nystatin. All the plates were incubated at 37°C for 24 h in triplicate until inhibition zones were visible.

PKS and NRPS Genes Screening and Phylogenetic Diversity Analysis

The highly conserved sequences of β-ketoacyl synthase (KS) domains are shared among all PKSs; thus, KS domains are useful in screening for PKSs in fungi. Similarly, the most conserved A domain can be used for PCR primer design to survey NRPSs gene diversity. Thus, KA [2] and LC series [27] primers were used to detect PKS genes in the fungal isolates (Supplement, Tables 2, 3). The PCR process was set as 4 min at 95°C; 35 cycles of 30 s at 95°C, 1 min at 50°C, 2 min at 72°C, and 7 min at 72°C. The PCR products were purified with E.Z.N.A.TM MicroElute Gel Extraction Kit, cloned into pEASY-T1 cloning Kit and submitted for sequencing. NRPS genes except the primers were screened using primer set AUG003–AUG007 [39] and the PCR process was set as 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, 3 min at 72°C, and 8 min at 72°C. PCR products were ligated into pEASY-Blunt Simple vector and transformed into Trans1-T1 Phage-Resistant Chemically Competent Cell using pEASY-Blunt Simple Cloning Kit (Transgen). The positive recombinants were screened by indicator plates with X-Gal, IPTG, and ampicillin by color-based recombinant selection method. Positive clones were sequenced using primers M13F and M13R by Shanghai Invitrogen Company. The obtained PKS and NRPS gene sequences were analyzed with BLASTX. Sequences were aligned against reference sequences by CLUSTAL X [44], and then the aligned dataset was used as input for phylogenetic analysis program. Phylogenetic trees based on gene sequences of KS domain of PKS and A domain of NRPS were constructed using neighbor-joining method in mega 4.0 combined with bootstrap analysis setting with 1,000 replications, respectively.

Nucleotide Sequence Accession Number

Fungal 18S rRNA gene sequences obtained in this study were deposited in GenBank under accession numbers HQ234220–HQ234243, HQ317206–HQ317210; Fungal PKS gene sequences were submitted to GenBank under accession numbers HQ234244–HQ234258; Fungal NRPS gene sequences were submitted to GenBank under accession numbers HQ234259–HQ234262.

Results

Phylogenetic Diversity of Sponge-Associated Cultivable Fungi

In total, 177 fungi isolates were obtained from 10 sponges in the South China Sea using 6 media formulations at three

different temperatures: 20°C, 25°C, and 30°C. According to the number of isolated fungi, the most fungi-rich sponges are *A. dispar* (X9, 35 isolates), *P. foliascens* (X6, 32 isolates), *M. fibrexilis* (X12, 25 isolates), *H. oculata* (X5, 23 isolates), and *Rhizaxinella* sp. (X10, 22 isolates). Among the tested six media, Martins medium and Marine fungi-isolated medium resulted in the most fungal isolates (45 and 44, respectively). There was no obvious difference among the three cultivation temperatures 20°C, 25°C, and 30°C.

Based on 18S rRNA gene-restriction fragment length polymorphism (RFLP) analysis, 24 different genotypes were observed; thus, 24 fungal representative isolates were selected for phylogenetic analysis. As shown in Table 1, all the representatives clustered into four taxonomic orders in the phylum *Ascomycota*: *Microascales*, *Phyllachorales*, *Hypocreales*, and *Eurotiales* (Fig. 1). Genera of *Aspergillus* and *Penicillium* in *Eurotiales* represented the dominant fungi (94/177). The second dominant isolates were *Hypocreales* (68 isolates) which were composed of at least six genera *Ascomycete*, *Fusarium*, *Isaria*, *Simplicillium*, *Trichoderma*, and *Volutella*. The obtained *Phyllachorales* (14 isolates) and *Microascales* (one isolate) fungi were affiliated with *Plectosphaerella* and *Pseudallescheria*, respectively.

Antimicrobial Activity of Sponge-Associated Cultivable Fungi

In the bioassay of fungal antimicrobial activity against *E. coli*, *P. fluorescens*, *S. aureus*, *B. subtilis*, *A. nigers*, and *C. albicans*, eight isolates exhibited antibacterial activity against *P. fluorescens*, while three against *B. subtilis*, especially X13–105, X12–94, and X13–118, displayed antibacterial activity against both *P. fluorescens* and *S. aureus* (Table 2). Interestingly, isolates X12–91 and X10–49, which had the same closest relative *Penicillium* sp.HAW-OCF5 (AY887947; Table 1), showed different antimicrobial activity.

