RESEARCH ARTICLE

Pezizomycotina dominates the fungal communities of South China Sea Sponges *Theonella swinhoei* and *Xestospongia testudinaria*

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Abstract

Compared with the knowledge of sponge-associated bacterial diversity and ecological roles, the fungal diversity and ecological roles of sponges remain largely unknown. In this study, the fungal diversity and protein synthesis potential in two South China Sea sponges *Theonella swinhoei* and *Xestospongia testudinaria* were investigated by rRNA vs. rRNA gene analysis. EF4/fung5 was chosen after a series of PCR tests to target fungal 18S rRNA and 18S rRNA gene. Altogether, 283 high-quality sequences were obtained, which resulted in 26 Operational taxonomic units (OTUs) that were assigned to *Ascomycota*, *Basidiomycota*, and *Blastocladiomycota*. At subphylum level, 77.3% of sponge-derived sequences were affiliated with *Pezizomycotina*. The fungal compositions of *T. swinhoei* and *X. testudinaria* were different from that of ambient seawater. The predominant OTU shared between two sponges was rare in seawater, whereas the most abundant OTUs in seawater were not found in sponges. Additionally, the major OTUs of sponge cDNA datasets were shared in two sponges. The fungal diversity illustrated by sponge cDNA datasets correlated well with that derived from sponge DNA datasets, indicating that the major members of sponge-associated fungi had protein synthesis potential. This study highlighted the diversity of *Pezizomycotina* in marine sponge–fungi symbioses and the necessity of investigating ecological roles of sponge-associated fungi.

Introduction

As ancient sessile-feeding metazoa, sponges (Porifera) have evolved over *c.* 600–800 million years and sponges have a close association with a wide variety of prokaryotic and eukaryotic microbes including bacteria, archaea, fungi and protists (Garson *et al.*, 1998; Hentschel *et al.*, 2006; Taylor *et al.*, 2007, 2012; Gao *et al.*, 2008; Lee *et al.*, 2010; Ding *et al.*, 2011; Schmitt *et al.*, 2011, 2012). Sponges not only harbor abundant symbionts but also offer enormous number of natural products (Taylor *et al.*, 2007). In particular, many cytotoxic compounds and an anti-microbial compound were isolated from sponge-associated fungi (D'Auria *et al.*, 1995; Höller *et al.*, 2000; Jadulco *et al.*, 2002; Hiort *et al.*, 2004; 4Proksch *et al.*, 2010). Investigations on sponge-associated fungi have been driven by the demands and development of marine drugs since 1994 (Cheng *et al.*, 1994), resulting frequently in detection or isolation of filamentous fungi and thousands of bioactive natural products (D'Auria *et al.*, 1995; Höller *et al.*, 2000; Jadulco *et al.*, 2002; Proksch *et al.*, 2010; Ding *et al.*, 2011; Zhou *et al.*, 2011; Yu *et al.*, 2013). However, compared with sponge-associated bacterial diversity, which is being drastically broadened by next generation sequencing (Lee *et al.*, 2010; Webster *et al.*, 2010; Schmitt *et al.*, 2011; Jackson *et al.*, 2012), knowledge of sponge-associated fungal diversity is still limited because the information is mainly obtained using cultivation-dependent strategies (Ding *et al.*, 2011; Zhou *et al.*, 2011; Yu *et al.*, 2013). Until now, few investigations have been carried out using culture-independent approaches to investigate the diversity of sponge-associated fungi (Gao *et al.*, 2008; Baker *et al.*, 2009).

Theonella swinhoei and *Xestospongia testudinaria* are two high-microbial-abundance (HMA) sponges, which are ubiquitous in the South China Sea. *Theonella swinhoei* is known to have great chemical diversity (Wilson *et al.*, 2014) and *X. testudinaria* harbors a highly complex bacterial community (Moitinho-Silva *et al.*, 2013), but little is known about their fungal diversity. It is worth investigating whether HMA sponges harbor diverse fungi, as a basis for understanding the sponge–fungi association. Given that fungi have frequently been isolated from South China Sea sponges (Ding *et al.*, 2011; Zhou *et al.*, 2011; Yu *et al.*, 2013), we hypothesized that diverse fungi exist in South China Sea sponges and are viable, e.g. with protein synthesis potential *in vivo*.

Ribosomal RNA (rRNA) gene-based strategy has been proven to be effective for characterizing the phylogenetic and taxonomic structures of microbial assemblages (Lee *et al.*, 2010; Webster *et al.*, 2010), but it has limitations when attempting to assess the metabolic potential. According to the review of Blazewicz *et al.* (2013), microbial rRNA infers not only taxonomic information but also the protein synthesis potential. Meanwhile, rRNA can be detected with high sensitivity since a cell generally contains thousands of ribosomes. In this study, both fungal 18S rRNA and 18S rRNA genes were analyzed to compare the fungi community structures of marine sponges *T. swinhoei* and *X. testudinaria*. Additionally, the total RNA of ambient seawater was used as a control to evaluate the differences in fungal communities between sponges and ambient seawater. These differences and the correlation between sponge DNA datasets and cDNA datasets highlighted the ecological roles of *Pezizomycotina* in sponge–fungi symbioses.

