

Assessment of fungal diversity in deep-sea sediments by multiple primer approach

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Abstract

Increasing evidence of the fungal diversity in deep-sea sediments has come from amplification of environmental DNA with fungal specific or eukaryote primer sets. In order to assess the fungal diversity in deep-sea sediments of the Central Indian Basin (CIB) at ~5,000 m depth, we amplified sediment DNA with four different primer sets. These were fungal-specific primer pair ITS1F/ITS4 (internal transcribed spacers), universal 18S rDNA primers NS1/NS2, Euk18S-42F/Euk18S-1492R and Euk18S-555F/Euk18S-1269R. One environmental library was constructed with each of the primer pairs, and 48 clones were sequenced per library. These sequences resulted in 8 fungal Operational Taxonomic Units (OTUs) with ITS and 19 OTUs with 18S rDNA primer sets respectively by taking into account the 2% sequence divergence cut-off for species delineation. These OTUs belonged to 20 distinct fungal genera of the phyla Ascomycota and Basidiomycota. Seven sequences were found to be divergent by 79-97 % from the known sequences of the existing database and may be novel. A majority of the sequences clustered with known sequences of the existing taxa. The phylogenetic affiliation of a few fungal sequences with known environmental sequences from marine and hypersaline habitat suggests their autochthonous nature or adaptation to marine habitat. The amplification of sequences belonging to Exobasidiomycetes and Cystobasidiomycetes from deep-sea is being reported for the first time in this study. Amplification of fungal sequences with eukaryotic as well as fungal specific primers indicates that among eukaryotes, fungi appear to be a dominant group in the sampling site of the CIB.

Key words: Uncultured fungi, 18S rRNA gene, ITS primers, deep-sea sediments, Central Indian Basin.

Introduction

Microbial communities existing in deep-sea oceanic environments account for a total cellular carbon content of $\sim 3 \times 10^{17}$ g (Whitman et al. 1998). These deep-sea microbial communities, mainly of bacteria, archaea, protists and fungi play an important role in the recycling of the nutrients (Snelgrove et al. 1997). Among these, bacteria and archaea have been studied in detail (Stackebrandt et al. 1993; Urakawa et al. 1999; Li et al. 1999; Takai and Horikoshi 1999; DeLong and Pace. 2001; Sogin et al. 2006; Hongxiang et al. 2008; Luna et al. 2009). A few recent studies have reported eukaryotic diversity from extreme environments such as hydrothermal vents (López-García et al. 2003, 2007), anoxic environments (Stoeck and Epstein 2003, Stoeck et al. 2003, 2006; Jebaraj et al. 2010) and deep-sea sediments (Bass et al. 2007; Edgcomb et al. 2011).

The eukaryotic diversity studies have been executed by molecular survey of ribosomal genes using culture-independent approach in most of the recent reports. An unexpected high diversity of eukaryotic rDNA sequences was reported from the deep-sea (López-García et al. 2001). Using eukaryotic specific primers, several studies have reported only a small fraction of total rDNA sequences affiliating with fungi in comparison with other eukaryotic lineages (López-García et al. 2001; Stoeck et al. 2003, 2006). However, Edgcomb et al. (2011), while using eukaryotic specific primers, reported fungi to be dominant in marine deep-sea subsurface. The use of multiple primer approach for studying diversity has revealed recovery of diverse fungal forms from oxygen depleted marine environments (Jebaraj et al. 2010).

Using culture-dependent approach, fungi were isolated and identified from the deep-sea sediments of the CIB (Raghukumar et al. 2004; Damare et al. 2006; Singh et al. 2010). Further Singh et al. (2011) analyzed the fungal diversity in deep-sea sediments using culture-independent approach by targeting universal 18S as well as fungal specific and universal ITS (internal transcribed spacers) regions of rRNA genes from three locations in the CIB. It is known that some of the primer pairs designed for amplification of fungal sequences may co-amplify non-fungal templates from environmental samples leading to inaccurate estimation of fungal diversity (Borneman and Hartin, 2000). In addition, the specific primers designed for amplification of fungal 18S rDNA and ITS regions may be biased towards certain fungal taxonomic groups. In order to overcome these problems, we used a multiple-primer approach to study fungal diversity in the present work. The efficiency of primers for amplification of fungal as well as other eukaryotic sequences from mixed environmental samples was evaluated by targeting ITS and 18S rDNA regions. The primer set used for ITS region was fungal specific, whereas all the three 18S rDNA primer sets used were universal. Fungal and eukaryotic 18S

rDNA region share some common sequences resulting in cross amplification by the primers designed for this region (Anderson et al. 2003). Such primers may vary in their specificity in amplifying different fungal taxonomic groups. Therefore, we hypothesized that using several 18S rDNA primer sets may help in estimating fungal diversity with greater accuracy.

Materials and methods

Sampling

Sediment sample was collected on the cruise ABP-26 on board the Russian research vessel Academic Boris Petrov in December 2006 from the station SVBC-33 at a depth of ~5,000 m. The seafloor at this site is characterized by the presence of homogenous, soft to slightly compact, dark brown coloured sediments with yellowish bands at shallower and mottling of dusky brown bands at deeper depths. The sediment was of siliceous nature. The sampling procedure described by Raghukumar et al. (2004) and Damare et al. (2006) was followed. Sediment was collected with an USNEL-type box corer of 50 cm³ size. The sample thus collected was undisturbed and compact. Sub-cores of sediment were collected using a sterile PVC cylinder of 5 cm inner diameter. The length of the sediment core obtained was 30 cm. Subsections of 2 cm down to a depth of 10 cm and thereafter every 5 cm length down to 30 cm depth were cut from the sediment core and directly introduced into sterile plastic bags to avoid exposure to aerial contamination. The sediment was stored at -20°C immediately after sampling. In order to monitor the contamination by air-borne fungi, fungal media plates were exposed to the air for 10 min on the deck of the research vessel where the cores were received and in the microbiology laboratory on board the research vessel.