Screening of PKS and NRPS Genes in Sponge-Associated Cultivable Fungi

Fifteen PKS genes (ca. 800 bp) were detected in seven fungal strains by KA-series primers or LC1/LC2c primer (Table 3). Nine reducing PKS KS fragments were detected by KA-series primers and six non-reducing PKS KS fragments by LC1/LC2c, especially both reducing PKSs and non-reducing PKSs were detected in X9–31, X10–49, X11–77, and X4–167 simultaneously. All the Beta-ketosynthase domain sequences from fungal isolates belong to type I and showed high sequence similarity to parts of *Penicillium chrysogenum* Wisconsin 54–1255 complete genome [45] and *Aspergillus fumigatus* Af293 polyketide synthase gene [28] (Table 3), which are involved in antibiotic biosynthesis. The PKS fragments X9-31-3, X10-49-1, X11-77-2, X12-91, X13-105, X4-167-1, X9-31-2, and X11-77-3 had similarity to sequences in clone PC00c11 (AM920436) and PC00c13 (AM920438) which is similar to lovastatin diketide synthase lovF of *Aspergillus terreus*, while X9-31-1, X10-49-2, X11-77-1, and X4-167-2 were close similar to the sequence found in clone Pc00c12 (AM920437) which has high similarity to nonaketide synthase lovB of *A. terreus*. The PKS fragments X12-94-1 and X4-167-3 were closely similar to part of *A. fumigatus* Af293 PKS gene (XM-742074) which is similar to *B. fuckeliana* polyketide synthase (PKS10) gene, and X12-94-2 was similar to part of *A. fumigatus* Af293 PKS gene (XM-748078) which is also similar to lovastatin nonaketide synthase of *A. terreus*. As shown in Fig. 2, the phylogenetic analysis of gene fragments of KS domain suggested that most of sponge-derived KS sequences obtained in this study gathered with the existing marine-derived PKS sequences; however, three of our sequences gathered into a unique clade with their most similar sequences and showed an obvious genetic distance to another clade.

In the case of NRPS, four NRPS genes fragments were found in isolates X11-77 and X12-91 by AUG003/AUG007 primer set. All of the fragments were also similar to parts of *P. chrysogenum* Wisconsin 54–1255 complete genome (Table 4, Fig. 3). The phylogenetic analysis based on A domain in NRPS showed that the sponge-derived NRPS had strong similar to the existed fungal NRPS genes. X11-77-1 was closely relative to ORF Pc13g02980 in clone Pc00c13 which is similar to mitochondrial inter-membrane space export machinery component Oxa1-*Saccharomyces cerevisiae*. X11-77-2 was closely similar to ORF Pc22g13340 which is similar to human aquaporin 3AQP3. X12-91-1 was closely relative to *P. chrysogenum* Wisconsin 54–1255 alpha-aminoadipyl-cysteinyl-valine synthetase pcbAB, and X12-91-2 was close to ORF Pc21g15100 which is similar to H^+-transporting ATPase vma-1 of *Neurospora crassa*. Hybrid PKS–NRPS gene was also detected in two strains of *P. chrysogenum*, isolates X11-77 and X12-91.

Discussion

The majority of fungi associated with sponges are mitosporic or anamorphic [31], and many morphologically indistinguishable fungal species have recently been isolated [10]; therefore, the identification of sponge-associated fungi is limited in using a morphology-based approach. Phylogenetic taxonomy has been accepted as the appropriate way to identify fungi [41]. Besides ITS region sequences [46],

Table 1 The isolated fungi and representatives after RFLP analysis and the phylogenetic affiliations of fungi associated with sponges