Materials and methods

Sample collection

The sponges *T. swinhoei* and *X. testudinaria* were collected by SCUBA diving within a 15-m radius at *c.* 10-depths near Yongxing Island (112°20′E, 16°50′N) in the South China Sea, and were morphologically identified by Prof. Jinhe Li at the Institute of Oceanology, Chinese Academy of Sciences. The sponges were placed into ziplock bags containing seawater. Samples from three individuals of each sponge species were cut into pieces thinner than 5 mm and smaller than 2 cm^3, including pinacoderm and choanoderm, rinsed twice with artificial seawater to remove the microbes loosely attached to the sponge surface and inner cavity, and then transferred to clean tubes containing 20 mL RNA Later® (Qiagen, Hilden, Germany). The total time between sponge collection and RNA Later® fixation was <15 min. All the sponge samples fixed with RNA Later® were kept at −80 °C until further processing. The seawater samples were collected at the same depth when the sponges were collected. The seawater samples were pooled (3 L in total) and filtered on 5-cm-diameter micro-pore (0.22 μm) filters. Each filter was then kept in 20 mL RNA Later® −80 °C until further treatment.

Nucleic acid extraction and cDNA synthesis

Sponge specimens were ground with liquid nitrogen using mortar and pestle. Then the total DNA and total RNA were extracted using AllPrep DNA/RNA mini kit (Qiagen). Extractions were performed separately for all three individuals of each specimen and then pooled. For seawater samples, the total RNA extraction was performed using RNeasy Mini Kit (Qiagen). After an hour-long digestion of genomic DNA, RNA samples were converted into single-strand cDNA with random hexamers using RevertAid First Strand cDNA Synthesis Kit (Thermo) according to the manufacturer's instructions. The RNA samples served as negative controls for subsequent PCRs. Nucleotide acid integrity was analyzed by agarose gel electrophoresis. Nucleic acid concentration was measured with the NanoDrop 2000c (PEQLAB Biotechnologie GmbH, Germany). DNA was stored at −20 °C and RNA at −80 °C before use.

PCR, cloning and sequencing

Multiple primers targeting fungal ITS region, SSU, and LSU rRNA were used (Table 1) (White *et al.*, 1990; Smit *et al.*, 1999; Borneman & Hartin, 2000; Díez *et al.*, 2001; Porter & Golding, 2012; Schoch *et al.*, 2012). Genomic DNA of an *Aspergillus terreus* strain, previously isolated by our group (Ding *et al.*, 2011), was used as a positive control for PCR. PCR was performed in triplicate as follows: 5 min denaturation at 94 °C, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing for 1 min (for temperature of each primer pair please see Table 1), and extending at 72 °C for 1 min. Final extension was achieved at 72 °C for 10 min. PCR products in expected lengths (listed in Table 1) were cut and purified by electrophoresis on a 1.2% (w/v) agarose gel using a gel purification kit (CWBIO, China) prior to cloning into the pCR2.1-TOPO vector and being transformed into One Shot Competent *Escherichia coli* cells using the TOPO TA cloning kit (Invitrogen). The positive recombinants were selected based on blue white colony screening and sequenced by Sangon Biotech (Shanghai, China) using M13F/R primers. The obtained sequences were edited with CHROMAS LITE version 2 (Technelysium).

Table 1. PCR primers and results

Primer	Length of PCR product (bp)	No. of sequenced clones: No. of fungal inserts	No. of sequenced clones: No. of sponge inserts	Annealing temperature (°C)	Reference
SSU					
nu-SSU-0817-5/nu-SSU-1196-3	379	57	35	54	Borneman & Hartin (2000)
nu-SSU-0817-5/nu-SSU-1536-3	719	17	37	56	
EF4/fung5	558	283	5	55	Smit *et al.* (1999)
EF4/EF3	1400	4	37	53	
EukA/EukB	1700	0	49	55	Díez *et al.* (2001)
ITS					
ITS1/ITS4	630	0	34	55	White *et al.* (1990)
ITS1/ITS2	300	0	29	56	
LSU					
LR0R/LR3	600	0	10	50	Poter & Golding (2012)
LR0R/LR5	950	0	17	48	Schoch *et al.* (2012)