DNA extraction, environmental PCR and clone library analyses

Environmental DNA was isolated from each frozen sub-section of the sediment cores under sterile conditions to avoid cross contamination. DNA was isolated from 0.5 g of the sediment sample from each subsection of the core using the Q-Bio gene Soil DNA extraction kit (MP Biomedicals, OH, USA) according to the manufacturer's instructions. The DNA samples from all the subsections were pooled together and was amplified using fungal-specific primer pair, ITS1F/ITS4 as well as universal 18S rDNA primers, NS1/NS2, Euk18S-42F/Euk18S-1492RE and Euk18S-555F/Euk18S-1269R (Table 1). The conditions for PCR included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 15 min. The PCR reaction mixture (50 µl) consisted of 50 µg bovine serum albumin (New England Biolab), 0.6 U *Taq* DNA polymerase (Bangalore Genei, India),

1.5 mM MgCl₂, dNTPs (0.2 mM each), primers (0.5 μM each), and 1 x PCR buffer (Roche, Switzerland). Reaction mixture without template DNA was used as a negative control, and sediments spiked with fungal DNA was used as a positive control. Amplified products were gel-purified and ligated with pGEM-T easy vector (Promega, USA) and transformed into *E. coli* cells (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Transformants were grown overnight at 37°C on Luria Bertani agar containing 100 μg ml⁻¹ of ampicillin. White colonies were screened for the presence of insert by single colony lysis PCR with M13 forward and reverse primers. PCR protocol included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. A total of 4 environmental gene libraries, one each from the four primer sets was obtained. Forty eight clones were screened from each library. Clones containing positive insert were further processed for plasmid isolation and purification using Millipore plasmid preparation kit (Millipore, USA). Sequencing of the plasmids was done at the National Centre for Cell Sciences, Pune, India, using the Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) according to the manufacturer's protocol and analyzed in a DNA Analyzer (3730 DNA Analyzer, Applied Biosystems, USA).

Phylogenetic analyses

Forward and reverse sequences were assembled using Chromas Pro version 1.34. Sequences obtained with ITS and 18S rDNA primers were analyzed separately. All the sequences were checked with Ribosomal Database Project for the presence of chimeras (Cole et al. 2004). These chimeric sequences were eliminated from subsequent analyses. Pairwise alignment of the sequences was carried out using Clustal W2 software (Thompson et al. 1994). Conserved motifs were identified, and sequences were trimmed manually. Clone sequences were grouped into operational taxonomic units (OTUs) by using sequence similarity cut-off value of 98% (O'Brien et al. 2005) by using MOTHUR software (Schloss et al. 2009). A representative sequence from each OTU was queried against NCBI-GenBank BLASTn search. Details are shown for the phylogenetic affiliation of the representative sequence from each OUT (Table 2). Multiple alignments were done for all the sequences along with their closest match in Clustal W. Gaps and ambiguously aligned sequences were removed from the further analyses. A phylogenetic analysis was conducted using distance setting maximum likelihood (ML) in MEGA 5.03 (Kumar et al, 2008) with 1000 bootstrap replicates. Individual phylogenetic trees were constructed with sequences obtained with ITS and each of the 18S rDNA primer set.

Nucleotide sequence accession numbers

The GenBank accession numbers of the nucleotide sequences determined in this study are presented in [Table 2](#).

Results

Physical parameters of the sampling site

The sediment sample collected for the present study was characterized by the presence of polymetallic nodules. The nodules abundance was found to be 0.4 Kg m⁻². The approximate salinity, temperature and pH were found to be 35 psu (practical salinity units), 3°C and 7.0 respectively. Among other parameters, the total proteins, carbohydrates and lipids were approximately 1.5, 1.0 and 1.0 mg g⁻¹ sediment respectively. Total bacterial counts (Acridine Orange Direct Counts) in this area ranged from 10⁷-10⁹ cells g⁻¹ dry sediment. Organic carbon ranged from 0.3-0.4 mg g⁻¹ dry sediment ([Sharma 2008](#)).

Analysis of libraries

No growth of aerial mycoflora was observed on media plates that were exposed on the deck and in the microbiology laboratory on board the research vessel. A total of 48 and 144 clones (3 x 48) from ITS and 18S primer pairs respectively, were sequenced from the environmental libraries. This resulted in 27 fungal OTUs, eight with ITS (17% relative frequency) and nineteen with 18S rDNA primer pairs (13 % relative frequency) after clustering on a basis of 98% sequence identity criterion. A total of 20 distinct fungal species were obtained from these 27 OTUs. The results are presented as **OTU_01 to OTU_08** obtained with fungal-specific ITS primer pair, **OTU_9 and OTU_10** with universal 18S rDNA primer pairs, NS1/NS2, **OTU_11 to OTU_20** with 18S rDNA primer pair Euk18S-42F/ Euk18S-1492RE and **OTU_21 to OTU_27** with 18S rDNA primer pairs Euk18S-555F/ Euk18S-1269R ([Table 2](#)).

None of the 18S rDNA primers amplified non-fungal eukaryotic sequences.. Out of the 48 and 144 total clones for ITS and 18S rDNA primer sets, 23 (47.9 %) and 39 (27.1 %) respectively were bacterial or chimeric in nature and thus were excluded from the analysis. The fungal OTUs mostly belonged to the phyla Ascomycota and Basidiomycota ([Table 2](#)). The number of singletons i.e. detected only once were four and ten respectively for the ITS and 18S rDNA primer sets ([marked with an *asterisk in Table 2](#)). The remaining OTUs were present ≥2 times in different libraries.

Diversity observed with different primer sets

The fungal specific primer set ITS1F/ITS4 amplified eight OTUs belonging to seven different classes of Ascomycota and Basidiomycota. Of these, the majority belonged to Basidiomycota (Fig 1a). A larger proportion of the sequences affiliated with the unidentified and uncultured fungal clones (Fig. 1a, Table 2). Among Basidiomycota, OTU_02 clustered with an uncultured clone belonging to the class Wallemiomycetes with 79 % similarity (Table 2). Two basidiomycetes, OTU_05 and OTU_07 affiliated with the sequences (Table 2) which have been isolated from sea water in the previous studies (NCBI data base).

Universal fungal 18S rDNA primer pair NS1/NS2 amplified sequences showing 99% similarity to two phylotypes i.e. uncultured *Malassezia* and *Aspergillus* clone, belonging to the class Exobasidiomycetes and Eurotiomycetes respectively (Table 2, Fig 2a).

The universal eukaryotic primer set 18S-42F/Univ1492RE, recovered fungal sequences belonging to ten different phylotypes and these belonged to six different classes of the phyla Ascomycota and Basidiomycota (Table 2). Three OTUs (OTU_15, OTU_17 and OTU_20) showed very low similarity (79, 80 and 94 %) with their closest relative in NCBI database and thus may be novel phylotypes. Most of the sequences amplified by this primer set clustered with uncultured fungal clones. OTU_15 and OTU_17 branched out forming a separate cluster within Basidiomycota (Fig. 3a).