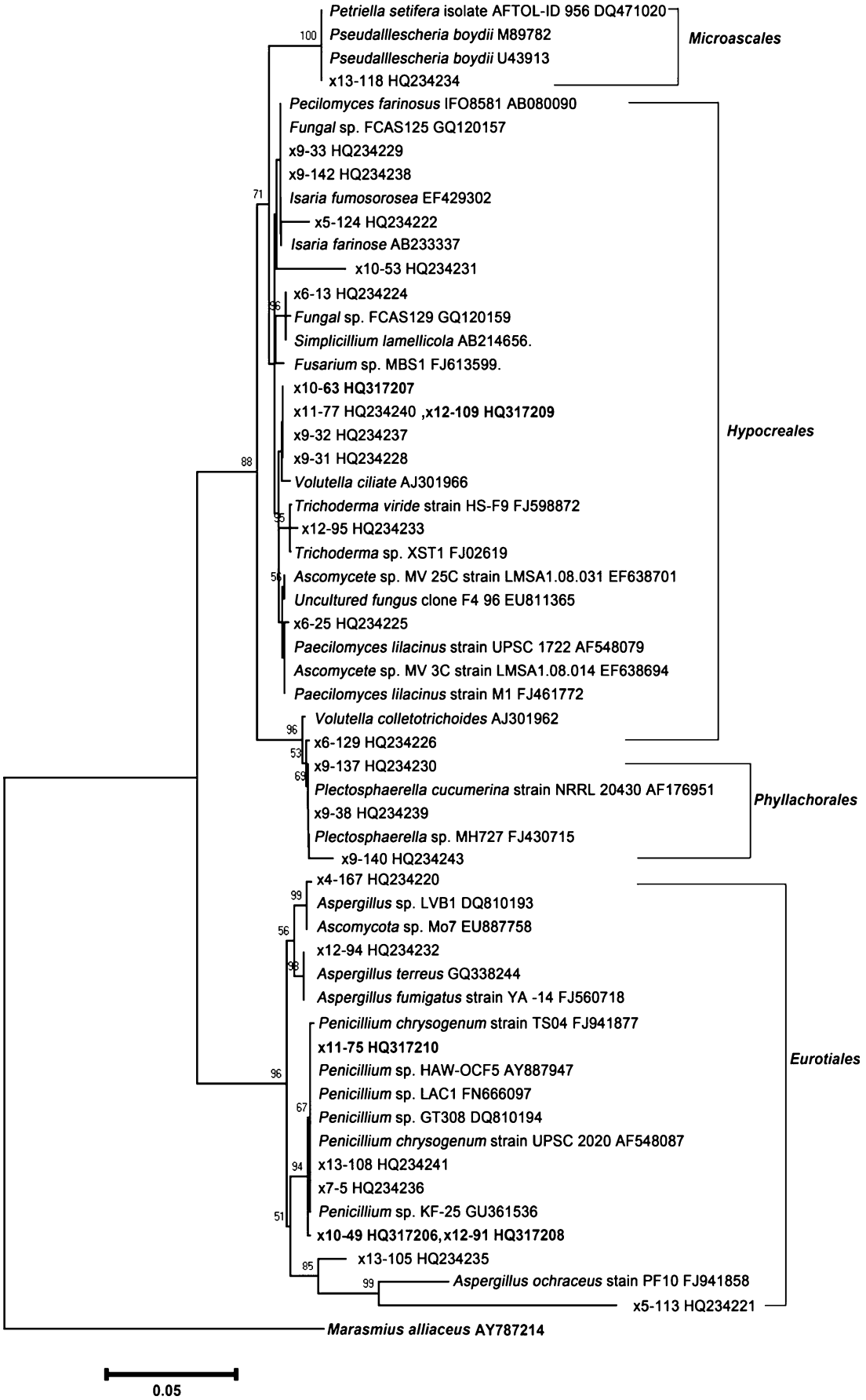
Strain	RFLP[a]			Taxon		Closest relative (accession no.)	Identity (%)	Source	Frequency isolated
	HifII	HeaIII	TaqI	Phylum	Order				
X4-167	3	2	2	*Ascomycota*	*Eurotiales*	*Aspergillus* sp.LVB1 (DQ810194)	99	Marine	4
X5-113	3	3	2			*Aspergillus ochraceus*(FJ941859)	87	Marine	3
X12-94	2	2	3			*Aspergillus terreus* (GQ338244)	100	Plant	1
X10-49 X12-91	2	2	2			*Penicillium* sp.HAW-OCF5 (AY887947)	99	Marine	47
X7-5	3	2	3			*Penicillium* sp.GT308(DQ810194)	99	Marine	1
X11-75	3	2	2			*Penicillium* sp.GT308(DQ810194)	100	Marine	30
X13-105	2	2	2			*Penicillium* sp.KF-25 (GU361536)	97	Plant	1
X13-108	3	2	3			*Penicillium chrysogenum* (FJ941877)	100	Marine	8
X6-13	0	3	3		*Hypocreales*	*Simplicillium lamellicola* (AB214656)	99	Soil	2
X6-129	2	3	2			*Volutella colletotrichoides* (AJ301962)	99	Plant	22
X6-25	3	3	2			*Ascomycete* sp. MV_3C strain(EF638694)	99	Marine	3
X9-32	3	2	3			*Volutella ciliata*(AJ301966)	98	Soil	4
X9-33	2	3	3			*Isaria farinosa*(AB233337)	99	Insect	12
X9-142	3	3	2			*Isaria farinosa*(AB233337)	98	Insect	2
X5-124	2	3	3			*Fungal* sp. FCAS125(GQ120157)	99	Marine	1
X10-53	3	3	3			*Isaria farinosa*(AB233337)	97	Insect	1
X9-31	0	3	3			*Fusarium* sp.(FJ613599)	99	Soil	1
X11-77 X12-109	0	3	2						14
X10-63	0	2	3						4
X12-95	2	3	3			*Trichoderma* sp.(FJ026619)	99	Plant	1
X9-38	0	2	2		*Phyllachorales*	*Plectosphaerella* sp.(FJ430715)	99	Soil	1
X9-137	3	3	2			*Plectosphaerella* sp.(FJ430715)	100	Soil	10
X9-140	3	3	2			*Plectosphaerella* sp.(FJ430715)	98	Soil	3
X13-118	2	3	2		*Microascales*	*Pseudallescheria boydii* (M89782)	99	Soil	1

[a] The number of bands in RFLP analysis

partial conservative region of 18S rRNA gene of fungus has been widely used in fungal taxonomy [47]. In this study, according to 18S rRNA gene analysis, most of the fungal genotypes were closely related to sequences in the NCBI database at 98–100% homology. A few isolates such as X5–113, X13–105, and X10–53 maybe potential novel fungal strains because of low similarity (below 97%) to their relatives, especially isolate X5–113 may represent a new species of fungal strains with only 87% homology to its closest relative *Aspergillus ochraceus* isolated from sponge *Clathrina luteoculcitella* [7].

Kohlmeyer et al. [19] divided marine fungi in two groups: (1) primary marine fungi derived from marine ancestors, that have not left their original marine environment; and (2) marine fungi which are thought to have evolved from terrestrial ancestors. Most of the isolates (97/177) had very close affiliation with marine-derived relatives, for example, isolate X13–108 had 100% identity to *P. chrysogenum* isolated from sponge *Holoxea* sp. by Ding et al. [7]; isolate X5–124 had 99% identity to Fungal sp. FCAS125 isolated from oxygen-depleted regions of the Arabian Sea [15]; isolate X6–133 was closely related to *Penicillium* sp. HAW-OCF5, which was isolated from marine sediment; the closest relatives of isolates X4–167, X11–75, and X7–5 were isolated from Yellow Sea, China. About 800 species of obligate marine fungi, most of which belong to ascomycetes, have been reported [14], *Ascomycete* sp. strain X6–25 was successfully isolated in this study

Figure 1 Neighbor-joining phylogenetic tree based on partial sequences of 18S rRNA genes (ca. 700–800 bp) of cultured fungi from 10 sponges in the South China Sea. *Numbers at branches* indicate Bootstrap values of neighbor-joining analysis (>50%) from 1,000 replicates. The *scale bar* represents 0.05 nucleotide substitutions per site