Statistical and phylogenetic analysis

A local fungal database was constructed for MOTHUR 1.29 (Schloss *et al.*, 2009) according to the fasta file of SILVA SSU database release 115 (Quast *et al.*, 2013). Low quality sequences and chimeras were removed with MOTHUR before subsequent analysis. Taxonomy assignment was done by 'assign_taxonomy.py' in QIIME using naïve Bayesian classifier (Caporaso *et al.*, 2010). Considering the low phylogenetic resolution of 18S rRNA at lower ranks, the default depth of classification was order-level. If a sequence could not be classified at order-level, the taxonomy assignment was made to the lowest rank that fit the confidence level (80%). Operational taxonomic units (OTUs) were determined based on 3% cutoff using MOTHUR 1.29. Richness was estimated by 'alpha_diversity.py' in QIIME. Beta-diversity was calculated using 'beta_diversity.py' based on weighted UNIFRAC method in QIIME. Non-metric multidimensional scaling (nMDS) and UPGMA tree were plotted in MATLAB2010. Fungal sequences (*c.* 550 bp) and their next relatives determined by BLAST homology search (National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov/Blast.cgi) were imported into MEGA 5.1, aligned using CLUSTALW implemented in MEGA5.1 (Tamura *et al.*, 2011). The alignment was manually corrected using the editor tool in MEGA 5.1. Maximum likelihood, maximum parsimony and neighbor-joining trees were calculated in MEGA 5.1. Maximum parsimony bootstraps (100 resamplings) were performed to further assess the stability of topology structures. The 18S rRNA gene sequence of *Zoophagus insidians* was chosen as the outgroup.

Nucleotide sequence accession numbers

The representative sequences of OTUs in this study were deposited in the GenBank database under accession numbers KM245896–KM245921.

Results

Feasibility of different primers

To reveal the phylogenetic diversity of fungi associated with marine sponges *T. swinhoei* and *X. testudinaria*, nine primer sets were used to amplify the rRNA genes and ITS regions of fungi. As shown in Table 1, all PCR experiments were successful, but LR0R/LR5, LR0R/LR3, EukA/EukB, and primers targeting ITS regions amplified sponge sequences rather than fungal sequences. EF4/fung5 showed high fungi-specificity, recovering 283 fungal 18S rRNA fragments (including 88 sequences from seawater samples) but only five sponge sequences. Nevertheless, nu-SSU-0817-5/nu-SSU-1196-3, nu-SSU-0817-5/nu-SSU-1536-3, and EF4/EF3 showed low fungi-specificity and generated considerable sponge sequences. Hence, based on the fungi-specificity and efficiency of primers, EF4/fung5 was chosen to assess the fungal diversity in sponges. For the cohesiveness of comparison, EF4/fung5 was used to amplify fungal 18S rRNA fragments from seawater cDNA as well. As a result, all amplicons from seawater cDNA were assigned to fungi.

Richness and distribution pattern of sponge-associated fungi

As summarized in Table 2, all libraries had enough coverage (> 90%) to generate reliable diversity information. As indicated by the Chao1 index, the fungal richness in seawater was the highest, followed by *T. swinhoei* and *X. testudinaria*. With respect to OTU diversity (combining richness and evenness, taking the relative abundances of species into account), the fungal diversity of *X. testudinaria* was less diverse than that of seawater or *T. swinhoei*.

The rarefied datasets were further analyzed using weighted UNIFRAC, ordinated by non-metric multidimensional scaling

(nMDS) and clustered by UPGMA. From the nMDS analysis (Fig. 1a), the fungal community in seawater was distinct from those of sponges. No clear ordination pattern was observed among sponge datasets, whereas a clear clustering pattern and OTU distribution were illustrated by UPGMA (Fig. 1b). Interestingly, sponge cDNA datasets were more similar to each other than to the corresponding DNA datasets based on UPGMA clustering. As showed in Table 3, the sponge cDNA datasets correlated well with the corresponding DNA datasets: (1) for *X. testudinaria*, all four OTUs in cDNA dataset existed in DNA dataset; (2) for *T. swinhoei*, only two cDNA-derived OTUs were not found in DNA dataset; (3) all sponge datasets shared the same predominant OTU (OTU2). Furthermore, several noteworthy differences across five datasets were observed: (1) the top three abundant OTUs in seawater – OTU8, OTU21 and OTU1 – did not exist in sponge datasets; (2) the most abundant OTU in sponge datasets (OTU2) was one of the rare taxa in seawater; (3) two major OTUs in cDNA datasets, OTU18 and OTU10, were rare in DNA datasets; (4) 11 OTUs that were exclusively found in sponges were not abundant OTUs, except for OTU19 and OTU24; (5) OTU related to *Basidiomycota* only existed in sponge DNA datasets.

Table 2. Sequencing results and statistical analysis

	SW	TD	TR	XD	XR
No. of sequences*	88	49	53	44	49
No. OTUs†	15	10	9	7	4
Goods_coverage‡	0.955	0.918	0.925	0.932	0.980
Chao1§	17	12	12	9	4
Shannon¶	3.323	1.849	2.227	1.615	1.627
Simpson¶	0.875	0.529	0.703	0.502	0.647

TD, *Theonella swinhoei* DNA-derived library; TR, *T. swinhoei* cDNA-derived library; XD, *Xestospongia testudinaria* DNA-derived library; XR, *X. testudinaria* cDNA-derived library; SW, seawater cDNA-derived library.

*Only high quality sequences (283 out of 359) were showed and submitted to subsequent analysis.