The other universal eukaryotic primer set EK555F/EK1269R, amplified seven fungal phylotypes clustering with four different classes of Ascomycota and Basidiomycota (Fig. 4a). Three phylotypes OTU_21, OTU_22 and OTU_26 showed < 97% similarity with existing sequences in the database and thus may be new (Table 2). Out of these three, OTU_21 and OTU_22 affiliated with the sequences which were also amplified by NS1/NS2 primer set but with a similarity of 99% with sequences in the public database (Table 2). These three OTUs clustered separately from the sequences of their closest relative, with OTU_26 showing a maximum divergence (Fig. 4a).

Proportional distribution of different taxonomic groups in various clone libraries

The proportional distribution (shown in percentage) of fungal taxonomic groups varied in different clone libraries (Figs. 1b to 4b). *Trichosporon asahii* (32 %) formed a major portion of sequences amplified by the ITS primer set (Fig. 1b). Two sequences, namely uncultured clones of *Aspergillus* and *Malassezia* sp. amplified with 18S rDNA primers set NS1/NS2, showed distribution of 75 and 25% respectively (Fig. 2b). Maximum number of distinct fungal sequences was obtained

with eukaryotic primer set, 18S-42F/Univ1492RE, out of which *Wallemia* sp. (28%) was the dominant phylotypes (Fig. 3b). The four phylotypes, *Aphyllophorales* sp., *Nectria mauritiicola*, unidentified isolate f4Fc56 and unidentified fungal clone S3 amplified by this primer set were amplified by ITS1F/ITS4 primer set as well (Figs. 1b & 3b). A majority of the sequences amplified by these two primer sets belonged to Basidiomycota. The major phylotypes amplified by the primer set EK555F/EK1269R belonged to the class Eurotiomycetes of the phylum Ascomycota (Fig. 4b). The two sequences, *Aspergillus* and *Malassezia* sp amplified by this primer pair were amplified by NS1/NS2 as well (Figs. 2b and 4b). There were no common OTUs shared by the primer sets 18S-42F/Univ1492RE and EK555F/EK1269R (Figs. 3b & 4b).

Discussion

The aim of the present study was to obtain maximum diversity of fungi from the deep-sea sediments. Therefore, four different sets of primers were used to amplify fungal sequences from a single core of deep-sea sediment from the Central Indian Basin. Previous studies on fungal diversity from the same area in three distantly located stations did not show a rich fungal diversity (Singh et al. 2011) and was perhaps due to primer bias towards specific fungal taxonomic groups and limited number of primers used. Therefore, an attempt was made to study fungal diversity from a single core using four primer sets which included three 18S rDNA primers besides one fungal-specific ITS primer set. The percentage frequency of fungal phylotypes obtained in the present study with ITS and 18S rDNA primer pairs was 17 and 13% respectively. This was higher than in the earlier study conducted in the same area (Singh et al. 2011), where the frequency obtained was 4 and 6% respectively with ITS and 18S rDNA primer pairs. Therefore, a multiple-primer approach appears to be better for assessment of fungal diversity. Anderson et al. (2003) obtained high diversity of fungi from grassland soil samples by using four ITS and 18S rDNA primer sets.

The primers used in the present study have been reported to amplify diverse forms of eukaryotic lineages from mixed environmental samples from various habitats (López-García et al. 2003; Takishita et al. 2005; Stoeck et al. 2006;; Edgcomb et al. 2011). However, the absence of non-fungal eukaryotic sequences in the present study was intriguing. All the recovered sequences showed affiliation either with Ascomycota or Basidiomycota phyla whereas, the same fungal specific primers have been shown to amplify sequences from Zygomycota and Chytridiomycota also (O'Brien et al. 2005; Lai et al. 2007; Nagano et al. 2010). Non-detection of these two groups either by culture-dependent (Singh et al. 2010) or by culture-independent approach (Singh et al. 2011) suggests their absence or low abundance in sediments of the sampling sites. Additionally, as suggested by Gao et al.

(2010) the fungal primers used may have low specificity towards amplification of 18S rDNA sequences of Zygomycota and Chytridiomycota from mixed environmental samples. Out of the eight fungal OTUs obtained with ITS primer set, 4 were singletons and out of the nineteen OTUs obtained with 18S rDNA primer sets, 10 were singletons. Thus, ~ 50 % of the sequences were singletons indicating low abundance of these phylotypes. It also points out that diversity analysis is far from saturation in the present study and a greater number of sampling and clone libraries is required.

The sequences recovered with ITS1F/ITS4 primer set showed high number of phylotypes belonging to Basidiomycota which is concordant with the previous studies where basidiomycetous yeasts were found to be dominating forms in various deep-sea environments (Takishita et al. 2006; Bass et al. 2007).

One of the noticeable points in the present study was amplification of only two major phylotypes of unidentified *Malassezia* and *Aspergillus* species by the 18S rDNA primer set NS1/NS2. However, PCR artefact or primer bias cannot be attributed to this because these two phylotypes contributed to a large share in the phylotypes amplified by EK555F/EK1269R primer pair too (Fig. 4b). These species have been reported as potential pathogenic forms of animals (Lai et al. 2007). In addition, *Aspergillus* species was reported to play an important role in denitrification process in anaerobic marine sediments off Goa (Jebaraj et al. 2010). This suggests a possible versatile role of fungi in major ecological processes in the deep-sea.

The eukaryotic primer sets, 18S-42F/Univ1492RE and EK555F/EK1269R amplified a total of 17 fungal OTUs. Among these, 5 phylotypes (OTU_11, OTU_14, OTU_17, OTU_19 and OTU_24) affiliated with the sequences reported from marine habitat. Out of these, OTU_11 affiliated with the fungal sp. NIOCC#F15, which was obtained in culture from the CIB sediments and was identified as *Sagenomella* species by amplification of its 18S rDNA gene (Singh et al. 2010). The other phylotypes which were obtained in culturable form were OTU_07 and OTU_19 (*Rhodotorula* spp.) and OTU_23 and OTU_26 (*Aspergillus* spp.) in the previous studies (Singh et al. 2010). Growth and spore germination of these species under simulated conditions of deep sea proved their adaptation to such extreme conditions (Singh et al. 2010). One of the sequences, (OTU_24), amplified with EK555F/EK1269R showed high similarity with *Wallemia sebi* which was isolated from hypersaline waters of saltern and was demonstrated to be very efficient halophilic and osmophilic species (Kunčič et al. 2010). This species was also isolated from marine sponges (Gao et al. 2008; Liu et al. 2010). These findings suggest presence of salt tolerant species under such extreme conditions. There were no

overlapping sequences amplified by these two universal eukaryotic primer sets suggesting that each primer pair amplified specific 18S rRNA gene sequences.