Petriella setifera isolate AFTOL-ID 956 DQ471020
Pseudallescheria boydii M89782
Pseudallescheria boydii U43913
x13-118 HQ234234
Microascales
Pecilomyces farinosus IFO8581 AB080090
Fungal sp. FCAS125 GQ120157
x9-33 HQ234229
x9-142 HQ234238
Isaria fumosorosea EF429302
x5-124 HQ234222
Isaria farinose AB233337
x10-53 HQ234231
x6-13 HQ234224
Fungal sp. FCAS129 GQ120159
Simplicillium lamellicola AB214656.
Fusarium sp. MBS1 FJ613599.
x10-63 HQ317207
x11-77 HQ234240 ,x12-109 HQ317209
x9-32 HQ234237
x9-31 HQ234228
Volutella ciliate AJ301966
Trichoderma viride strain HS-F9 FJ598872
x12-95 HQ234233
Trichoderma sp. XST1 FJ02619
Ascomycete sp. MV 25C strain LMSA1.08.031 EF638701
Uncultured fungus clone F4 96 EU811365
x6-25 HQ234225
Paecilomyces lilacinus strain UPSC 1722 AF548079
Ascomycete sp. MV 3C strain LMSA1.08.014 EF638694
Paecilomyces lilacinus strain M1 FJ461772
Hypocreales
Volutella colletotrichoides AJ301962
x6-129 HQ234226
x9-137 HQ234230
Plectosphaerella cucumerina strain NRRL 20430 AF176951
x9-38 HQ234239
Plectosphaerella sp. MH727 FJ430715
x9-140 HQ234243
Phyllachorales
x4-167 HQ234220
Aspergillus sp. LVB1 DQ810193
Ascomycota sp. Mo7 EU887758
x12-94 HQ234232
Aspergillus terreus GQ338244
Aspergillus fumigatus strain YA -14 FJ560718
Penicillium chrysogenum strain TS04 FJ941877
x11-75 HQ317210
Penicillium sp. HAW-OCF5 AY887947
Penicillium sp. LAC1 FN666097
Penicillium sp. GT308 DQ810194
Penicillium chrysogenum strain UPSC 2020 AF548087
x13-108 HQ234241
x7-5 HQ234236
Penicillium sp. KF-25 GU361536
x10-49 HQ317206,x12-91 HQ317208
x13-105 HQ234235
Aspergillus ochraceus stain PF10 FJ941858
x5-113 HQ234221
Eurotiales
Marasmius alliaceus AY787214
100
71
96
88
95
56
96
53
69
99
56
98
96
67
94
51
85
99
0.05

(Table 1). Though most of the fungal isolates (97/177) showed very close affiliation with marine-derived relatives, as the results of other researchers [13, 21, 23, 46], they belonged to ubiquitous genera common to terrestrial habitats, suggesting they may be of terrestrial origin. But all the sponge-derived fungi were able to sporulate on media with ASW, suggesting that these fungi could at least be classified as "marine fungi."

Gao et al. [8] observed abundant fungi in the phylum *Ascomycota* in Hawaiian marine sponges *Suberites zeteki* and *Mycale armata* using culture-independent molecular method, meanwhile, *Ascomycota* fungi have been proven to be relatively easy to cultivate in vitro [3, 21, 31, 46]. Similarly, in this study, 10 genera (*Aspergillus*, *Ascomycete*, *Fusarium*, *Isaria*, *Penicillium*, *Plectosphaerella*, *Pseudonectria*, *Simplicillium*, *Trichoderma*, and *Volutella*) in four orders (*Eurotiales*, *Hypocreales*, *Microascales*, and *Phyllachorales*) of phylum *Ascomycota* were isolated from 10 species marine sponges in the South China Sea. Generally, fungal isolates from sponges are classified into three groups: "sponge-generalists"—found in all sponge species, "sponge-associates"—found in more than one sponge species, and "sponge-specialists"—found only in one sponge species [21]. In this study, though these sponges were collected in the same location, the isolated fungal number and species from different sponges were different. The different fungal phylogenetic diversity associated with different sponges in the same area of South China Sea also suggested that these fungi are not simply resulting from seawater column during the filter-feeding process. Though the fungal communities of different sponges were different, the phylogenetic diversity analysis showed that order *Eurotiales* especially genera *Penicillium*, *Aspergillus*, and order *Hypocreales* dominated the cultivable fungi in all the ten South China Sea sponges. This was in agreement with that of South China Sea sponges *C. luteoculcitella* and *Holoxea* sp. [7] as well as sponges from Hawaiian, Ireland, and other geographic locations [3, 13, 21, 31, 46], which suggested *Eurotiales* and *Hypocreales* fungi as sponge-generalists fungi. On the other hand, among the tested 10 sponges, *Phyllachorales* fungi mainly genus *Plectosphaerella* were observed only in a few sponges such as *P. foliascens* (X6) and *A. dispar* (X9), which were also detected in Hawaiian sponge *S. zeteki* [46]; *Microascales* fungi were found only in sponge *P. heteroraphis* (X13), which were also previously isolated from South China Sea sponge *C. luteoculcitella* [7]. Therefore, *Phyllachorales* and *Microascales* fungi may be sponge associates. It is essential to mention that *Microascales* have only been isolated from South China Sea sponges [7], indicating the potential different fungal community of South China Sea sponges from that of other geographic location sponges [3, 8, 13, 21, 23, 31, 46].