†The observed OTU numbers which were close/equal to Chao1 indices suggested the sequencing effort was sufficient to generate reliable diversity information, which was supported by Goods_coverage (> 90%) as well.

‡Goods_coverage indicated the coverage of each library.

§Chao1, nonparametric richness estimator, indicated the richness of each dataset.

¶Shannon and Simpson are the diversity index. High Shannon/Simpson values imply high diversity.

Taxonomy assignments and phylogenetic analysis

Three fungal phyla, *Ascomycota*, *Basidiomycota* and *Blastocladiomycota*, were found in this study. According to Table 4, more than half of the OTUs (15/26) could not

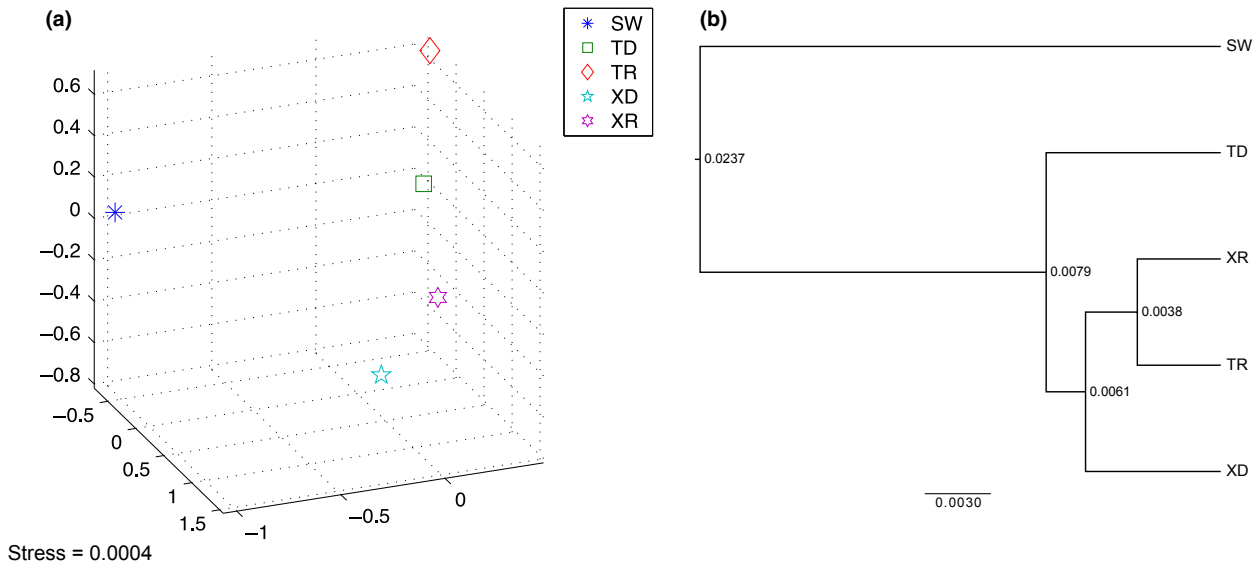

Fig. 1. Comparison of fungal community structures across all datasets. (a) Ordination analysis of fungal 18S rRNA gene clone libraries data based on weighted UNIFRAC measure of OTU abundances visualized by nMDS. Stress value = 0.0004 < 0.2 indicated the ordination was reliable. (b) UPGMA clustering pattern based on weighted UNIFRAC analysis. The jackknife value at each node was 1.0 based on 100 repetitions at 44 sequences per sample. The scale bar indicates the distance between clusters in UNIFRAC units. Bray–Curtis dissimilarity values are shown on the nodes of the tree. TD, *T. swinhoei* DNA-derived library; TR, *T. swinhoei* cDNA-derived library; X, *Xestospongia testudinaria* DNA-derived library; XR, *X. testudinaria* cDNA-derived library; SW, seawater cDNA-derived library.