None of the fungal phylotypes except *Trichosporon asahii*, obtained during the present study matched with the phylotypes obtained from the cores at different locations in the CIB, during the same sampling period (Singh et al. 2011). This suggests a spatial variation in distribution of fungal phylotypes, even in not so dynamic habitat such as deep-sea at ~5,000 m depth. The sequences belonging to Exobasidiomycetes amplified by all the four primer sets is being reported here for the first time from the deep-sea environment. Its presence in anoxic sediments off Goa (Jebaraj et al. 2010) and in coastal Hawaiian waters (Gao et al. 2010) was reported and is known to consist of species which are plant pathogens (Begerow et al. 2006). *Rhodotorula* sp. belonging to the subphylum Cystobasidiomycetes, reported in the present study is known to be a mycoparasite (Bauer and Oberwinkler 1991).

Proportional distribution of fungal sequences recovered with different primer sets varied (Figs. 1b to 4b). Among the four primer sets, the universal eukaryotic primer set 18S-42F/Univ1492RE amplified maximum number of diverse fungal phylotypes. These results are in contrast to the previous studies (López-García et al. 2003; Stoeck et al. 2006) where only a few fungal OTUs were obtained. Only a few sequences overlapped between different primer sets resulting in detection of high diversity of fungal phylotypes by multiple-primer approach (Fig 1b to 4b).

A total of seven OTUs affiliating with a percentage similarity of <97% with the existing sequences reported in the database were obtained in the present study. This indicates the efficient nature of the primers used to amplify such novel forms present in deep-sea sediments. These novel forms comprised mostly of the sequences matching with *Aspergillus*, *Malassezia* and unidentified fungal clones suggesting their possibility of being novel marine variants of the existing species. Jebaraj et al. (2010) also reported recovery of novel fungal sequences by using multiple primer approach.

The polymetallic nodules reported in the CIB are rich in Ba, Co, Cu, Fe, Mg and Mn (Nath et al, 1989). The recovery of fungi from this area suggests their tolerance to metals. In fact, Connell et al. (2009) have reported metal-tolerant yeasts producing siderophores from deep-sea basalt rocks in Vailulu'u Seamount, Samoa. These findings indicate active role of fungi in biogeochemical cycles in the deep sea.

In conclusion, the use of multiple primer approach enabled the recovery of diverse fungal phylotypes from mixed environmental sediment sample of the Central Indian Basin. The high diversity obtained in spite of less number of clones screened suggested the abundance of fungi under such extreme conditions. Some of the fungal sequences obtained in the present work have been earlier reported from marine environment. This supports their presence and ecological role in various biological processes in this environment. The presence of halophilic forms like *Wallemia* sp. from culture-independent study should be proved by culture-dependent approach using various culturing techniques which may enhance our understanding of salt tolerance in deep-sea fungi. Fungi such as *Sagenomella* species obtained by culture-dependent as well as culture-independent approach could be used as a model organism to understand pressure tolerance in fungi.

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References

- Anderson IC, Campbell CD, Prosser JI (2003) Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environ Microbiol* 5: 36-47
- Bass D, Howe A, Brown N, Barton H, Demidova M, Michelle H, Li L, Sander H, Watkinson SC, Willcock S, Richards TA (2007) Yeast forms dominate fungal diversity in the deep oceans. *Proc R Soc B* 274: 3069-3077
- Bauer R, Oberwinkler F (1991) The colacosomes: new structures at the host-parasite interface of a mycoparasitic basidiomycete. *Bot Acta* 104: 53-57
- Begerow D, Stoll M, Bauer RA (2006) phylogenetic hypothesis of *Ustilaginomycotina* based on multiple gene analyses and morphological data. *Mycologia* 98:906-916
- Borneman J, Hartin RJ (2000) PCR primers that amplify fungal rRNA genes from environmental samples. *Appl Environ Microbiol* 66: 4356–4360
- Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM, Garrity GM, Tiedje JM (2004) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res* 33: D294–D296

- Connell L, Barrett A, Templeton A, Staudigel H (2009) Fungal diversity associated with an active deep-sea volcano: Vailulu'u Seamount, Samoa. *Geomicrobiology J* 26: 597-605
- Damare S, Raghukumar C, Raghukumar S (2006) Fungi in deep-sea sediments of the Central Indian Basin. *Deep-Sea Res PT -1* 53:14-27
- DeLong EF, Pace NR (2001) Environmental Diversity of Bacteria and Archaea *Syst Biol* 50:470-478
- Edgcomb VP, Beaudoin D, Gast R, Biddle JF, Teske A (2011) Marine subsurface eukaryotes: the fungal majority. *Environ Microbiol* 13: 172-183
- Gao Z, Johnson ZI, Wang G (2010) Molecular characterization of the spatial diversity and novel lineages of mycoplankton in Hawaiian coastal waters. *ISME J* 4: 111-120
- 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
- Hongxiang X, Min W, Xiaogu W, Junyi Y, Chunsheng W (2008) Bacterial diversity in deep-sea sediment from north eastern Pacific Ocean *Acta Ecol Sinica* 28:479-485
- 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 Microb Ecol* 71:99-412
- Kumar S, Nei M, Dudley J, Tamura K (2008) MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* 9: 299-306
- Kunčič MK, Kogej T, Drobne D, Gunde-Cimerman N (2010) Morphological response of the halophilic fungal genus *Wallemia* to high salinity. *Appl Environ Microbiol* 76:329-337
- Lai X, Cao L, Tan H, Fang S, Huang Y, Zhou S (2007) Fungal communities from methane hydrate-bearing deep-sea marine sediments in South China Sea. *ISME J* 1:756–762
- Li L, Kato C, Horikoshi K (1999) Bacterial diversity in deep-sea sediments from different depths. *Biodivers Conserv* 8:659-677
- Liu WC, Li CQ, Zhu P, Yang JL, Cheng 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
- López-García P, Philippe H, Gail F, Moreira D (2003) Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the mid-Atlantic Ridge. *Proc Natl Acad Sci USA* 2:697-702
- López-García P, Rodríguez-Valera F, Pedros-Alio C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature* 409:603–607