A putative instance of horizontal gene transfer involving adjacent, discrete KS, acyl carrier protein, and NRPS domains of the epothilone Type I polyketide biosynthetic gene cluster from the myxobacterium *Sorangium cellulosum* was identified by Lopez [24]. Schmitt et al. [37] found an ancient horizontal gene of PKS transfer event from an actinobacterial source into ascomycete fungi. In this study, in the comparison between the phylogenetic diversity of 18S rRNA genes and KS domain sequences, a lateral gene transfer event was indicated: the 18S rRNA gene defined isolate X4–167 as an *Aspergillus* member, but two *P. chrysogenum* KS domain sequences have been detected in isolate X4–167. All these suggest the horizontal gene transfer as a proposed mechanism in the evolution of biosynthetic gene clusters.

Table 2 Bioassay of antimicrobial activity of fungi associated with sponges

Strain	Indicator					
	E. coli	*P. fluorescens*	*S. aureus*	*B. subtilis*	*A. nigers*	*C. albicans*
X12-91	–	–	–	+	–	–
X11-75	–	–	–	+	–	–
X4-167	–	–	–	+	–	–
X12-95	–	++	–	–	–	–
X12-109	–	+	–	–	–	–
X13-105	–	+	+++	–	–	–
X10-49	–	+	–	–	–	–
X12-94	–	++	+	–	–	–
X6-13	–	+++	–	–	–	–
X10-63	–	+	–	–	–	–
X13-118	–	++	+	–	–	–

+++ large inhibition zone (r>5 mm); ++ middle inhibition zone (5 mm≥r>2 mm); + small inhibition zone (2 mm≥r>1 mm); – no inhibition zone (r≤1 mm)

Table 3 Detection of KS domain of PKS of fungi associated with sponges and their closest relatives

PKS fragment	Primer pair	Closest relative (accession no.)	Identity (%)
X10-49-1	KAF1/KAR1	*Penicillium chrysogenum* Wisconsin 54–1255 complete genome, contig Pc00c11(AM920436)	99
X12-91			99
X13-105			98
X4-167-1	LC1/LC2c		99
X11-77-2			98
X9-31-3	KAF1/KAR2		98
X10-49-2	KAF1/KAR1	*Penicillium chrysogenum* Wisconsin 54–1255 complete genome, contig Pc00c12(AM920437)	98
X4-167-2	LC1/LC2c		99
X11-77-1			99
X9-31-1	KAF1/KAR2		99
X11-77-3	LC1/LC2c	*Penicillium chrysogenum* Wisconsin 54–1255 complete genome, contig Pc00c13(AM920438)	98
X9-31-2	KAF1/KAR2		98
X12-94-1	KAF1/KAR1	*Aspergillus fumigatus* Af293 PKS,AFUA_8G00370 (XM_742074)	98
X4-167-3	LC1/LC2c		99
X12-94-2	KAF1/KAR1	*Aspergillus fumigatus* Af293 PKS, AFUA_1G17740 (XM_748048)	99

The suffix number after the strain number means the serial number of PKS fragments sequences