Table 3. OTU distribution and BLAST results

OTU	XD	XR	TR	TD	SW	Next relative	ACCN	Identity (%)
OTU8	0	0	0	0	18	*Balansia henningsiana*	AY545727	99
OTU21	0	0	0	0	16	*Eupenicillium limosum*	EF411061	98
OTU1	0	0	0	0	14	*Peziza proteana*	AY544703	97
OTU17	1	0	0	0	9	*Balansia henningsiana*	AY545727	98
OTU18	1	11	7	1	8	*Mycosphaerella punctiformis*	DQ471017	100
OTU10	1	14	12	3	6	*Alternaria alternata*	DQ678031	99
OTU7	0	0	0	0	6	*Peziza proteana*	AY544703	97
OTU22	0	0	0	0	4	*Botryotinia fuckeliana*	AY544695	98
OTU2	26	23	25	33	1	*Aspergillus fumigatus*	AB008401	99
OTU23	0	0	0	0	1	*Neofabraea malicorticis*	AY544706	95
OTU4	0	0	0	0	1	*Xylaria hypoxylon*	AY544692	97
OTU14	0	0	0	0	1	*Aureobasidium pullulans*	DQ471004	94
OTU20	0	0	0	0	1	*Cryptococcus gastricus*	DQ645513	97
OTU5	0	0	0	0	1	*Eutypa lata*	DQ836896	94
OTU9	0	0	0	0	1	*Catenomyces* sp.	AY635830	99
OTU11	0	0	1	0	0	*Pleospora herbarum*	DQ767648	96
OTU12	0	0	2	0	0	*Catenomyces* sp.	AY635830	99
OTU13	0	0	1	1	0	*Orbilia auricolor*	DQ471001	97
OTU15	1	0	0	3	0	*Tricholoma aestuans*	AY757267	93
OTU16	0	0	1	1	0	*Petriella setifera*	DQ471020	98
OTU19	9	0	0	4	0	*Cladosporium* sp.	AY016351	99
OTU24	4	0	0	0	0	*Cochliobolus heterostrophus*	AY544727	99
OTU25	1	1	0	0	0	*Dothiora cannabinae*	DQ479933	99
OTU26	0	0	1	1	0	*Meria laricis*	DQ471002	94
OTU3	0	0	0	1	0	*Cladosporium herbarum*	DQ678022	98
OTU6	0	0	3	1	0	*Chaetomium globosum*	AY545725	99

XD, *Xestospongia testudinaria* DNA-derived library; XR, *X. testudinaria* cDNA-derived library; TD, *Theonella swinhoei* DNA-derived library; TR, *T. swinhoei* cDNA-derived library; SW, seawater cDNA-derived library.

be classified at order level, e.g. the top two abundant OTUs (OTU8 and OTU21) in seawater and the most abundant OTU in sponges (OTU2). Due to the uneven classification depth, it was not feasible to compare the fungal communities at order level. However, to summarize at subphylum level, 148 sponge-derived sequences were affiliated with various taxa of *Pezizomycotina*, constituting 77.3% of sponge-derived datasets. Particularly, 53% of the sponge-derived *Pezizomycotina* sequences came from sponge cDNA datasets. In the phylogenetic tree (Fig. 2) we did not find any clusters exclusively comprising sponge-derived sequences. OTU2, the predominant OTU in sponge samples, fell into the *Aspergillus* branch; and OTU10, another major OTU in sponge cDNA datasets, fell into *Pleosporales* branch with strong support (bootstrap value = 95), along with OTU24 and two sponge-derived fungal isolates.

Discussion

Methodological concerns

Variation of taxa abundances in DNA and cDNA were generally treated as a reflection of the differences in cell activity/metabolic rate between the taxa. Yet according to the recent review of Blazewicz *et al.* (2013), the correlation between rRNA abundance and cell activity is not always validated in environmental samples and rRNA measurements should be viewed as potential of protein synthesis. Nonetheless, this approach has been proved to be of value in analyzing active sponge-associated bacteria, especially when ambient seawater was used as a control (Moitinho-Silva *et al.*, 2013). Indeed, it would be safer to view rRNA as a measurement of protein synthesis potential rather than the evidence of cell activities. But as a matter of common sense, extracellular RNA molecules are highly unstable. Thus, regardless of whether the fungal cells are dormant or active *in vivo*, the detectable 18S rRNA indicates the completeness of cells, which ensures the basic activities of microbes and their ecological functions. Hence, the ecological roles of sponge-associated fungi should not be neglected/underestimated. It is worth exploring the functional gene diversity of sponge-associated fungi in future studies.

PCR primers play a crucial role in the molecular assessment of environmental microbes, thus the evaluation of primer specificity and efficiency is necessary. The specificity of the primer pairs is vital and allows selective or

Table 4. Higher-rank lineages information of each OTU

OTU	Phylum	Subphylum	Class	Subclass	Order
OTU8	*Ascomycota*				
OTU21	*Ascomycota*				
OTU18	*Ascomycota*				
OTU13	*Ascomycota*				
OTU19	*Ascomycota*				
OTU25	*Ascomycota*				
OTU26	*Ascomycota*				
OTU22	*Ascomycota*	*Pezizomycotina*			
OTU5	*Ascomycota*	*Pezizomycotina*	*Sordariomycetes*		
OTU6	*Ascomycota*	*Pezizomycotina*	*Sordariomycetes*		
OTU15	*Basidiomycota*	*Ustilaginomycotina*	*Exobasidiomycetes*		
OTU20	*Basidiomycota*	*Agaricomycotina*	*Tremellomycetes*		
OTU3	*Ascomycota*	*Pezizomycotina*	*Dothideomycetes*	*Dothideomycetidae*	
OTU2	*Ascomycota*	*Pezizomycotina*	*Eurotiomycetes*	*Eurotiomycetidae*	
OTU16	*Ascomycota*	*Pezizomycotina*	*Sordariomycetes*	*Hypocreomycetidae*	
OTU23	*Ascomycota*	*Pezizomycotina*	*Eurotiomycetes*	*Chaetothyriomycetidae*	*Chaetothyriales*
OTU14	*Ascomycota*	*Pezizomycotina*	*Eurotiomycetes*	*Chaetothyriomycetidae*	*Chaetothyriales*
OTU17	*Ascomycota*	*Pezizomycotina*	*Sordariomycetes*	*Hypocreomycetidae*	*Hypocreales*
OTU1	*Ascomycota*	*Pezizomycotina*	*Pezizomycetes*		*Pezizales*
OTU7	*Ascomycota*	*Pezizomycotina*	*Pezizomycetes*		*Pezizales*
OTU10	*Ascomycota*	*Pezizomycotina*	*Dothideomycetes*	*Pleosporomycetidae*	*Pleosporales*
OTU11	*Ascomycota*	*Pezizomycotina*	*Dothideomycetes*	*Pleosporomycetidae*	*Pleosporales*
OTU24	*Ascomycota*	*Pezizomycotina*	*Dothideomycetes*	*Pleosporomycetidae*	*Pleosporales*
OTU4	*Ascomycota*	*Pezizomycotina*	*Sordariomycetes*	*Xylariomycetidae*	*Xylariales*
OTU9	*Blastocladiomycota*		*Blastocladiomycetes*		*Blastocladiales*
OTU12	*Blastocladiomycota*		*Blastocladiomycetes*		*Blastocladiales*