- López-García P, Vereshchaka A, Moreira D (2007) Eukaryotic diversity associated with carbonates and fluid-seawater interface in Lost City hydrothermal field. *Environ Microbiol* 9:546-554
- Luna GM, Stumm K, Pusceddu A, Danovaro R (2009) Archaeal diversity in deep-sea sediments estimated by means of different terminal-restriction fragment length polymorphisms (T-RFLP) Protocols. *Curr Microbiol* 59:356-361
- Nagano Y, Nagahama T, Hatada Y, Nunoura T, Takami H, Miyazaki J, Takai K, Horikoshi K (2010) Fungal diversity in deep-sea sediments-the presence of novel fungal groups. *Fungal Ecol* 3:316-325
- Nath BN, Rao VP, Becker KP (1989) Geochemical evidence of terrigenous influence in deep-sea sediments up to 8°S in the Central Indian Basin. *Marine Geol* 87: 301-313
- O'Brien H, Parrent JL, Jackson JA, Moncalvo J, Vilgalys R (2005) Fungal community analysis by large-scale sequencing of environmental samples. *Appl Environ Microbiol* 71:5544-5550
- Raghukumar C, Raghukumar S, Sheelu G, Gupta SM, Nath BN, Rao BR (2004) Buried in time: culturable fungi in a deep-sea sediment core from the Chagos Trench, Indian Ocean. *Deep-Sea Res PT 1* 51:1759-1768
- Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres W, Thallinger GG, Horn DJV, Weber CF (2009) Introducing mothur: open source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75:7537-7541
- Sharma R (2008) Project report: Benthic environmental variability in the Central Indian Basin-III, Cruise #ABP26, National Institute of Oceanography, Goa, India, pp. 1-170
- Singh P, Raghukumar C, Verma P, Shouche Y (2010) Phylogenetic diversity of culturable fungi from the deep-sea sediments of the Central Indian Basin and their growth characteristics. *Fungal Divers* 40:89-102
- Singh P, Raghukumar C, Verma P, Shouche Y (2011) Fungal community analysis in the deep-sea sediments of the Central Indian Basin by culture-independent approach. *Microb Ecol* 61:507-517
- Snelgrove PVR, Blackburn TH, Hutchings P, Alongi D, Grassle JF, Hummel H, King G, Koike I, Lamshead PJD, Ramsing NBV, Solis-Weiss, Freckman DW (1997) The importance of marine sediment biodiversity in ecosystem processes. *Ambio* 26: 578-582
- Sogin M, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ (2006) Microbial diversity in the deep sea and underexplored "rare biosphere". *Proc Natl Acad Sci USA* 103:12115-12120
- Stackebrandt E, Liesack W, Goebel BM (1993) Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *J FASEB* 7:232-236

- Stoeck T, Epstein S (2003) Novel eukaryotic lineages inferred from small-subunit rRNA analyses of oxygen depleted marine environments. *Appl Environ Microbiol* 69:2657–2663
- Stoeck T, Hayward B, Taylor GT, Varela R, Epstein SS (2006) A multiple PCR-primer approach to access the microeukaryotic diversity in environmental samples. *Protist* 157:31–43
- Stoeck T, Taylor GT, Epstein SS (2003) Novel eukaryotes from the permanently anoxic Cariaco Basin (Caribbean Sea). *Appl Environ Microbiol* 69:5656–5663
- Takai K, Horikoshi K (1999) Genetic diversity of Archaea in deep-sea hydrothermal vent environments. *Genetics* 152:1285-1297
- Takishita K, Miyake H, Kawato M, Maruyama T (2005) Genetic diversity of microbial eukaryotes in anoxic sediment around fumaroles on a submarine caldera floor based on the small-subunit rDNA phylogeny. *Extremophiles* 9:185–196
- Takishita K, Tsuchiya M, Reimer JD, Maruyama T (2006) Molecular evidence demonstrating the basidiomycetous fungus *Cryptococcus curvatus* is the dominant microbial eukaryote in sediment at the Kuroshima Knoll methane seep. *Extremophiles* 10:165–169
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673-4680
- Urakawa H, Kita-Tsukamoto K, Ohwada K (1999) Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiol* 145:3305-3315
- Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: The unseen majority. *Proc Acad Natl Sci USA* 95:6578-6583

Legends to the figures

Fig. 1A Maximum likelihood (ML) phylogenetic tree for the fungal OTUs constructed based on fungal ITS gene sequences obtained using fungal specific primer set ITS1F/ITS4. Topology was built using Mega v.5.03 from a ClustalW 1.83 alignment. Numbers below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle.

1B. Proportional distribution of different fungal taxa in the clone library constructed with fungal specific primer set ITS1F/ITS4.

Fig. 2A ML phylogenetic tree for the fungal OTUs constructed based on fungal 18S rDNA gene sequences obtained using universal fungal 18S rDNA primer set, NS1/NS2. Topology was built using Mega v.5.03 from a ClustalW 1.83 alignment. Numbers below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle.

2B. Proportional distribution of different fungal taxa in the clone library constructed with 18S rDNA primer set NS1/NS2.

Fig. 3A ML phylogenetic tree for the fungal OTUs constructed based on 18S rDNA gene sequences obtained using universal eukaryotic 18S rDNA primer set, Euk18S-42F/Euk18S-1492RE. Topology was built using Mega v.5.03 from a ClustalW 1.83 alignment. Numbers below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle.

3B. Proportional distribution of different fungal taxa in the clone library constructed with 18S rDNA primer set Euk18S-42F/Euk18S-1492RE .

Fig. 4A ML phylogenetic tree for the fungal OTUs constructed based on 18S rDNA gene sequences obtained using universal eukaryotic 18S rDNA primer set, Euk18S-555F/Euk18S-1269R. Topology was built using Mega v.5.03 from a ClustalW 1.83 alignment. Numbers below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle.

4B. Proportional distribution of different fungal taxa in the clone library constructed with 18S rDNA primer set Euk18S-555F/Euk18S-1269R Uncultured *Malassezia* clone (22.2%) includes OTU_21 (11.1%) and OTU_27 (11.1%).