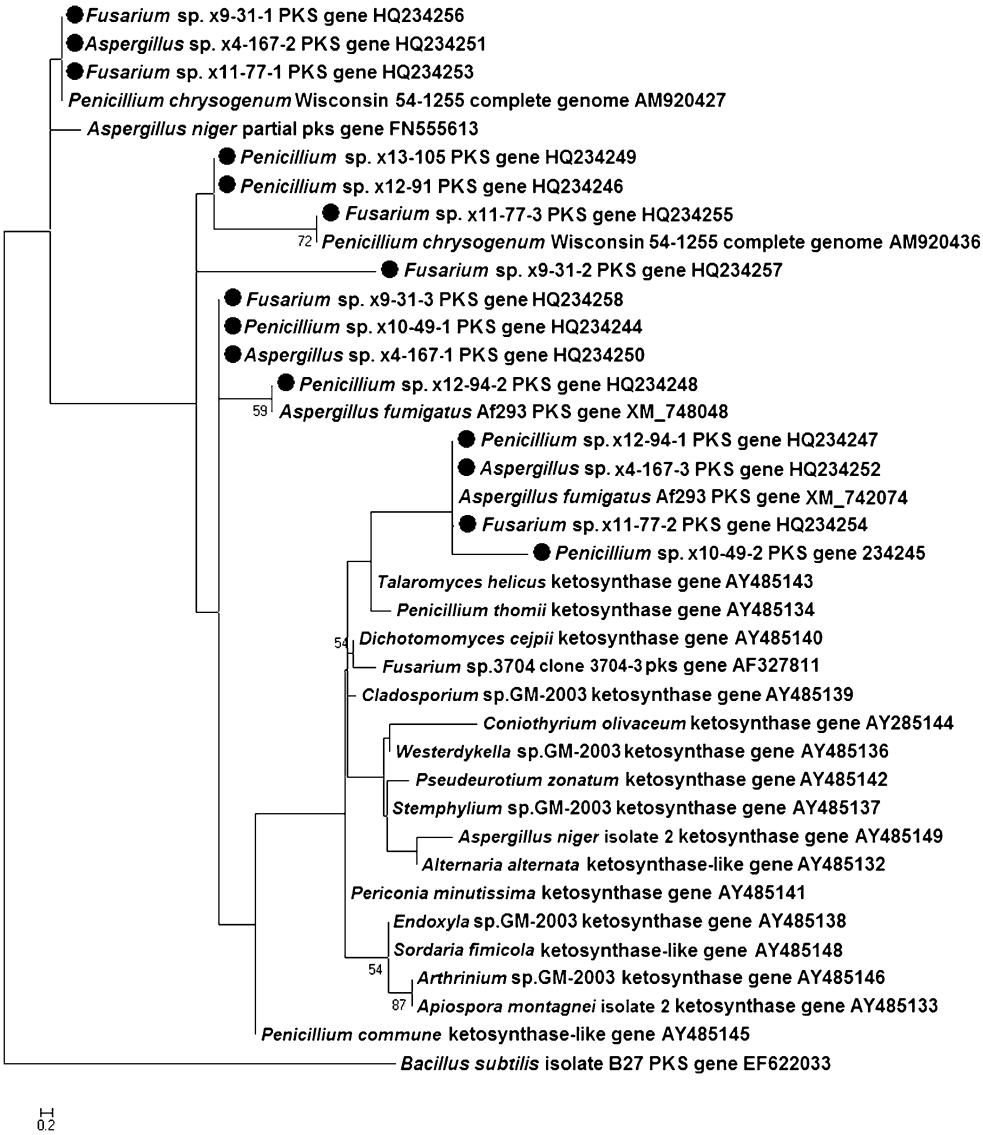


Figure 2 Polyketide synthase phylogenetic tree of sponge-derived fungi based on gene sequences of KS domain of PKS using Neighbor-joining method. *Numbers at branches* indicate Bootstrap values (>50%) from 1,000 replicates. The *scale bar* represents 0.2 nucleotide substitutions per site. The *black cycle* represents nucleotide sequences obtained in this study

Table 4 Detection of A domain of NRPS of fungi associated with sponges and their closest relatives

NRPS fragment	Primer pair	Closest relative (accession no.)	Identity (%)
X11-77-1	AUG003/AUG007	*Penicillium chrysogenum* Wisconsin 54–1255 complete genome, contig Pc00c13(AM920438)	99
X11-77-2	AUG003/AUG007	*Penicillium chrysogenum* Wisconsin 54–1255 complete genome, contig Pc00c22(NW_003020081)	99
X12-91-1	AUG003/AUG007	*Penicillium chrysogenum* Wisconsin 54–1255 alpha-aminoadipyl-cysteinyl-valine synthetase pcbAB(XM_002569068)	98
X12-91-2	AUG003/AUG007	*Penicillium chrysogenum* Wisconsin 54–1255 complete genome, contig Pc00c11(AM920436)	99

The suffix number after the strain number means the serial number of NRPS fragments sequences

Type I PKSs produce a vast array of biomedically important secondary metabolites such as antibiotic erythromycin, the immunosuppressant FK506 or the antiparasitic avermectin derivatives [30]. Almost all the characterized fungal PKSs belong to the type I (multifunctional, multidomain) enzyme class, which is generally divided into two subclasses: NR and reducing. NR PKSs include those synthesizing various fungal pigments, melanin, and aflatoxin while the reducing PKSs are involved in the synthesis of PKS compounds with various chemical reductions in structure [2]. In addition, the hybrid PKS–NRPS gene family is broadly distributed among the ascomycetous fungi, typically consisting of a type I iterative PKS module and a single-module NRPS. In this study, all the detected PKSs in sponge-associated fungi belong to type I including nine reducing and six non-reducing PKS KS fragments. The isolated sponge-associated fungi have a wide array of PKSs suggesting their potentials in the biosynthesis of different PKS compounds. For example, fungal isolates X9-31, X10-49, X11-77 and X4-167 had a lovastatin diketide synthase and a lovB nonaketide synthase, while X4-167 had another PKS similar to botryotinia fuckeliana polyketide synthase. Particularly, PKS or NRPS genes were detected in the fungal isolates with antimicrobial activity, for example isolates X4-167, X10-49, X12-91, X12-94 and X13-105, implying their potentials in the production of antimicrobial PKS or NRPS compounds. However, some isolates that had no PKS or NRPS genes, for example X6-13, X10-63, X11-75, X12-95, X12-109 and X13-118, also showed antimicrobial activity, which may resulted from other secondary metabolites instead of PKS/NRPS compounds.

It was the first time to detect PKS and NRPS genes in sponge-associated fungi, which extended our knowledge of marine sponge-associated fungi. Fungi associated with sponges are suggested to play ecological roles such as nutrient transfer and chemical defense [6]. Some sponge-derived fungi are able to produce novel antimicrobial compounds [1, 35]. The detected PKS and NRPS genes suggested the potentials of sponge-associated fungi in the

