

Fungal community analysis in the deep-sea sediments of the Central Indian Basin by culture-independent approach

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Abstract

Few studies have addressed the occurrence of fungi in deep-sea sediments, characterized by elevated hydrostatic pressure, low temperature and fluctuating nutrient conditions. We evaluated the diversity of fungi at three locations of the Central Indian Basin (CIB) at a depth of ~5000 m using culture-independent approach. Community DNA isolated from these sediments was amplified using universal and fungal-specific ITS and universal 18S rDNA primer pairs. A total of 39 fungal operational taxonomic units (OTUs), with 32 distinct fungal taxa were recovered from 768 clones generated from 16 environmental clone libraries. The application of multiple primers enabled the recovery of eight sequences that appeared to be new. The majority of the recovered sequences belonged to diverse phylotypes of Ascomycota and Basidiomycota. Our results suggested the existence of cosmopolitan marine fungi in the sediments of CIB. This study further demonstrated that diversity of fungi varied spatially in the CIB. Individual primer set appeared to amplify different fungal taxa occasionally. This is the first report on culture-independent diversity of fungi from the Indian Ocean.

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

Running title: Uncultured fungal diversity in deep-sea sediments

Introduction

Over 70 % of the earth is covered by oceans and ocean bottom which is covered with sediments of varying texture, form a major habitat for benthic organisms. Most marine sediments are located in a cold, dark, high hydrostatic pressure habitat where food comes from distant surface waters [33]. Several studies have described the presence of diverse forms of microbes from this habitat [24, 34, 36]. Besides bacteria fungi also play a major role in the global biogeochemical cycle of nutrients and have been reported from various terrestrial [11] and marine habitats [16], including deep-sea environments [8]. There is a growing interest in understanding the microbial diversity in extreme environments. However, diversity of fungi, their ecological role and abundance in the deep-sea which is one such environment, is poorly understood. Sporadic reports of fungi cultured from deep-sea sediments from various locations exist. Species of *Phoma*, *Lodderomyces*, *Malassezia*, *Cryptococcus*, *Cylindrocarpon*, *Hortaea*, *Pichia*, *Aspergillus* and *Candida* have been cultured from methane-hydrate bearing deep-sea sediments collected from water depths in South China down to ~3000 m [19]. While culturable fungi obtained from deep-sea sediments of the Central Indian Basin from ~5000 m depth were identified by classical morphology [8], those isolated by Singh et al., [32] were identified by molecular taxonomy. In both the cases, the fungi obtained showed growth under elevated hydrostatic pressure and low temperature. These studies may not depict the actual diversity of fungi in deep-sea sediments because of limitations in culturing methods and viability of fungal propagules. A few studies applying targeted environmental sequencing have reported existence of fungal species in deep-sea Antarctic plankton [23]. Using fungal-specific 18S rRNA gene libraries, Bass et al. [2] showed yeasts to be the dominant forms at several locations in the deep oceans. Presence of fungal signatures in hydrothermal sediments of the Mid-Atlantic ridge was reported by analysis of 18S rDNA sequences amplified with eukaryote-specific primers [22] whereas culturable fungi, identified with universal ITS primers from several hydrothermal vent sites were reported recently [5]. While studying microeukaryotic diversity in an acidic iron river, Gadanho and Sampaio [10] reported new fungal phylotypes with fungal-specific primers that were not detected when universal eukaryotic primers were used. These studies suggested that fungal diversity could have been underestimated in such extreme environments. In view of the above, we constructed gene libraries with universal and fungal-specific primer sets to assess the fungal diversity in the deep-sea sediments of the CIB through a culture-independent approach in the present study. Such primers have been known to amplify a variety of fungal sequences from mixed environmental samples [26]. Further, fungal community composition analysis was carried out for distantly located stations as well as two depths at each of these stations.

Methods

Sampling

Sediment samples were collected on the cruise ABP-26 on board the Russian research vessel Akademik Boris Petrov in December 2006 from three stations namely, SVBC-04 (Station A), SVBC-31 (Station B) and SVBC-37 (Station C) of the Central Indian Basin (Fig. 1) at a depth of ~5000 m (Table 1). The seafloor of the Central Indian Basin (CIB) is characterized by the presence of homogenous, soft to slightly compact, dark brown colored sediments with yellowish colored sediments at deeper depths. The station SVBC-37 was in a seamount area and the sediment texture was calcareous. The other two stations were characterized by the presence of siliceous sediments. The sampling procedure described by Raghukumar *et al.* [29] and Damare *et al.* [8] was followed. The samples were collected with an USNEL-type box corer of 50 cm³ size. Thus, the collected sediments were mostly undisturbed and compact. The average length of sediment cores obtained from these locations was ~30 cm. Sub-cores of sediments were collected using a alcohol-sterilised PVC cylinder of 5 cm inner diameter. 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 cores and directly introduced into sterile plastic bags to avoid any aerial contamination. The sediments were stored at -20°C immediately after sampling. DNA was isolated from each frozen sub-section of the sediment cores under sterile conditions to avoid cross contamination. The DNA samples from the subsections were pooled into two parts of 0-15 and 15-30 cm for each of the three stations in order to minimize the number of clone libraries. Thus, finally a total of 6 DNA samples were processed. In order to monitor the contamination by air-borne fungi, control media plates were exposed 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 during every sampling.

DNA extraction, Environmental PCR and clone library construction

DNA was isolated from 0.5 g of the sediment sample using the Q-Bio gene Soil DNA extraction kit (MP Biomedicals, Ohio, U.S.) according to the manufacturer's instructions. DNA samples from the three stations were amplified using fungal-specific ITS1F/ITS4 [13], primer pair a, as well as universal ITS1/ITS4, primer pair b and universal 18S rDNA NS1/NS2 primer set [41], primer pair c. 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 sec, annealing at 55° C for 30 sec 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 in Luria-Bertani broth containing 100 µg ml⁻¹ of Ampicillin. The presence of insert was confirmed by PCR with M13 forward and reverse primers. One µl of the broth containing the clone was added to 25 µl of PCR reaction mixture. PCR protocol included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min followed by a final extension at 72 °C for 5 min. 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). A total of 18 environmental gene libraries were constructed from the DNA samples from 3 stations, each with two depths and three primer pairs (3x2x3). A total of 48 clones were screened from each library.

Phylogenetic analyses

Forward and reverse sequences were assembled using Chromas Pro version 1.34 (Technelysium Pty Ltd, Tewantia, Queensland, Australia). Sequences obtained with ITS and 18S rDNA primers were analysed separately. All the sequences were checked with Ribosomal Database Project [7] for the presence of chimeras. These chimeric sequences were eliminated from subsequent analyses. Pairwise alignment of the sequences was carried out using Clustal W2 software [37]. Conserved motifs were identified and sequences were trimmed manually. Clones were grouped into operational taxonomic units (OTUs) by using sequence similarity cut-off value of 98% [26] by using MOTHUR software version 1.4.1 [30]. The results are presented as **OTU_01 to OTU_09** (blue fonts) obtained with fungal-specific ITS primer pair, **OTU_10 to OTU_22** (green fonts) retrieved with universal ITS primer pair and **OTU_23- to OTU_39** (red fonts) with universal 18S rDNA primer pair (Table 2).