The final depth of taxonomy is underlined.

enriching amplification of fungal rRNA genes from environmental DNA (Pang & Mitchell, 2005). Small subunit rRNA (SSU rRNA) has been used in marine fungi studies for a long time and is well presented in the current database, which is convenient for higher-rank level comparison and identification (Richards *et al.*, 2012). Nonetheless, as fungal SSU rRNA has very low resolution in lower ranks, internal transcription space (ITS) and large subunit rRNA (LSU rRNA) were also used as a phylogenetic marker in this study (Schoch *et al.*, 2012). In total, nine primer sets were selected to amplify the fungal sequences from sponge tissues, but only one primer pair was suitable for this aim. ITS regions have been successfully used to detect sponge-associated fungi (Gao *et al.*, 2008) and are becoming a universal barcode for fungal phylogenetic diversity research (Schoch *et al.*, 2012). One major concern in targeting fungal ITS regions is that there is no ITS region in mature rRNA, but Rajala *et al.* (2011) proved that fungal ITS primers could detect the related sequences in rRNA precursor. However, current ITS primers showed very low fungi-specificity in our study (Table 1). Gao *et al.* (2008) tested over 10 primer sets on Hawaiian sponge *Suberites zeteki*, including three ITS primer sets. According to their observations, ITS1/ITS4 was the only suitable primer set, whereas ITS1/ITS2 offered no fungal sequences. Hence, considering the inference of sponge ITS, the fungal ITS region may not be a universal barcode for studying fungal diversity in sponges. Among seven primer sets targeting SSU and LSU, EF4/fung5 yielded the best results, although this primer set had lower coverage in *Basidiomycota* compared to EF4/EF3 (Smit *et al.*, 1999). A very recent pyrosequencing survey of fungal and protistan diversity in sponges using eukaryotic universal primers (3NDf & euk_v4_R2) showed that amplicons of sponge 18S rRNA gene composed over 80% of total reads (> 99.5% in *T. swinhoei*), which made it an inefficient way to target fungal diversity (He *et al.*, 2014). Together with previous researches, our results showed that when dealing with different sponge species, the specificity of primers may vary and the inference of sponges rRNA genes cannot be ignored, in which case, the coverage of primers might not be the prior concern.

According to the recent review from Lindahl *et al.* (2013), fungal 18S rRNA had very limited phylogenetic resolution at lower ranks and identical sequences could be found in hundreds of other species across entire fungi, which meant it was not always accurate to classify a fungi sequence based on a perfect BLAST hit. Thus, in this study we applied a composition-based method (naïve Bayes classifier) to address the taxonomic assignment (Wang