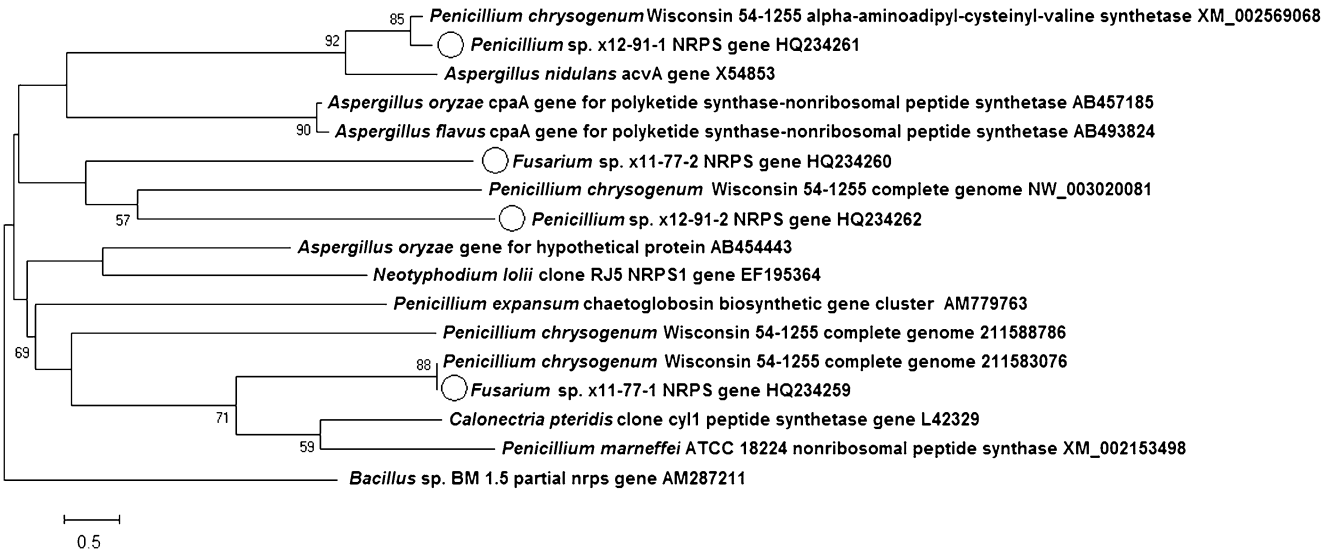


Figure 3 Non-ribosomal peptide synthase phylogenetic tree of sponge-derived fungi based on gene sequences of A domain of NRPS using Neighbor-joining method. *Numbers at branches* indicate Bootstrap values (>50%) from 1,000 replicates. The *scale bar* represents 0.5 nucleotide substitutions per site. The blank cycle represents nucleotide sequences obtained in this study

biosynthesis of related bioactive metabolites with chemical defense role for sponge at gene level to some extent. The observed antimicrobial activity of sponge-associated fungi in this study provided a directive evidence for the possible participation of these fungi in the antibiotic defense for sponge host [26].

Acknowledgements This work was supported by the High-Tech Research and Development Program of China (2011AA09070203), and National Natural Science Foundation of China (NSFC) (41076077, 30821005). The authors are grateful for the supply of sponges by Prof. Houwen Lin at Second Military Medical University, P. R. China.

References

1. Amagata T, Morinaka BI, Amagata A, Tenney K, Valeriote FA, Lobkovsky E, Clardy J, Crews PA (2006) Chemical study of cyclic depsipeptides produced by a sponge-derived fungus. J Nat Prod 69:1560–1565
2. Amnuaykanjanasin A, Punya J, Paungmoung P, Rungrod A, Tachaleat A, Pongpattanakitshote S, Cheevadhanarak S, Tanticharoen M (2005) Diversity of type I polyketide synthase genes in the wood-decay fungus *Xylaria* sp. BCC 1067. FEMS Microbiol Lett 251:125–136
3. Baker P, Kennedy J, Dobson A, Marchesi J (2009) Phylogenetic diversity and antimicrobial activities of fungi associated with *Haliclona simulans* isolated from Irish coastal waters. Mar Biotechnol 11:540–547
4. Blunt JW, Copp BR, Hu WP, Munro MHG, Northcote PT, Prinsep MR (2009) Marine natural products. Nat Prod Rep 26:170–244
5. Borneman J, Hartin RJ (2000) PCR primers that amplify fungal rRNA genes from environmental samples. Appl Environ Microbiol 66:4356–4360
6. Bugni TS, Ireland CM (2004) Marine-derived fungi: a chemically and biologically diverse group of microorganisms. Nat Prod Rep 21:143–163
7. Ding B, Yin Y, Zhang FL, Li Z (2010) Recovery and phylogenetic diversity of culturable fungi associated with marine sponges *Clathrina luteoculcitella* and *Holoxea* sp. in the South China Sea. Mar Biotechnol. doi:10.1007/s10126-010-9333-8
8. 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
9. Gesner S, Cohen N, Ilan M, Yarden O, Carmeli S (2005) Pandangolide 1a, a metabolite of the sponge-associated fungus *Cladosporium* sp., and the absolute stereochemistry of pandangolide 1 and iso-Cladospolide B. J Nat Prod 68:1350–1353
10. Grunig C, Brunner P, Du A, Sieber T (2007) Suitability of methods for species recognition in the *Phialocephala fortinii-Acephala applanata* species complex using DNA analysis. Fungal Genet Biol 44:773–788
11. Hentschel U, Usher KM, Taylor MW (2006) Marine sponges as microbial fermenters. FEMS Microbiol Ecol 55:167–77
12. Hochmuth T, Piel J (2009) Polyketide synthases of bacterial symbionts in sponges-Evolution-based applications in natural products research. Phytochemistry 70:1841–1849
13. Höller U, Wright AD, Matthee GF, Konig GM, Draeger S, Aust H, Schulz B (2000) Fungi from marine sponges: diversity, biological activity and secondary metabolites. Mycologia 104:1354–1365
14. 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 Diversity Press, Hong Kong, pp 172–204
15. Jebaraj CS, Raghukumar C, Behnke A, Stoeck T (2010) Fungal diversity in oxygen-depleted regions of the Arabian Sea revealed by targeted environmental sequencing combined with cultivation. FEMS Microbiol Ecol 71:399–412
16. Jiang S, Sun W, Chen M, Dai S, Zhang L, Liu Y, Lee K, Li X (2007) Diversity of culturable actinobacteria isolated from marine sponge *Haliclona* sp. Antonie Leeuwenhoek 92:405–416
17. Kennedy J, Codling CE, Jones BV, Dobson ADW, Marchesi JR (2008) Diversity of microbes associated with the marine sponge, *Haliclona simulans*, isolated from Irish waters and identification of polyketide synthase genes from the sponge metagenome. Environ Microbiol 10:1888–1902
18. Kim T, Fuerst J (2006) Diversity of polyketide synthase genes from bacteria associated with the marine sponge *Pseudoceratina clavata*: culture-dependent and culture-independent approaches. Environ Microbiol 8:1460–1470
19. Kohlmeyer J, Kohlmeyer E (1979) Marine Mycology: The Higher Fungi. Academic, London
20. König G, Kehraus S, Seibert S, Abdel-Lateff A, Müller D (2006) Natural products from marine organisms and their associated microbes. Chembiochem 7:229–238
21. Li Q, Wang G (2009) Diversity of fungal isolates from three Hawaiian marine sponges. Microbiol Res 164:233–241
22. 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
23. Liu WC, Li CQ, Zhu P, Yan JL, Cehng KD (2010) Phylogenetic diversity of culturable fungi associated with two marine sponges: *Haliclona simulans* and *Gelliodes carnosa*, collected from the Hainan Island coastal waters of the South China Sea. Fungal Divers 42:1–15
24. Lopez JV (2003) Naturally mosaic operons for secondary metabolite biosynthesis: variability and putative horizontal transfer of discrete catalytic domains of the epothilone polyketide synthase locus. Mol Gen Genomics 270:420–431
25. Maldonado M, Cortadellas N, Trillas MI, Ruetzler K (2005) Endosymbiotic yeast maternally transmitted in a marine sponge. Biol Bull 209:94–106
26. Müler WEG, Brümmer F, Batel R, Müler IM, Schröder HC (2003) Molecular biodiversity. Case study: Porifera (sponges). Naturwissenschaften 90:103–120
27. Nicholson T, Rudd B, Dawson M, Lazarus C, Simpson T, Cox R (2001) Design and utility of oligonucleotide gene probes for fungal polyketide synthases. Chem Biol 8:157–178
28. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C et al (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 438:1151–1156
29. Piel J (2004) Metabolites from symbiotic bacteria. Nat Prod Rep 21:519–538
30. Piel J, Hui D, Fusetani N, Matsunaga S (2004) Targeting modular polyketide synthases with iteratively acting acyltransferases from metagenomes of uncultured bacterial consortia. Environ Microbiol 6:921–927
31. Pivkin M, Aleshko S, Krasokhin V, Khudyakova Y (2006) Fungal assemblages associated with sponges of the southern coast of Sakhalin Island. Russ J Mar Biol 32:207–213
32. Pruesse E, Quast C, Knittel K, Fuchs B, Ludwig W, Peplies J, Glockner F (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196
33. Raghukumar C (2008) Marine fungal biotechnology: an ecological perspective. Fungal Divers 31:19–35
34. Rot C, Goldfarb I, Ilan M, Huchon D (2006) Putative cross-kingdom horizontal gene transfer in sponge (Porifera) mitochondria. BMC Evol Biol 6:71