A representative sequence from each OTU was queried against NCBI-GenBank BLASTn search [1]. Out of the 18 environmental gene libraries, 2 showing biased amplification of a single sequence type due to PCR artefact were eliminated from the further analyses. Multiple alignments were done for all the sequences along with their closest match in ClustalW [37]. Gaps and ambiguously aligned sequences were removed from the further analyses. A phylogenetic analysis was conducted using distance setting (Maximum Parsimony) in MEGA 4 [17] with 1000 bootstrap replicates. Phylogenetic trees were constructed with sequences obtained with ITS (primer pairs **a** and **b**) and 18S rDNA primer set (primer pair **c**) individually.

Diversity analyses

All diversity analyses were carried out using MOTHUR software. Shannon-Wiener diversity indices were calculated for OTUs obtained from each library constructed with three individual primer sets. Species effort curves were constructed to determine the sensitivity of the observed richness to the number of clones sampled. Community composition was plotted as relative abundance of taxonomic sub-classes at different stations and depths.

Nucleotide sequence accession numbers

Representative sequences of each OTUs were deposited in NCBI-GenBank under the accession numbers GU370716 to GU370774.

Results

Physico-chemical properties of the sediments

The area of sampling was characterized by the presence of polymetallic nodules. The nodule abundance ranged 0-10.7 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 ranged from 0-1.5, 0-1 and 0-1 mg g⁻¹ dry sediment respectively. Total bacterial 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 (unpublished data).

Environmental libraries

A total of 768 clones were sequenced from the 16 environmental libraries. Of the resultant sequences, 257 sequences were found to be fungal, resulting in a total of 39 OTUs, after clustering on a basis of 98% sequence identity criterion. The other 511 clones (~66.5%) were either bacterial, eukaryotic or chimeric in nature and thus were excluded from the study. The 39 fungal OTUs comprised 32 distinct fungal taxa (Table 2).

Analyses of fungal diversity obtained with different primer sets

Out of the 39 fungal OTUs, 4 were exclusively amplified by primer set **a**, 6 with primer set **b** and 15 by the primer set **c** (Table 2). Ten OTUs were simultaneously recovered with primer pairs **a** and **b** (Table 2) whereas, 4 with **b** and **c**. The number of singletons i.e. detected only once in libraries constructed with primer pair **a**, **b** and **c** were 4, 3 and 5 respectively (marked with an * in Table 2). Apart from these singletons, the remaining OTUs were represented ≥ 2 times in different libraries.

The fungal specific primer set **ITS1F/ITS4 (primer pair a)** and universal fungal primers **ITS1/ITS4 (primer pair b)** amplified sequences exclusively from a diverse group of fungi belonging to Ascomycota and Basidiomycota (Figs. 2 and 3). Primer pair **a** and primer pair **b** recovered 7 and 10 OTUs respectively belonging to Ascomycota. Both the ITS primer sets detected Sordariomycetes, Dothideomycetes, and Saccharomycetes (Figs. 2 and 3). In addition, the primer set **b** amplified members of Eurotiomycetes as well. Among Ascomycota, the primer pair **a** and the primer pair **b** amplified 1 (**OTU_01**) and 2 (**OTU_10**, **OTU_16**) new sequence types respectively having similarities less than 97 % to the known fungal taxa (Table 2).

Primer pair **a** and primer pair **b** recovered 2 and 3 OTUs belonging to Basidiomycota respectively. This group was found to include members from Tremellomycetes, Microbotryomycetes and Ustilaginomycetes. Out of these only Tremellomycetes was the common clade shared by both the primer sets (Fig. 3). Primer pair **b** amplified two new sequence type of basidiomycota (**OTU_17**, **OTU_18**).

Out of the total 22 OTUs recovered with these two primers sets, eight OTUs affiliated with sequences reported from marine environment (Table 2). Out of these eight OTUs, three were new sequence types. Five fungal taxa were simultaneously recovered with universal and fungal-specific ITS primer sets (Table 2).

A major part of the sequences recovered with universal 18S rDNA primer set **NS1/NS2** (**primer pair c**) belonged to Ascomycota (Fig. 4). The number of OTUs belonging to Ascomycota and Basidiomycota were 14 and 3 respectively. Out of these, three new sequence types showing less than 97 % similarity with the existing data base, **OTU_25** and **OTU_27** (with less than 50 % bootstrap value) and **OTU_39** (with bootstrap value of 91 %) branched deeply within Eurotiomycetes (Fig. 4). **OTU_34** belonging to the subclass Wallemiomycetes clustered with *Wallemia sebi* and **OTU_38** clustered with an unclassified species of fungus with a 99% bootstrap value (Fig. 4). Only 2 fungal taxa namely *Aspergillus penicilloides* and *A. restrictus* were simultaneously amplified with universal ITS and 18S rDNA primers (Table 2).

Fungal diversity at different stations

Fungal taxa were more or less unevenly distributed at different stations (Table 3). Some of these OTUs were found exclusively in station A, B or C. Distribution of fungal taxa in stations A, B and C was 10, 21 and 16, respectively, indicating richness of diversity in Station B. Species of *Sagenomella*, *Aspergillus penicilloides*, *Stenella musicola* and *Candida* sp. occurred in high frequency (Table 3).

Distribution of different subclasses and species richness

Seven and five major subclasses of fungi were recovered with ITS and 18S rDNA primers respectively (Fig. 5). Compositional difference was observed between stations and occasionally with different depths of the same station. In a few instances, individual primer sets amplified different subclasses from the environmental clone libraries. For example, Sordariomycetes, Tremellomycetes and Ustilaginomycetes were amplified with ITS primer sets, whereas Wallemiomycetes was amplified only with 18S rDNA primer set. (Figs. 2-4). Members of Eurotiomycetes were present in all the stations. Members of Sordariomycetes and Dothideomycetes were found to be the major forms in station A and C, whereas yeasts were dominant in station B. Sequences belonging to Wallemiomycetes were found only in Station C. Shannon-Wiener diversity values (H) were 2.5, 3.0 and 4.5 for libraries constructed with universal ITS, fungal specific ITS and universal 18S rDNA primers respectively. The species effort curves for libraries with all the primers were found to show positive slope and thus no evidence of saturation was found (data not shown).