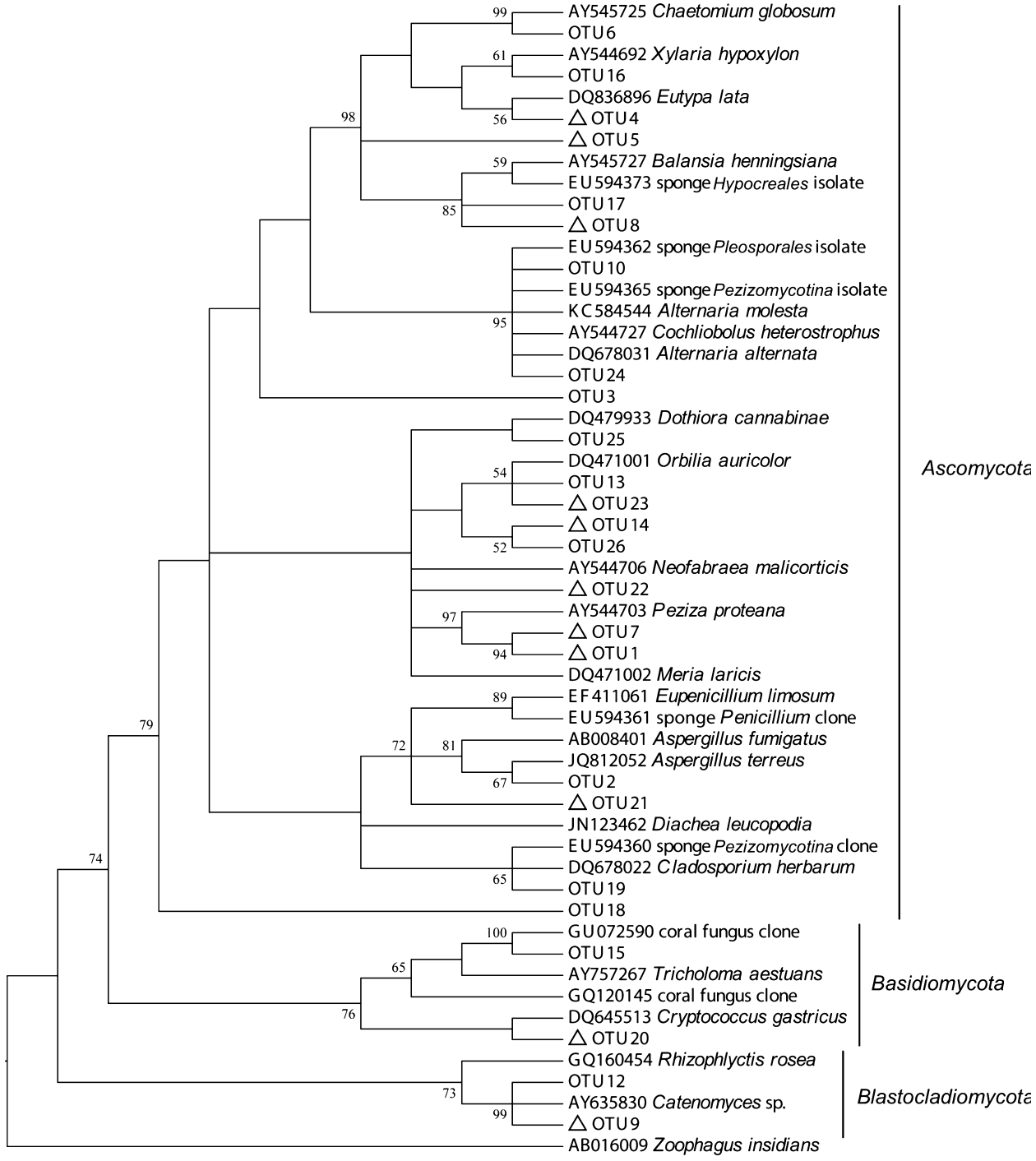

Fig. 2. Phylogenetic tree based on V2–V3 region of fungal 18S rRNA gene sequences (*c.* 550 bp) built by maximum-likelihood method using K2 + G as model: maximum parsimony tree and neighbor-joining tree (Supporting Information, Fig. S1) provided similar topology structure. Bootstrap values (100 replicates) higher than 50% were shown. OTUs that were only found in seawater were marked with triangles. *Zoophagus insidians* was chosen as the outgroup.

et al., 2007; Liu *et al.*, 2012). Using this strategy, we found that more than half of the OTUs could not be classified at order level even though some OTUs shared high identity with the references. For instance, OTU18 could only be assigned to *Ascomycota*, even though it shared 100% identity with *Mycosphaerella punctiformis* (Table 3).

According to phylogenetic analysis (Fig. 2), OTU18 formed a unique branch with no closely related sequences, which supported the classification result instead of the BLAST hit. OTUs without order-level affiliation might represent novel marine fungi, especially OTU8, OTU18, and OTU2, but we need other approaches, for example metagenomics and single-cell sequencing, to resolve their phylogeny and investigate their functions.

Dominance of *Pezizomycotina* in *T. swinhoei* and *X. testudinaria*

Pezizomycotina is the largest subphylum of *Ascomycota* and includes the vast majority of filamentous species, with roles in numerous ecological processes and symbioses (Spatafora *et al.*, 2006). In this study, over 75% of sponge-derived sequences were related to *Pezizomycotina* (Table 3), particularly the top two abundant OTUs (OTU2 and OTU10) shared by two sponge cDNA datasets. A similar dominance of *Pezizomycotina* in cDNA-derived library was observed in *Haliclona simulans* (Baker *et al.*, 2009). Noticeably, OTU2, the predominant OTU in sponges, was rare in ambient seawater (Table 3), suggesting that this OTU was more viable in sponges than in seawater. Similarly, although OTU10 was not rare in the seawater dataset, its relative abundance was lower than in sponge cDNA datasets (6.8% vs. 25.5%). Besides the two major OTUs, three less abundant *Pezizomycotina* OTUs – OTU6, OTU11, and OTU16 – were found in the *T. swinhoei cDNA* dataset, which suggested the protein synthesis potential of these rare taxa. Additionally, dominance of *Pezizomycotina* sponge-associated fungal communities was also reported in culture-dependent studies on South China Sea sponges (Ding *et al.*, 2011; Yu *et al.*, 2013) and Irish coastal sponges (Baker *et al.*, 2009).

