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Fungal diversity in deep-sea sediments revealed by culture-dependent and culture-independent approaches

Purnima Singh¹, Chandralata Raghukumar^{1*}, Ram Murti Meena¹, Pankaj Verma² and Yogesh Shouche²

¹National Institute of Oceanography (Council for Scientific and Industrial Research), Dona Paula, Goa 403 004, India

²National Centre for Cell Sciences, Lab-III, Pune University Campus, Ganeshkhind, Pune 411 007, India

*Corresponding author's Email: lata_raghukumar@rediffmail.com

Abstract

Diversity of fungi from deep biosphere is recently gaining an increasing attention. We investigated fungal diversity in two sediment cores ~40 cmbsf (cm below seafloor) at a depth of ~5000 m in the Central Indian Basin (CIB) by culture-dependent as well as culture-independent approaches. This resulted in recovering a total of 19 culturable fungi and 46 operational taxonomic units (OTUs) respectively. Two of the cultured fungi showed similarity to *Hortaea werneckii* and *Aspergillus versicolor* and 11 OTUs from environmental libraries showed high divergence (86-97 %) from the existing sequences in the GenBank (NCBI database). Some of the fungi such as *Cerrena*, *Hortaea* and *Aspergillus* sp. were recovered by culture-dependent as well as culture-independent approaches. In conclusion, culture-dependent and culture-independent approaches detected a total of 12 distinct fungal genera and 42 OTUs respectively from 2 sediment cores indicating presence of a high fungal diversity in deep-sea sediments.

Keywords

Marine fungi, SSU rRNA gene, ITS rRNA gene, operational taxonomic unit, environmental clone libraries, Central Indian Basin

Introduction

Fungi in marine ecosystem are involved in the decomposition and mineralization of organic matter (Hyde 1989; Hyde et al. 1998). They have also been reported as parasites, symbionts or pathogens (Kohlmeyer and Kohlmeyer 1979) and as endobionts in marine organisms (Wegley et al. 2007; Ding et al. 2011). Recently, diversity of fungi from various extreme habitats such as deep-sea hydrothermal vents (Le Calvez et al. 2009; Burgaud et al. 2009, 2010), deep-sea waters and sediments (Bass et al. 2007; Nagano et al. 2010; Edgcomb et al. 2011) and methane hydrate-bearing deep-sea sediments (Takishita et al. 2006; Lai et al. 2007; Nagahama et al. 2011; Thaler et al. 2011) were reported. Diversity was analyzed by either culture-dependent or culture-independent methods in the above studies resulting in the identification of highly novel as well as phylotypes closely related to known fungi. However, a combination of these two approaches may provide deeper insight into the diversity assessment as reported by Jebaraj et al. (2010). By culture-dependent method, fungi belonging to 13 classes, comprising 5 of the phylum Ascomycota and 8 of Basidiomycota have been reported from deep-sea environment (Nagahama & Nagano 2012). These authors have further compiled the diversity of fungi by culture-independent method into 14 classes comprising 5 of Ascomycota, 8 of Basidiomycota and 1 Chytridiomycota. Besides, 2 phylotypes belonging to basal clades of LKM11 and KD14 have been also reported. These two basal clades comprised of eukaryotic members, earliest divergent from fungal lineages which may provide insight in the evolutionary history of fungi in deep-sea environment (Nagahama et al. 2011). Considering the role of marine fungi as a source of useful bioactive compounds (Bhadury et al. 2006; Konishi et al. 2010) and their involvements in various biogeochemical cycles (Gadd et al. 2007) the actual diversity of fungi in deep-sea sediments needs to be assessed.

Earlier studies revealed diversity of fungi from deep-sea sediments of the Central Indian Basin (CIB) by culture-dependent (Damare et al. 2006; Singh et al. 2010) approach wherein several culture media and isolation techniques were used. A total of 22 distinct fungi were obtained during these studies (Raghukumar et al. 2010). Fungal diversity was assessed by culture-independent approach from three sediment cores of the CIB (Singh et al. 2011a). A total of 39 fungal operational taxonomic units (OTUs), with 32 distinct fungal phylotypes were recovered using three primer pairs. In another study four different primer pairs were used in amplifying sediment DNA to assess the fungal community (Singh et al. 2011b). A total of 27 OTUs with 20 distinct fungal phylotypes were recovered from a single sediment core by this approach. In the present study we analysed diversity of fungi by culture-

dependent as well as culture-independent approaches in the same sediment samples of 2 cores from the CIB. The fungi isolated in culture were identified by amplification and sequencing of 18S rRNA gene. Sporulating cultures were identified by microscopic method also. Using culture-independent approach, 8 environmental gene libraries were constructed after amplification of sediment DNA with fungal specific ITS and universal 18S rRNA gene primer sets.