Discussion

Diversity with multiple primers

Primer sets for the amplification of two different segments of rRNA gene were chosen because 18S rDNA is supposed to be conserved in nature and thus discrimination between closely related species is difficult to be resolved. On the other hand, ITS region of rRNA gene is known to give better resolution of taxonomic species, but phylogenetic analyses of unknown sequences cannot be resolved due to its less conserved nature [26]. Therefore, to overcome this problem and get a better diversity estimate, we resorted to amplify both of these segments from environmental DNA samples. The use of different primers enabled the recovery of larger and varied number of fungal OTUs from deep-sea sediments than would have been possible otherwise [15, 26]. Thus, a total of 22 and 17 OTUs were recovered exclusively with ITS and 18S rDNA primer sets, respectively. Primer sets for ITS and 18S rDNA region exclusively identified 15 fungal taxa each. Two fungal taxa were commonly recovered with ITS and 18S rDNA primer sets (Table 2). Jebaraj *et al.*, [15] have also recovered a large number of fungal OTUs from anoxic sediments using multiple primer approach. In contrast, yeasts were found to be dominating forms in deep oceans including distantly related forms in a study conducted by the amplification of SSU region with fungal-specific 18S rDNA primers [2]. In the previous study employing culture-dependent approach, only 12 filamentous fungi and 8 species of yeasts from a total of 188 sediment samples from 20 stations of CIB were isolated [32]. Therefore diversity observed through culture-independent method (32 distinct species) with multiple primer sets was ~ 38 % higher than that obtained through culture-dependent (20 fungal species) approach from the same geographical location. The need to use different primer sets is also highlighted by the fact that the phylogenetic affinities of OTUs recovered with different primer sets seemed to be different (Table 2). In the present study, Asco- and Basidiomycota were the most common phylogenetic sequences identified with ITS and 18S rDNA primers as observed by O'Brien *et al* [26] using the same primer sets. In contrast to our results, O'Brien *et al* [26] recovered sequences belonging to Chytridiomycota and Zygomycota with these primer pairs. Lai *et al* [19] also reported the presence of Asco- and Basidiomycota from methane hydrate seeps using only the fungal specific ITS primer pair.

Multiple sequence analysis approach resulted in identifying eight OTUs showing less than 97% similarity to the existing fungal taxa and thus appeared to be novel. Out of these, 6 were affiliated to Ascomycota and 2 to Basidiomycota. Thus, these new sequences add to the existing database for probable novel fungal signatures.

Spatial distribution and heterogeneity

This study suggests that fungal diversity may be heterogeneous in deep-sea sediments as some of the subclasses were restricted to a few stations (Fig. 5). Biological communities in deep sea have shown dependence on variables such as substrate availability and type, biogeochemistry, nutrient input, productivity and hydrological conditions on regional scale [20]. Substrate heterogeneity was shown to play an important role in structuring nematode diversity in deep-sea sediments [40]. These authors reported nematode assemblages that varied in hard nodule substratum from soft sediments beneath the nodules. Such small-scale habitat heterogeneity may also impact fungal diversity in deep-sea sediments. Sequences belonging to Chytridiomycota and Zygomycota were recovered by using fungal specific 18S rDNA primers from marine sources [2]. Therefore, their absence in the present study suggests that their distribution may be site-specific. Microbial diversity has been shown to be impacted by temporal and spatial dimensions of deep-sea [34].

Phylogenetic affiliation with marine fungal taxa

Out of 39 OTUs, 12 OTUs showed affiliation with sequences already reported from marine sources (Table 2) thus adding support to the view that these were possibly typically marine. Out of these 12, 2 OTUs representing *Sagenomella* sp, and the yeast *Rhodosporidium* sp. were recovered in culture from the CIB in the earlier study [32]. These two cultures showed growth under simulated deep-sea conditions of 200 bar pressure, temperature of 5⁰C and salinity of 35 PSU suggesting either their adaptation to the deep-sea conditions or their autochthonous existence. This is the first report of occurrence of *Wallemia sebi* from deep-sea sediments. It is a halophilic and osmophilic fungus isolated from hypersaline water of salterns and is described to have successfully adapted to life in extremely saline environments [18]. Its sequence was detected in a marine sponge from Hawaii [12] and was also isolated in culturable form from different species of marine sponges of the South China Sea [21] suggesting its marine nature. Cantrell *et al.* [6] identified a series of halotolerant fungi, including *Aspergillus*, *Penicillium*, *Cladosporium* and *Hortaea* sp. from solar salterns using ITS1/ITS4 primer set. Using the same primer set, some of these were recovered in the present study also. We are reporting here for the first time recovery of sequences showing affiliation with Microbotryomycetes, and Tremellomycetes from deep-sea sediments. The percentage abundance of these subclasses was very low in comparison with other groups suggesting their low diversity under deep-sea conditions. The class Microbotryomycetes (represented by *Rhodotorula*, *Rhodosporidium*) includes mycoparasites, phytopathogens and putative saprotrophs with a diversity of

micromorphological characters. Many species contain organelles, termed colacosomes, that are associated with mycoparasitism [3]. The recovery of other pathogenic yeasts like *Candida* and *Debaromyces*, suggests that deep-sea sediments may act as reservoir of such forms. Pathogenic black yeast in mussel and other animal parasitic forms have been reported from hydrothermal vents [25, 39]. These results are in accordance with earlier observation that oceans are incubators and conveyor belt for pathogenic microorganisms [14].

Terrestrial fungi in the deep-sea sediments

A large proportion (~ 69%) of fungal sequences recovered in high frequency (Table 3) matched with terrestrial taxa, supporting the earlier hypothesis [8, 9, 29] that they are transported into the deep sea and subsequently adapt to the extreme conditions. One of the most common terrestrial fungus *Aspergillus* has been recovered both by culture-dependent [4, 8] and culture-independent methods from various marine habitats [2, 15, 19]. Therefore the ecological role and adaptations of these ubiquitous terrestrial forms in such extreme conditions need to be further investigated. Takishita *et al.* [36] and Shao and Sun *et al.* [31] have emphasized the role of sedimentation in accumulating facultative marine fungi in deep-sea sediments. Irrespective of their mode of transport, these facultative marine fungi offer an excellent tool to study physiology of deep-sea adapted fungi. Fungi like *Sagenomella* which is reported for the first time from deep-sea by culture-dependent [32] as well as culture-independent approach from the same site can be one such model organism. Several of these terrestrial taxa were isolated from marine sponges too and they exhibited in vitro anti-fungal properties [28] anti-tumor and anti-bacterial activities [21] suggesting that terrestrial taxa found in the marine environment could be a rich source of new biologically active natural products [21].

In conclusion, the multiple primer approach used in the present study provided an insight to the diversity of fungi in the deep-sea sediments of the CIB. The lack of saturation level with all the primer sets (data not shown) indicates that the actual diversity of fungi must be greater than detected here. Therefore, exhaustive sampling to obtain near-accurate estimation of fungal diversity in deep-sea is recommended. In addition, designing more fungal specific primers to identify novel species obtained by culture-independent approach is required. It is not appropriate to speculate that all the fungal diversity obtained by using DNA amplification represent viable fungi because low temperature conditions of deep-sea environment will favour preservation of DNA. Thus, it is possible that only a fraction of the uncultured fungi will be viable. Culture-independent approach provides valuable indication of the presence of the organism/community and in turn information

gained could be utilized for their recovery in culturable form using targeted culturing methods. The methods to isolate the yet-to be-cultured fungi from deep-sea sediments such as improvement of isolation media, incubation techniques should be evolved so that rare and slow-growing forms could be obtained.