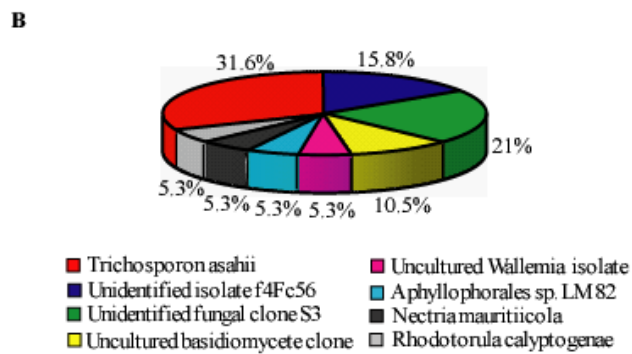
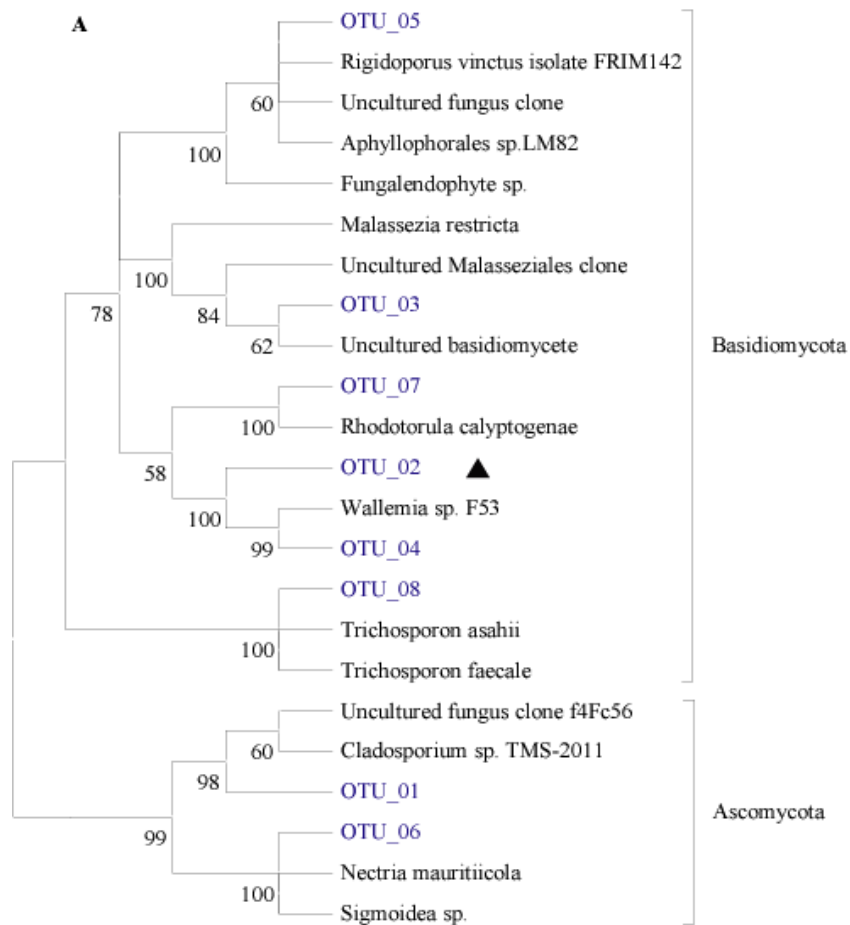


Fig. 1

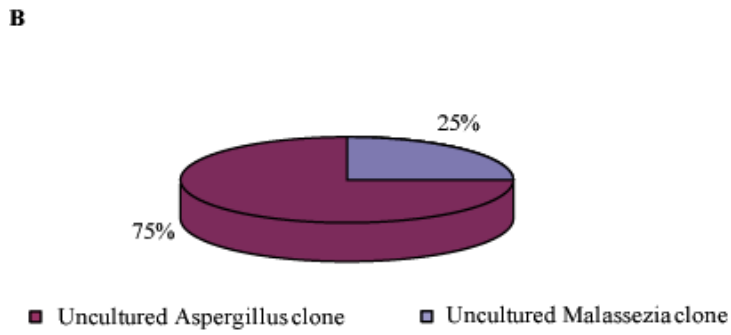
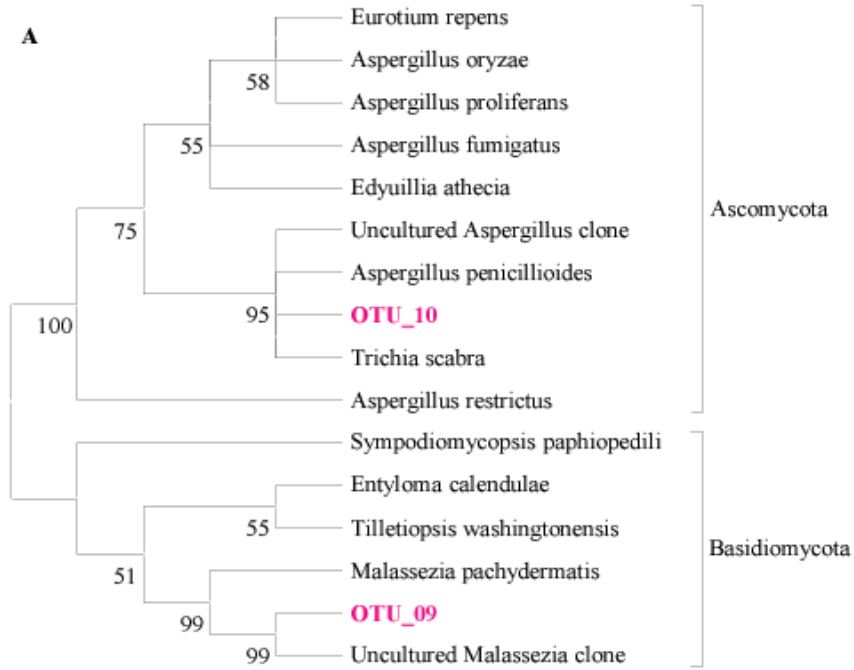