35. 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
36. Schirmer A, Gadkari R, Reeves C, Ibrahim F, Delong E, Hutchinson C (2005) Metagenomic analysis reveals diverse polyketide synthase gene clusters in microorganisms associated with the marine sponge *Discodermia dissoluta*. Appl Environ Microbiol 71:4840–4849
37. Schmitt I, Lumbsch HT (2009) Ancient horizontal gene transfer from bacteria enhances biosynthetic capabilities of fungi. PLoS ONE 4:e4437
38. Siegl A, Hentschel U (2010) PKS and NRPS gene clusters from microbial symbiont cells of marine sponges by whole genome amplification. Environ Microbiol Rep 2:507–513
39. Slightom JL, Metzger BP, Luu HT, Elhammer AP (2009) Cloning and molecular characterization of the gene encoding the aureobasidin A biosynthesis complex in *Aureobasidium pullulans* BP-1938. Gene 431:67–79
40. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
41. Taylor J, Jacobson D, Kroken S, Kasuga T, Geiser D, Hibbet D, Fisher M (2000) Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31:21–32
42. Taylor MW, Hill RT, Piel J, Thacker RW, Hentschel U (2007) Soaking it up: the complex lives of marine sponges and their microbial associates. ISME J 1:187–190
43. Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev 71:295–347
44. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL-X windows interface:flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
45. Van Den Berg M, Albang R, Albermann K, Badger JH, Daran J, Driessen AJM, Garcia-Estrada C, Fedorova ND, Harris DM, Heijne WHM et al (2008) Genome sequencing and analysis of the filamentous fungus *Penicillium chrysogenum*. Nat Biotechnol 26:1161–1168
46. 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
47. Wu Z, Tsumura Y, Blomquist G, Wang X (2003) 18S rRNA gene variation among common airborne fungi, and development of specific oligonucleotide probes for the detection of fungal isolates. Appl Environ Microbiol 69:5389–5397
48. Zhang W, Li Z, Miao X, Zhang F (2009) The screening of antimicrobial bacteria with diverse novel non-ribosomal peptide synthetase (NRPS) genes from South China Sea sponges. Mar Biotechnol 11:346–355
49. Zhang W, Zhang FL, Li ZY, Miao XL, Meng QP, Zhang XS (2009) Investigation of sponge-associated cultivable bacteria with polyketide synthase genes and antimicrobial activity in the South China Sea. J Appl Microbiol 107:567–575