Biological diversity in this extreme ecosystem may have enormous potential in the development of new products such as pharmaceuticals, molecular probes, enzymes, cosmetics, nutritional supplements and agrichemicals [35]. A recently described antibiotic-resistance enzyme from the deep-sea bacterium *Oceanobacillus iheyensis* isolated from 1050 m depth in the Pacific Ocean [38] and a novel λ -Carrageenase with no similarity to any reported protein, obtained from a deep-sea bacterium isolated from Suruga Bay, Japan at a depth of 2409 m [27] are some of the examples. Deep-sea sediments may harbour new fungal taxa that are tolerant to low temperature and elevated hydrostatic pressure which may lead to discovery of new metabolites or enzymes that are useful for novel biotransformation processes.

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References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403-410
2. 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
3. Bauer R, Oberwinkler F (1991). The colacosomes: new structures at the host- parasite interface of a mycoparasitic basidiomycete. *Bot Acta* 104: 53-57

4. Burgaud G, Calvez TL, Arzur D, Vandenkoornhuyse P, Barbier G (2009) Diversity of culturable marine filamentous fungi from deep-sea hydrothermal vents. *Environ Microbiol* 11: 1588–160
5. Calvez TL, Burgaud G, Mahe S, Barbier G, Vandenkoornhuyse P (2009) Fungal Diversity in Deep-Sea Hydrothermal Ecosystems. *Appl Environ Microbiol* 75: 6415-6421
6. Cantrell SA, Casillas-Martinez L, Molina M (2006) Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. *Mycol Res* 110: 962-970
7. 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
8. 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
9. Damare SR, Nagarajan M, Raghukumar C (2008) Spore germination of fungi belonging to *Aspergillus* species under deep-sea conditions. *Deep-Sea Res PT I* 55: 670-678
10. Gaddanho M, Sampaio JP (2006) Microeukaryotic diversity in the extreme environments of the Iberian Pyrite Belt: a comparison between universal and fungi-specific primer sets, temperature gradient gel electrophoresis and cloning. *FEMS Microbiol Ecol* 57: 139-148
11. Gadd G, Watkinson SC, Dyer PS (2007) *Fungi in the environment*. Cambridge, UK: Cambridge University Press.
12. 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
13. Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Mol Ecol* 2: 113-118
14. Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, Samuel MD (2002) Climate warming and disease risks for terrestrial and marine biota. *Science* 296: 2158-2162
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 Microb Ecol* 71: 99-412
16. Jones EBG, Sakayaroj J, Suetrong S, Somrithipol S, Pang KL (2009) Classification of marine Ascomycota anamorphic taxa and Basidiomycota. *Fungal Divers* 35: 1-187.

17. 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
18. Kuncic KM, Kogej M, Drobne D, Gunde-cimerman N (2010) Morphological response of the halophilic fungal genus *Wallemia* to high salinity. *Appl Environ Microbiol* 76: 329-337
19. 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
20. Levin LA, Etter RJ, Rex MA, Gooday AJ, Smith CR, Pineda J, Stuart CT, Hessler RR, Pawson D (2001) Environmental influences on regional deep-sea species diversity. *Annu Rev Ecol Syst*, 132: 51–93
21. 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
22. Lopez-Garcia 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
23. Lopez-Garcia P, Rodriguez-Valera F, Pedros-Alio C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature* 409: 603–607
24. Moon-van der Staay SY, DeWachter R, Vaulot D (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* 409: 607–610
25. Moreira D, Lopez-Garcia P (2003) Are hydrothermal vents oases for parasitic protists? *Trends Parasitol* 19: 556–558
26. 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
27. Ohta Y, Hatada Y (2006) A novel enzyme, λ -Carrageenase, isolated from a deep-sea bacterium. *J Biochem* 140: 475-481
28. Paz Z, Komon-Zelazowska M, Druzhinina IS, Aveskamp MM, Shnaiderman A, Aluma Y, Carmeli S., Ilan M, Yarden O (2010) Diversity and potential antifungal properties of fungi associated with Mediterranean sponge. *Fungal Divers* 42: 17-26
29. 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

30. 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* Doi: 10.1128/AEM.01541-09
31. Shao Z, Sun F (2007) Intracellular sequestration of manganese and phosphorous in a metal-resistant fungus *Cladosporium cladosporioides* from deep-sea sediment. *Extremophiles* 11: 435–443
32. 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
33. Snelgrove PVR, Blackburn TH, Hutchings P, Alongi D, Grassle JF, Hummel H, King G, Koike I, Lamshead PJD, Ramsing NB, Solis-Weiss V, Freckman DW (1997) The importance of marine sediment biodiversity in ecosystem processes *Ambio* 26: 578-582
34. 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
35. Synnes M (2007) Bioprospecting of organisms from deep sea: scientific and environmental aspects. *Clean Techn Environ Policy* 9: 53-59
36. 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
37. 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
38. Toth M, Smith C, Frase H, Mobashery S, Vakulenko S (2010) An antibiotic-resistance enzyme from a deep-sea bacterium. *J Am Chem Soc* 132: 816-823
39. Van Dover CL, Ward ME, Scott JL, Underdown J, Anderson B, Gustafson C, Whalen M, Carnegie RB (2007) A fungal epizootic in mussels at a deep-sea hydrothermal vent. *Marine Ecol* 28: 54–62
40. Vanreusel A, Fonseca G, Danovaro R (2010) Contribution of deep-sea macrohabitat heterogeneity to global nematode diversity. *Marine Ecol* 31: 6-20
41. White TJ, Bruns TD, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *Academic press* 315-324

Legends to the figures

Fig. 1. Geographic location of the sampling site in the Central Indian Basin.

Fig. 2. Maximum parsimony phylogenetic tree based on fungal ITS gene sequences of phylum Ascomycota after amplification with primer set, **ITS1F/ITS4 (fungal specific, marked with blue color)** and **ITS1/ITS4 (universal ITS, marked with green color)**. Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle.

Fig. 3. Maximum parsimony phylogenetic tree based on fungal ITS gene sequences of phylum Basidiomycota after amplification with primer set, **ITS1F/ITS4 (fungal specific, marked with blue color)** and **ITS1/ITS4 (universal ITS, marked with green color)**. Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle.

Fig. 4. Maximum parsimony phylogenetic tree based on fungal 18S rDNA gene sequences of phylum Ascomycota and Basidiomycota after amplification with universal 18S rDNA primer set, **NS1/NS2 (Universal 18S rDNA, marked with Red color)**. Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle.

Fig. 5. Proportional distribution of different taxonomic groups in various clone libraries. Percentage occurrence of sequences of each taxonomic group is indicated on x-axis. Clone libraries are represented on y-axis. The clone libraries are represented in bold, designated with Station number A (SVBC-04), B (SVBC-31) and C (SVBC-37) followed by depth of sediment sample (15 or 30 cm), primer pair used were **a (ITS1F/ITS4)**, **b (ITS1/ITS4)** and **c (NS1/NS2)**. The clone libraries B-15-a and C-30-b showed biased amplification of a single sequence type due to PCR artefact and were eliminated from the analyses.