Comparison of fungal diversity between sponge holobionts and seawater

In the first molecular detection of sponge-associated fungi, DGGE fingerprints demonstrated that the fungal diversity in sponges was different from that in the ambient seawater (Gao *et al.*, 2008). Our study echoed this finding (Fig. 1). In terms of rRNA vs. rRNA gene analysis, a recent research showed that the 'sponge-specific' bacteria could be detected in seawater, albeit with relatively low rRNA abundance (Moitinho-Silva *et al.*, 2013). Similarly, in our research, the predominant OTU (OTU2) in sponge cDNA datasets was rare in seawater dataset and the abundant OTUs, e.g. OTU8, OTU21, in seawater were not found in sponge datasets (Table 3), suggesting the selective enrichment of sponge-associated fungi, as observed previously in sponge-associated bacteria studies (Webster *et al.*, 2010).

Richards *et al.* (2012) pointed out that the filamentous fungi were poorly represented in marine clone libraries and *Dikarya* yeasts appeared to dominate the known diversity of marine fungi. But together with the molecular evidence from Gao *et al.* (2008) and Baker *et al.* (2009), filamentous fungi, mostly *Pezizomycotina*, were prevalent in the clone libraries of marine sponges. In previous studies, 21 orders of *Ascomycota* (*Boliniales*, *Botryosphaeriales*, *Capnodiales*, *Chaetosphaeriales*, *Claromycetales*, *Diaporthales*, *Dothideales*, *Eurotiales*, *Helotiales*, *Hypocreales*, *Microascales*, *Moniliales*, *Mucorales*, *Onygenales*, *Phyllachorales*, *Pleosporales*, *Polyporales*, *Saccharomycetales*, *Sordariales*, *Trichosphaeriales*, and *Xylariales*) and eight orders of *Basidiomycota* (*Agaricales*, *Agaricostilbales*, *Corticiales*, *Malasseziales*, *Polyporales*, *Sporidiobolales*, *Tremellales*, and *Wallemiales*) were found in marine sponges (Höller *et al.*, 2000; O'Brien *et al.*, 2005; Wang, 2006; Gao *et al.*, 2008; Proksch *et al.*, 2008, 2010; Wang *et al.*, 2008; Baker *et al.*, 2009; Li & Wang, 2009; Liu *et al.*, 2010; Paz *et al.*, 2010; Ding *et al.*, 2011; Rozas *et al.*, 2011; Wiese *et al.*, 2011; Zhou *et al.*, 2011; Suryanarayanan, 2012; Thirunavukkarasu *et al.*, 2012; Yu *et al.*, 2013), of which only two orders (*Saccharomycetales* and *Malasseziales*) were not filamentous fungi. Nonetheless, global sampling, deep sequencing, and application of multiple phylogenetic markers are necessary and will be greatly beneficial for revealing the diversity pattern of sponge-associated fungi.

Our understanding of the function of marine fungi is still quite limited (Richards *et al.*, 2012; Wang *et al.*, 2012). So far it has not been determined whether the symbiosis of sponge and fungi is a parasitic one (Richards *et al.*, 2012). However, there are several lines of evidence that can give us some ideas about the fungal roles in the sponge–fungi symbioses. On one hand, sponge-associated fungi display diverse biological activities, which make them the most prolific sources for bioactive compounds (Höller *et al.*, 2000; Proksch *et al.*, 2010). In particular, *Ascomycota* contributes more than 70% of natural products originated from sponge-associated fungi (Thomas *et al.*, 2010). Within *Pezizomycotina*, *Capnodiales*, *Eurotiales*, and *Pleosporales* are able to produce natural products with multiple cytotoxic activities (D'Auria *et al.*, 1995; Höller *et al.*, 2000; Jadulco *et al.*, 2002; Hiort *et al.*, 2004). In view of the antimicrobial and cytotoxic activities we found based on culture-dependent approaches (Ding *et al.*, 2011; Yu *et al.*, 2013), sponge-associated *Pezizomycotina* might be involved in the chemical defense of the sponge host. On the other hand, marine fungi are thought to be major contributors to the decomposition of organic matter (e.g. chitin, lignin) in coastal and

marine surface environments (Mann, 1988). Hence, the roles of fungi in the nutrient cycling within sponges cannot be ignored. Last but not least, fungi in water columns are of importance within the microbial food web in the coastal oceans in many ways, e.g. controlling energy flow, regulating food web dynamics, and with spores serving as food for zooplankton (Wang *et al.*, 2012). Thus, it would be worth investigating in the near future the roles of sponge-associated fungi in the food webs within marine sponges.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogenetic tree based on V2–V3 region of fungal 18S rRNA gene sequences (*c.* 550 bp) built by neighbor-joining method.