Fig. 2

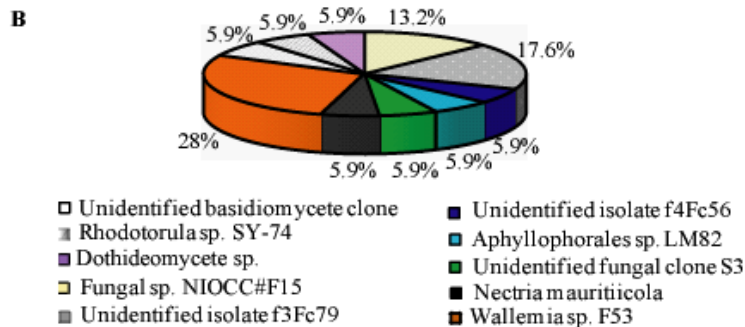
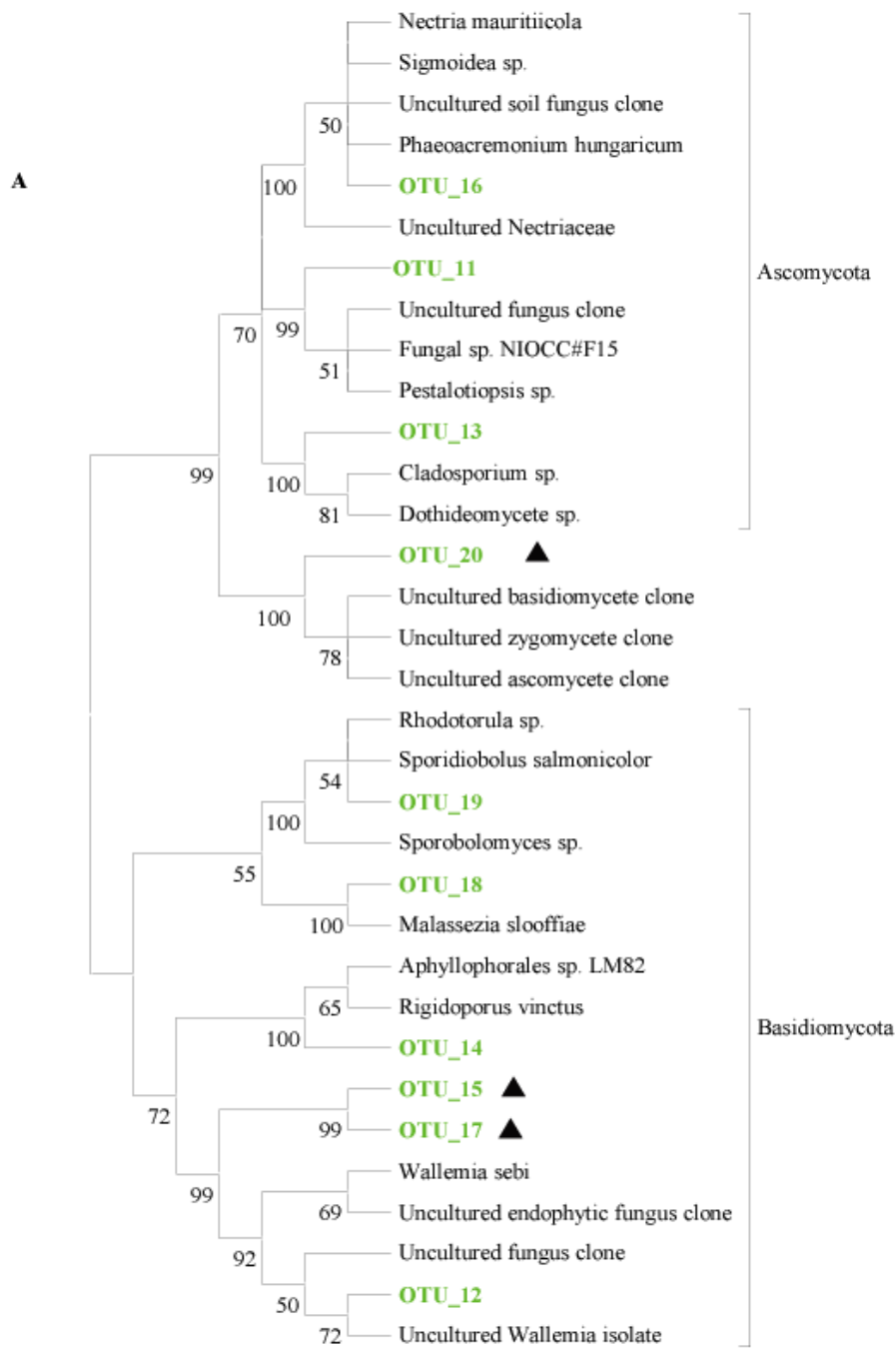


Fig. 3

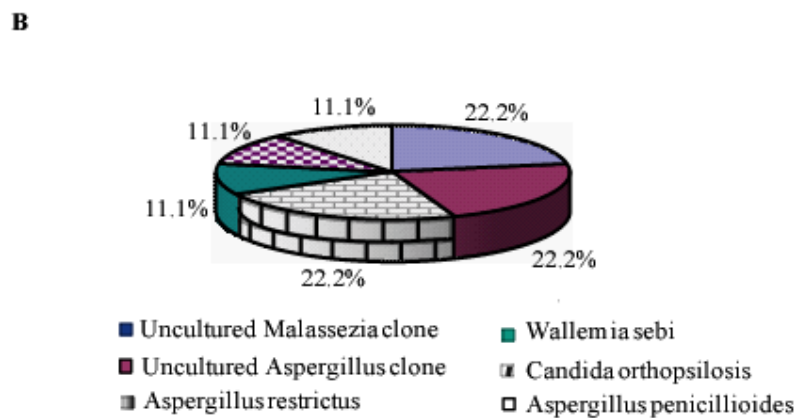
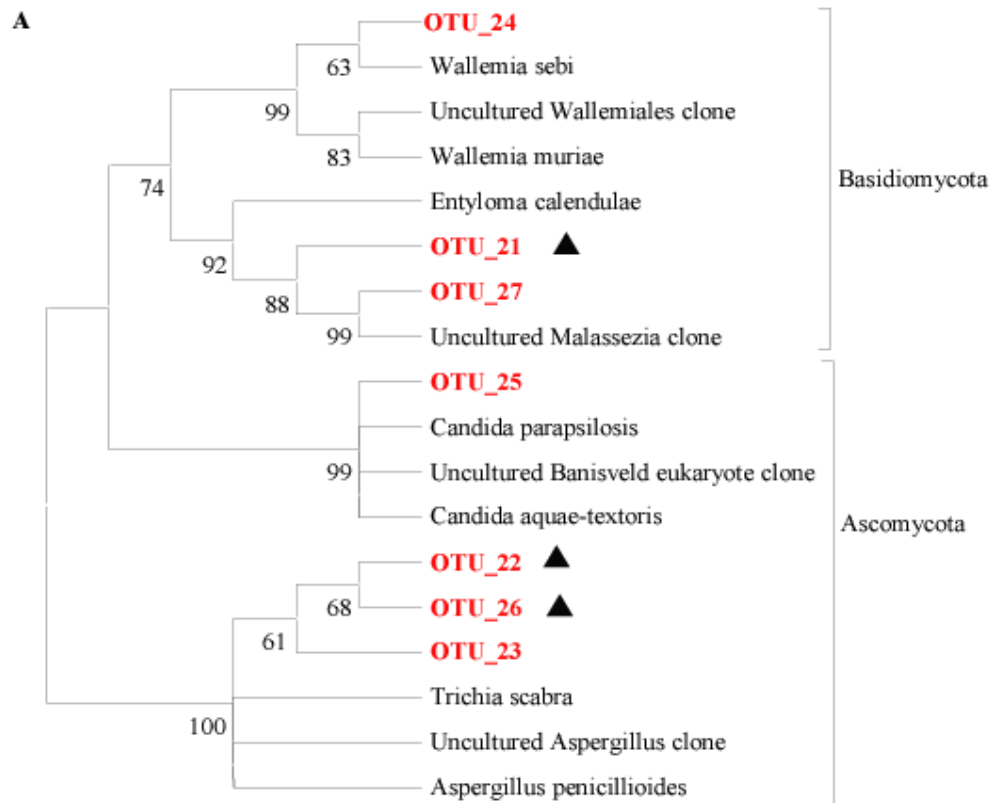