Table 1. Sampling locations in the Central Indian Basin.

Sample number	Touch positions		Water depth (m)	Sediment texture
	Lat (°S)	Long (°E)		
SVBC-04 (Station A)	13 00.000	76 29.950	5377	Siliceous
SVBC-31 (Station B)	12 00.000	74 59.900	5153	Siliceous
SVBC-37 (Station C)	12 59.980	74 59.960	3992	Calcareous, Seamount area

Table 2. Phylogenetic affiliation of the Fungal OTUs obtained with different primer sets (Sequences showing <97 % similarity values are represented in bold).

Serial no.	Phylum	Closest relative (GenBank Accession no.)	% similarity	Originally reported habitat	OTUs with Fungal Specific ITS primers	OTUs with Universal ITS primers	OTUs with Universal 18S rDNA primers
1	Ascomycota	<i>Sagenomella</i> sp. (FJ357782)	99	Deep sea sediment of CIB	OTU_02	OTU_11	----
2	Ascomycota	<i>Dothideomycete</i> sp. (EU680535)	99	Moth exoskeleton	----	OTU_21	----
3	Ascomycota	<i>Aspergillus penicillioides</i> (DQ985959)	97	Lacaziosis patient	----	OTU_15	OTU_23
4	Ascomycota	<i>Aspergillus restrictus</i> (AB008407)	99	Soil	----	----	OTU_24
5	Ascomycota	<i>Hortaea</i> sp. F 47 (FJ755827)	95	Marine sponge	OTU_01	OTU_10	----
6	Ascomycota	<i>Stenella musicola</i> (EU514294)	99	<i>Musa acuminata</i>	OTU_04	OTU_13	----
7	Ascomycota	<i>Candida</i> sp. F15 (FJ755821)	100	Coastal waters of china	OTU_05	OTU_14	----
8	Ascomycota	<i>Aspergillus restrictus</i> (AB002079)	94	Not known	----	OTU_16	OTU_27
9	Ascomycota	<i>Debaryomyces yamadae</i> (AB054266)	98	Not known	----	OTU_19*	----
10	Ascomycota	<i>Pichia jadinii</i> (EU568927)	99	Pilot-scale Municipal compost	OTU_06*	----	----
11	Ascomycota	<i>Nodulisporium</i> sp. (EF600033)	98	Stroma of <i>Epichloe typhina</i>	OTU_07*	----	----
12	Ascomycota	<i>Aspergillus niger</i> (GQ228449)	96	Soil from Kalabagh iron ore deposit	----	----	OTU_25
13	Ascomycota	<i>Candida glucosophila</i> (AB013519)	97	Not known	----	----	OTU_28
14	Ascomycota	<i>Aspergillus fumigatus</i> (GQ169424)	99	Fossa cheese	----	----	OTU_29*
15	Ascomycota	<i>Aspergillus</i> sp. (FJ864683)	99	Root of <i>Angelica sinensis</i>	----	----	OTU_30*
16	Ascomycota	<i>Phoma herbarum</i> (AY293777)	99	Not known	----	----	OTU_32
17	Ascomycota	<i>Aspergillus unguis</i> (EF067336)	99	Culture collection	----	----	OTU_35
18	Ascomycota	<i>Ulospora bilgramii</i> (DQ384071)	98	Dung	----	----	OTU_37*
19	Ascomycota	<i>Capnodium coffeae</i> (DQ247808)	93	Maize rhizosphere Soil	----	----	OTU_39*
20	Ascomycota	<i>Bionectriaceae</i> sp. (FJ821507)	97	Basidioma of <i>Phellinus gilvus</i>	----	OTU_22*	----
21	Ascomycota	fungal sp. ARIZ L365 (FJ612791)	99	Tree seed	OTU_08*	----	----
22	Ascomycota	Uncultured (GQ120138)	98	Arabian Sea sediment	----	----	OTU_26
23	Ascomycota	Uncultured (AB074653)	98	Arabian Sea sediment	----	----	OTU_31*
24	Ascomycota	Uncultured (AJ515945)	97	Maize rhizosphere Soil	----	----	OTU_33
25	Ascomycota	<i>Candida parapsilosis</i> (GQ395610.1)	99	Feeding production sample	----	OTU_20*	----
26	Basidiomycota	<i>Malassezia pachydermatis</i> (DQ457640)	96	Not known	----	OTU_17	----
27	Basidiomycota	<i>Trichosporon asahii</i> (AB369919)	99	Skin of patient	OTU_03	OTU_12	----
28	Basidiomycota	<i>Wallemia sebi</i> (AY741380)	97	Hypersaline water of salterns	----	----	OTU_34
29	Basidiomycota	<i>Rhodospodium sphaerocarpum</i> (EF643587)	100	Deep-sea of pacific ocean	OTU_09*	----	----
30	Basidiomycota	fungal sp. FCAS11 (GQ120154)	97	Arabian Sea sediment	----	----	OTU_38
31	Basidiomycota	Uncultured (AJ515927)	99	Contaminated aquifer sediment	----	----	OTU_36
32	Basidiomycota	Uncultured (FJ889114)	94	Hawaiian coastal waters	----	OTU_18	----

Primer pairs are represented by different colors- **Blue: ITS1F/ITS4 (Fungal specific), Green: ITS1/ITS4 (Universal ITS), Red: NS1/NS2 (Universal 18S rDNA)**

Singletons are marked with *

Table 3. Distribution of closest identified fungal taxa in different stations.

OTU nos.	Closest relative (GenBank Accession no.)	Number of clones recovered			Total number of clones recovered
		Station A	Station B	Station C	
OTU_02, OTU_11	<i>Sagenomella</i> sp.(FJ357782)	33	11	27	71
OTU_15, OTU_23	<i>Aspergillus penicillioides</i> (DQ985959)	21	20	1	42
OTU_05, OTU_14	<i>Candida</i> sp. F15 (FJ755821)	8	26	0	34
OTU_04, OTU_13	<i>Stenella musicola</i> (EU514294)	6	10	7	23
OTU_16, OTU_27	<i>Aspergillus restrictus</i> (AB002079)	4	6	4	14
OTU_25	<i>Aspergillus niger</i> (GQ228449)	3	6	4	13
OTU_01, OTU_10	<i>Hortaea</i> sp. F 47 (FJ755827)	2	0	4	6
OTU_28	<i>Candida glucosophila</i> (AB013519)	0	3	2	5
OTU_24	<i>Aspergillus restrictus</i> (AB008407)	3	2	0	5
OTU_03, OTU_12	<i>Trichosporon asahii</i> (AB369919)	3	1	0	4
OTU_26	Uncultured Ascomycota (GQ120138)	4	0	0	4
OTU_21	<i>Dothideomycete</i> sp. (EU680535)	0	3	0	3
OTU_18	Uncultured Basidiomycota sp. (FJ889114)	0	3	0	3
OTU_35	<i>Aspergillus unguis</i> (EF067336)	0	0	3	3
OTU_36	Uncultured soil basidiomycete (AJ515927)	0	0	3	3
OTU_38	fungal sp. FCAS11 (GQ120154)	0	0	3	3
OTU_17	<i>Malassezia pachydermatis</i> (DQ457640)	0	3	0	3
OTU_32	<i>Phoma herbarum</i> (AY293777)	0	0	2	2
OTU_33	Uncultured soil Ascomycete (AJ515945)	0	0	2	2
OTU_34	<i>Wallemia sebi</i> (AY741380)	0	0	2	2
OTU_19	<i>Debaryomyces yamadae</i> (AB054266)	0	1	0	1
OTU_06	<i>Pichia jadinii</i> (EU568927)	0	1	0	1
OTU_07	<i>Nodulisporium</i> sp. (EF600033)	0	1	0	1
OTU_29	<i>Aspergillus fumigatus</i> (GQ169424)	0	1	0	1
OTU_30	<i>Aspergillus</i> sp. (FJ864683)	0	1	0	1
OTU_37	<i>Ulospora bilgramii</i> (DQ384071)	0	0	1	1
OTU_39	<i>Capnodium coffeae</i> (DQ247808)	0	0	1	1
OTU_22	<i>Bionectriaceae</i> sp. (FJ821507)	0	1	0	1
OTU_08	fungal sp. ARIZ L365 (FJ612791)	0	1	0	1
OTU_31	Uncultured fungal clone (AB074653)	0	0	1	1
OTU_20	<i>Candida parapsilosis</i> (GQ395610.1)	0	1	0	1
OTU_09	<i>Rhodospidium sphaerocarpum</i> (EF643587)	0	1	0	1

Primer pairs are represented by different colors- **Blue: ITS1F/ITS4 (Fungal specific), Green: ITS1/ITS4 (Universal ITS), Red: NS1/NS2 (Universal 18S rDNA)**

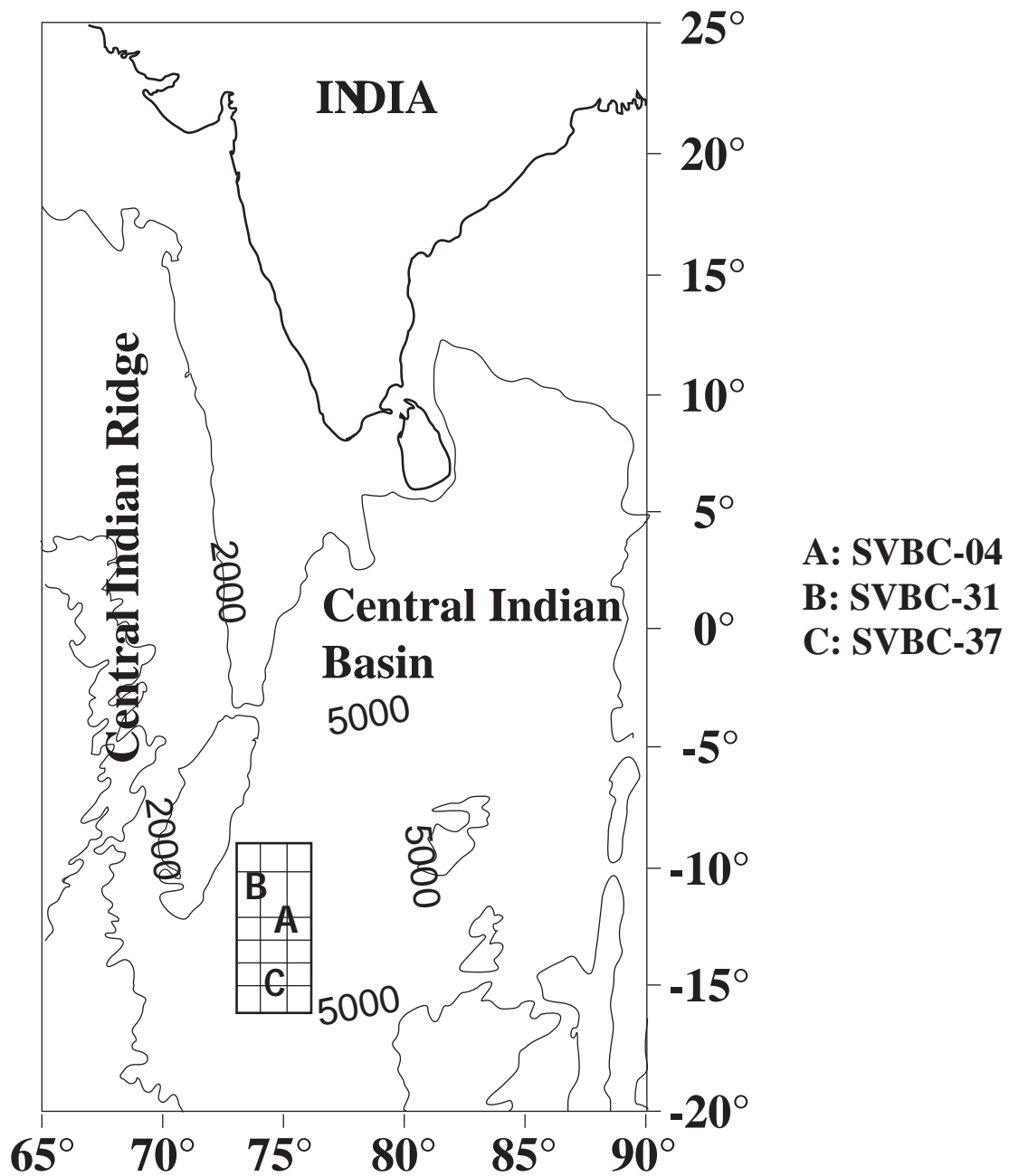


Fig 1.