Fig. 4

Table 1. Details of the primers used.

Primer name	Sequences	Reference
ITS1F	5'-CTGGTCATTTAGAGGAAGTAA-3'	Lai et al. 2007
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	Lai et al. 2007
NS1	5'-GTAGTCATATGCTTGTCTC-3'	O Brien et al. 2005
NS2	5'-GGCTGCTGGCACCAGACTTGC-3'	O Brien et al. 2005
Euk18S-42F	5'-CTCAARGAYTAAGCCATGCA-3'	Lopez-Garcia et al. 2003
Euk18S-1492R	5'-ACCTTGTTACGRCTT-3'	Stoeck et al. 2006
Euk18S-555F	5'-AGTCTGGTGCCAGCAGCCGC-3'	Lopez-Garcia et al. 2003
Euk18S-1269R	5'-AAGAACGGCCATGCACCAC-3'	Lopez-Garcia et al. 2003

Tab Table 2. Phylogenetic affiliations of the OTUs obtained with different primer sets for station SVBC-33.

Singletons are represented with *

OTU no.	Primer set used	Accession no.	Best BLAST hit				Isolation source	% Similarity
			Taxon	Phylum	Class	Accession no.		
OTU_01		HM572248	Unidentified isolate f4Fc56	Ascomycota	Dothideomycetes	EU680535	Filter dust	99
OTU_02		GU370751	Unidentified fungal clone S3	Basidiomycota	Wallemiomycetes	FJ820491	Air sample	79
OTU_03		GU370752	Uncultured basidiomycete clone	Basidiomycota	Exobasidiomycetes	AM901800	House dust	99
OTU_04*		GU370753	Uncultured <i>Wallemia</i> isolate	Basidiomycota	Wallemiomycetes	GU931736	House dust	99
OTU_05*	ITS1F/	GU370754	<i>Aphyllophorales</i> sp. LM82	Basidiomycota	Agaricomycetes	EF060457	Sea water	98
OTU_06*	ITS4	GU370755	<i>Nectria mauritiicola</i>	Ascomycota	Sordariomycetes	AJ557830	Not known	99
OTU_07*		GU370756	<i>Rhodotorula calyptogenae</i>	Basidiomycota	Cystobasidiomycetes	EU669878	Sea water	99
OTU_08		HM572249	<i>Trichosporon asahii</i>	Basidiomycota	Tremellomycetes	AB369919	Not known	99
OTU_09	NS1/	HM572252	Uncultured <i>Malassezia</i> clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	99
OTU_10	NS2	HM572251	Uncultured <i>Aspergillus</i> clone	Ascomycota	Eurotiomycetes	FJ393417	Gut of HBV infected patients	99
OTU_11		GU370757	<i>Fungal</i> sp. NIOCC F15	Ascomycota	Sordariomycetes	FJ357782	Deep-sea sediments	99
OTU_12		GU370758	Unidentified isolate f3Fc79	Basidiomycota	Wallemiomycetes	GU721564	Filter dust	99
OTU_13*		GU370759	Unidentified isolate f4Fc56	Ascomycota	Dothideomycetes	EU680535	Filter dust	99
OTU_14*		GU370760	<i>Aphyllophorales</i> sp. LM82	Basidiomycota	Agaricomycetes	EF060457	Sea water	98
OTU_15*	18S-42F/	GU370761	Unidentified fungal clone S3	Basidiomycota	Wallemiomycetes	FJ820491	Air sample	79
OTU_16*	Univ149	GU370762	<i>Nectria mauritiicola</i>	Ascomycota	Sordariomycetes	AJ557830	Not known	99
OTU_17	2RE	GU370763	<i>Wallemia</i> sp. F53	Basidiomycota	Wallemiomycetes	FJ755832	Marine sponge	80
OTU_18*		GU370765	Unidentified basidiomycete clone	Basidiomycota	Exobasidiomycetes	AM901883	House dust	99
OTU_19*		GU370766	<i>Rhodotorula</i> sp. SY-74	Basidiomycota	Microbotryomycetes	AB025984	Deep-sea	99
OTU_20*		GU370767	Dothideomycete sp.	Ascomycota	Dothideomycetes	EU680530	Leaf	94
OTU_21		GU370768	Uncultured <i>Malassezia</i> clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	96
OTU_22		GU370769	Uncultured <i>Aspergillus</i> clone	Ascomycota	Eurotiomycetes	FJ393417	Gut of HBV infected patients	97
OTU_23	EK555F/	HM587247	<i>Aspergillus restrictus</i>	Ascomycota	Eurotiomycetes	AB008407	Not known	99
OTU_24*	EK1269	GU370771	<i>Wallemia sebi</i>	Basidiomycota	Wallemiomycetes	AY741380	Hypersaline water of saltern	98
OTU_25*	R	GU370772	<i>Candida orthopsilosis</i>	Ascomycota	Saccharomycetes	FN812686	Not known	99
OTU_26*		GU370773	<i>Aspergillus penicillioides</i>	Ascomycota	Eurotiomycetes	AB002077	Not known	91
OTU_27		GU370774	Uncultured <i>Malassezia</i> clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	99