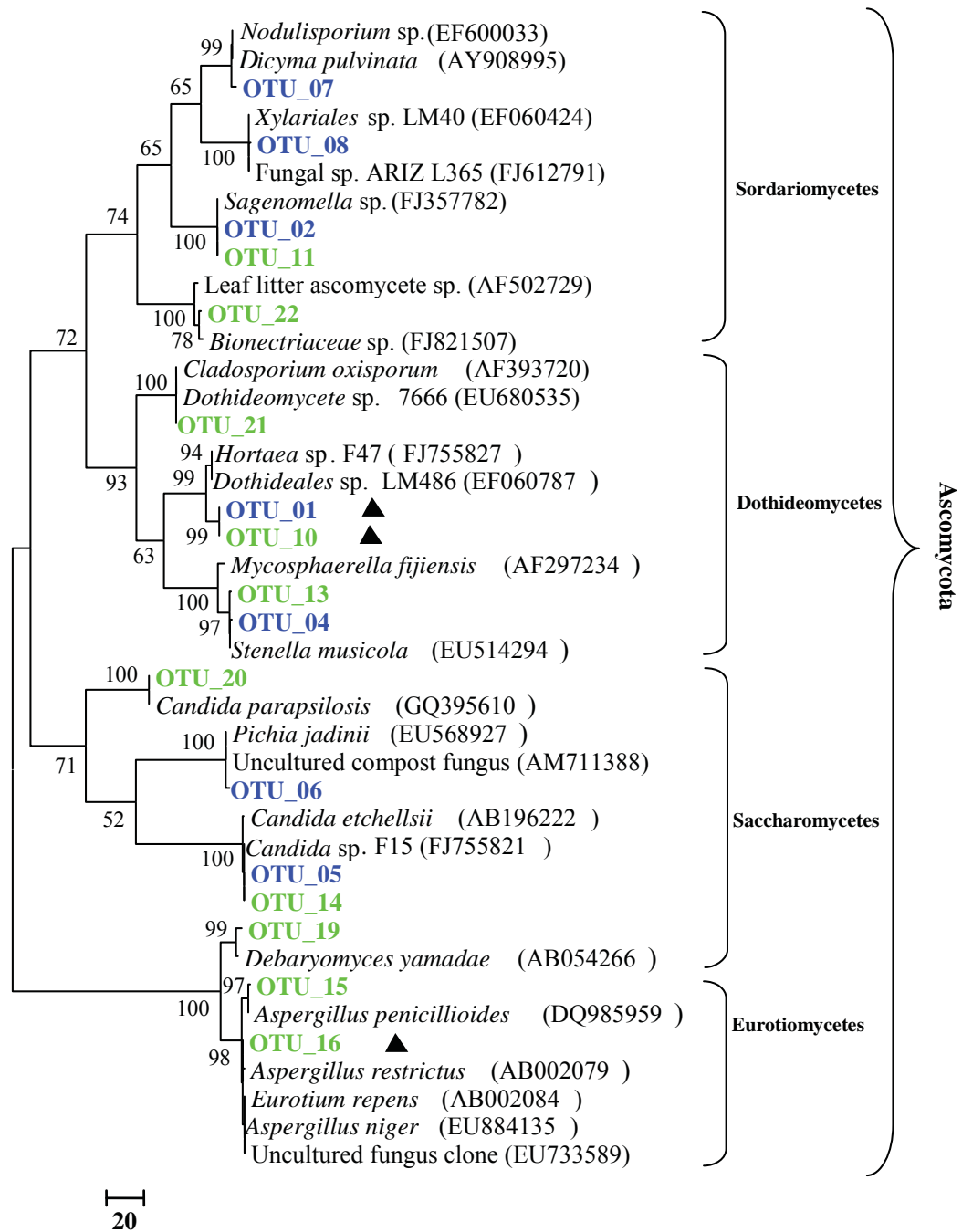


Fig 2.

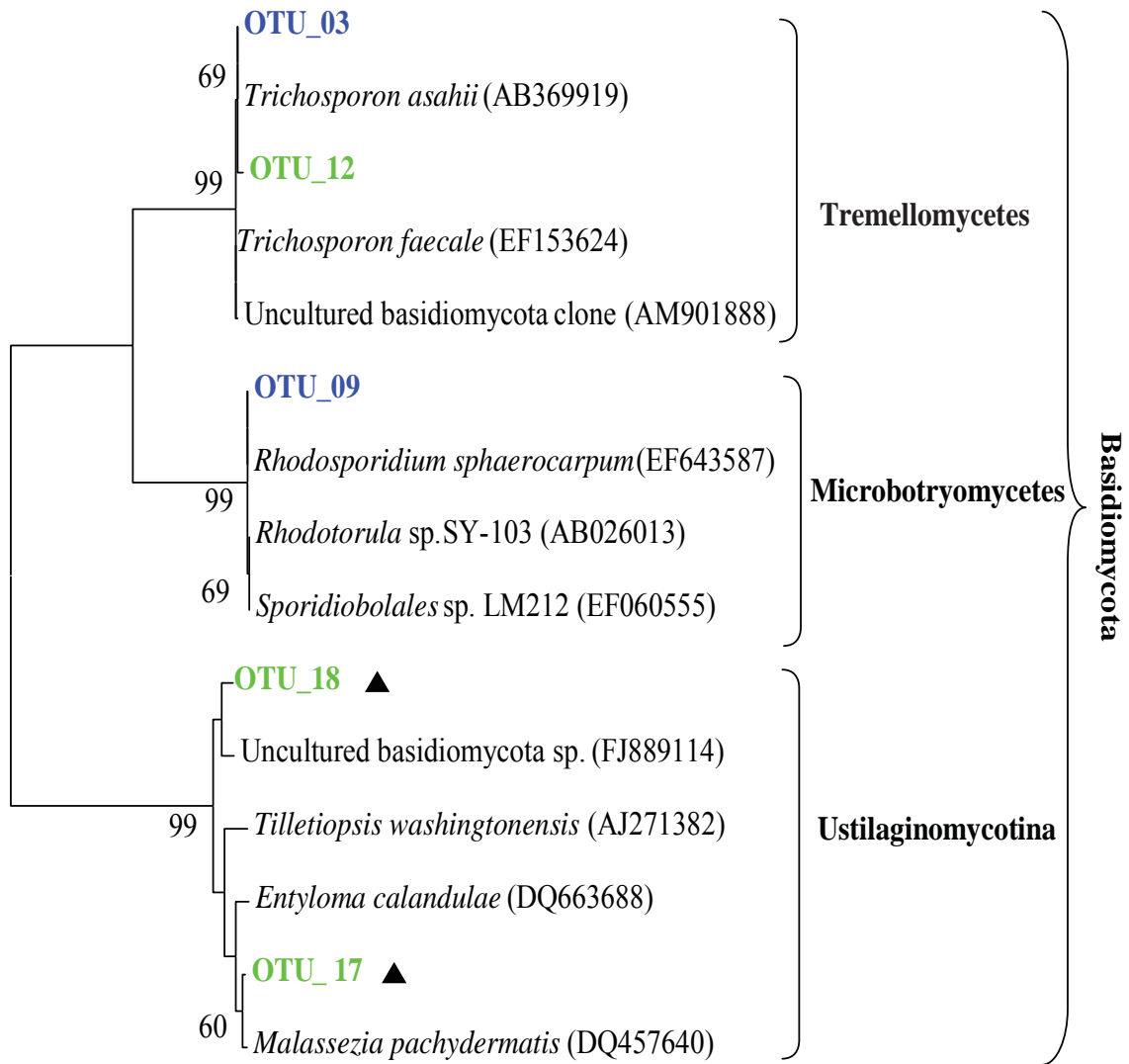


Fig 3.

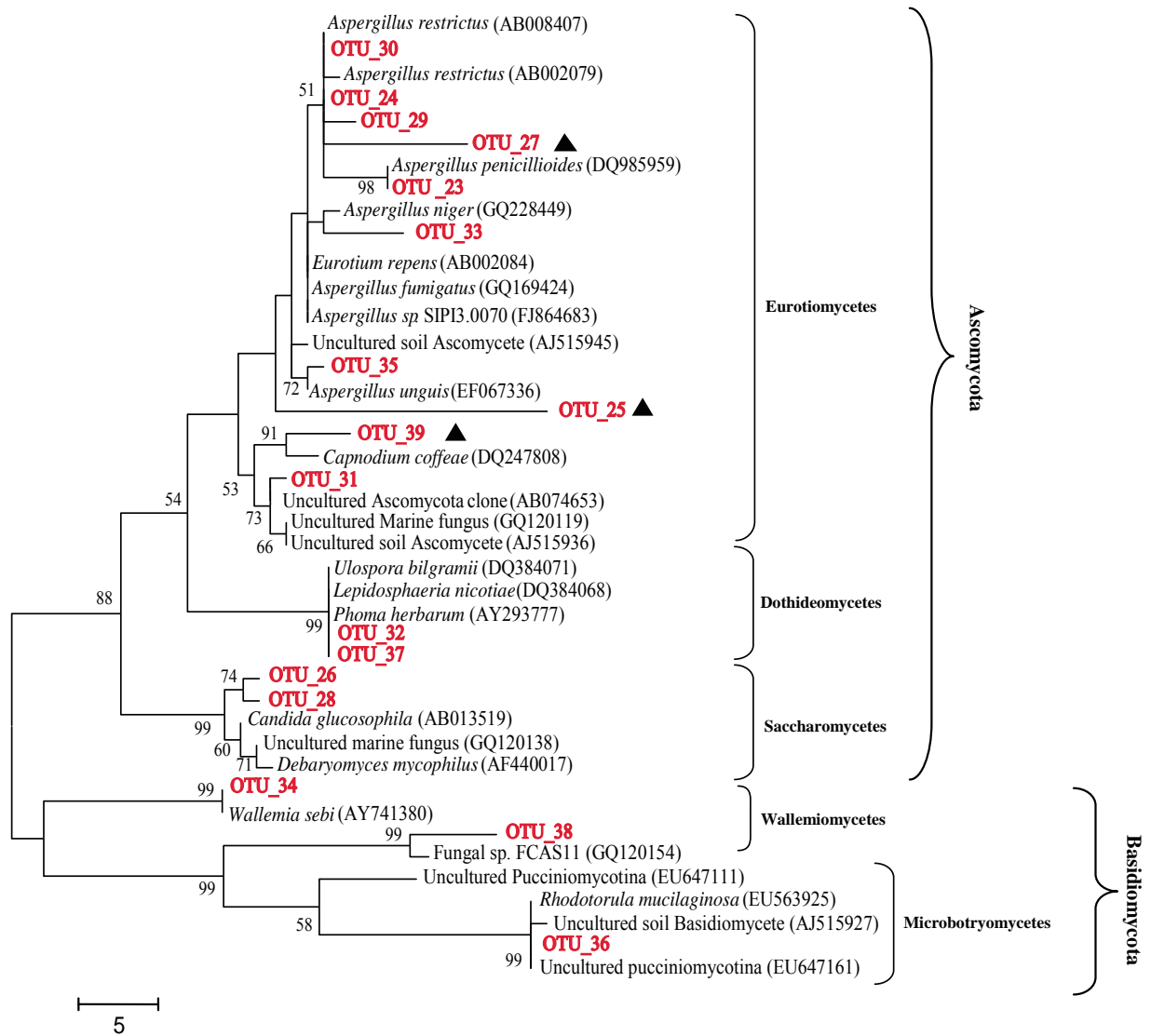


Fig 4.

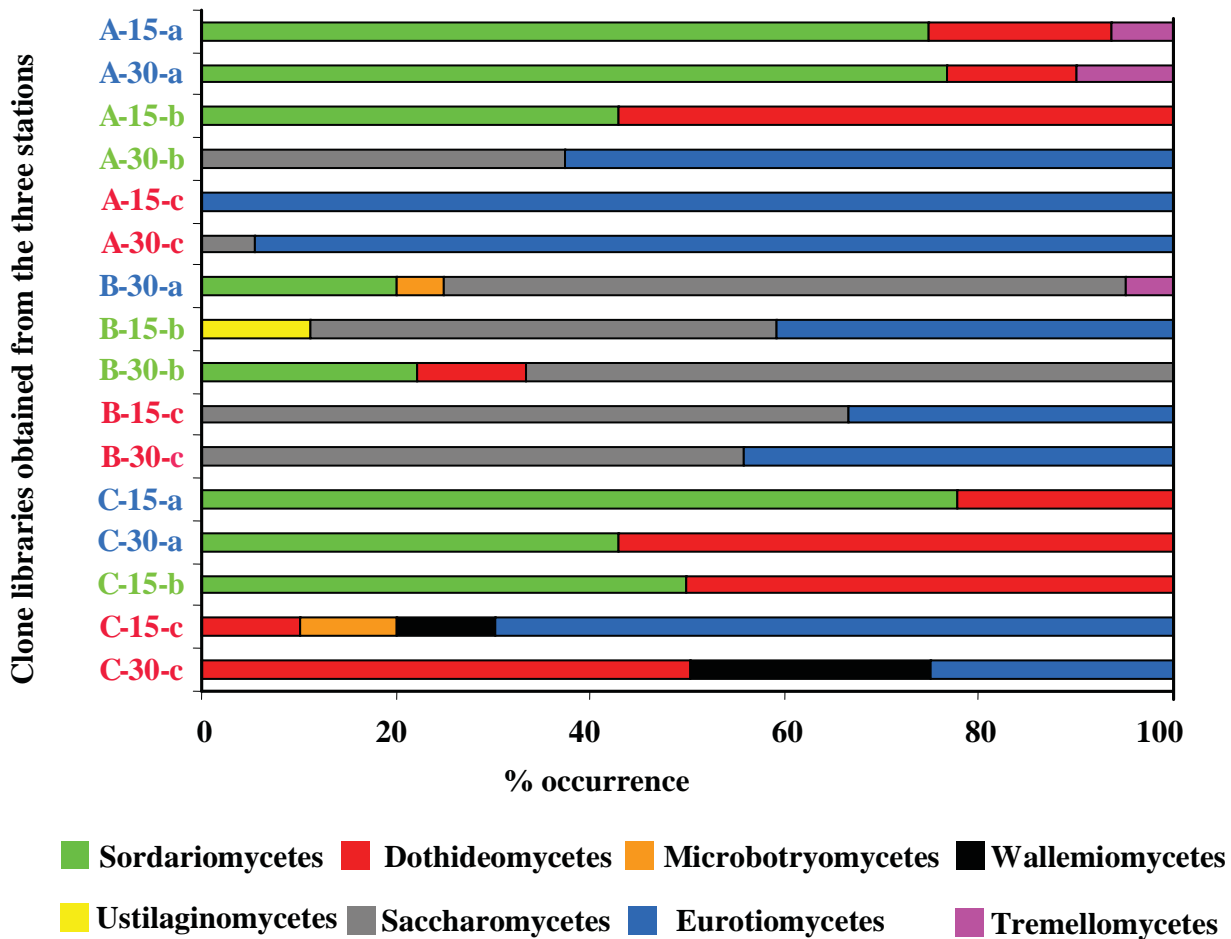


Fig 5.