Materials and Methods

Sampling methods

Sediment samples were collected during the cruise #ABP-38 from two locations of the CIB in September 2009 on board of the research vessel *Akademik Boris Petrov*. These were sediment cores #IVBC-18C (12.59°S, 74.30°E) and IVBC-20A (11.59°S, 75.29°E) at ~5100 m depth. The samples were collected with an USNEL type box corer of 50 cm³ size (Raghukumar et al. 2004). The collected sediments were mostly undisturbed and compact. The average length of sediment cores obtained from these locations was ~40 cm. Sub-cores of sediments were collected using an alcohol-sterilized PVC cylinder of 5-cm inner diameter from the centre of the box corer. Subsections of 2 cm down to a depth of 10 cm and thereafter every 5 cm length down to 40 cm depth were cut from the sediment cores and directly introduced into sterile plastic bags to avoid any aerial contamination. Fractions of these sediments were stored at -20°C immediately after sampling for DNA extraction.

Isolation of fungi

An aliquot of freshly collected sediment from the central part of the subsection was removed with an alcohol-sterilized spatula and placed in sterile vials for isolation of fungi on board the research vessel. Six different media, 1) Malt Extract Agar (MEA), 2) Czapek Dox Agar (CDA), 3) Corn Meal Agar (CMA), 4) Malt Extract Broth (MEB), 5) Sabouraud Dextrose Agar (SDA) and 6) Potato Dextrose Agar (PDA) were used for isolating fungi (Damare et al. 2006). The total organic carbon (TOC) in these sediments was reported to be in the range of 0.3-0.4 mg g⁻¹ dry weight (Sharma 2010). In contrast, the TOC content in coastal sediments of the Arabian Sea off Goa was 3-4% (Jebaraj & Raghukumar 2009). Therefore, the above media were used at 1/5 strength to simulate the low nutrient conditions of deep-sea sediments. The media were prepared in seawater and supplemented with penicillin and streptomycin (0.1 g each in 100 ml medium) to inhibit bacterial growth. Four different methods were employed for

isolation of fungi from the sediments. They were: 1) Particle plating method of [Bills & Polishook \(1994\)](#) wherein ~1 g of sediment was sieved sequentially through 2 meshes of 200 and 100 micron size using sterile seawater. Particles which passed through 200 microns but were retained on 100-micron mesh were spread on plates containing various media as mentioned above, 2) Dilution plating ([Damare et al. 2006](#)) wherein ~0.1 g of sediment was suspended in 9 ml of sterile seawater and vortexed for 1 min. An aliquot of 100 µl was spread on various media plates. 3) Pressure Incubation wherein ~0.1 g of sediment was suspended in 4 ml of MEB (1:5 diluted and amended with antibiotics) and sealed in plastic bags. These bags were incubated in pressure vessel (Tsurumi & Seiki Co., Japan) as described in detail ([Damare et al. 2006](#)). At the end of the incubation period the bags were opened and 100 µl was spread on five different media plates. 4) Low temperature incubation wherein ~0.1 g of sediment was directly suspended in 5 ml of MEB and incubated at 5°C for 30 days. Fungal filaments, which appeared in this broth were subcultured on MEA plates. All the media plates were incubated at 5°C for 30 days. Airborne fungi present in the ship laboratory and deck, contaminating the culture plates were monitored by exposing all the five different media plates for 10 min on the deck of the research vessel where the sediment cores were received, the microbiology laboratory on board and the inoculation hood. Contaminants that appeared on these control media plates were identified and such strains with identical morphology isolated from deep-sea sediments were eliminated. After isolation, the deep-sea fungi were maintained on MEA slants at 5°C and numbered as NIOCC (National Institute of Oceanography Culture Collection).

Isolation of DNA from the cultures and sediments

Fungi isolated by various techniques were identified by amplification and sequencing of their 18S region of SSU rRNA gene. Mitosporic fungi were also identified by classical morphological taxonomy. The fungi were grown in MEB for 4-5 days for DNA isolation. Yeasts were grown in yeast extract peptone and dextrose (YPD) medium and shaken at 170 rpm for 3-4 days. Mycelia and cells were harvested, lyophilized and crushed to a fine powder. Isolation of fungal DNA was carried out following the method of [Singh et al. \(2010\)](#).

Sediment DNA was isolated from each frozen sub-section of the sediment cores under sterile conditions to avoid cross-contamination ([Singh et al. 2011a](#)). DNA was isolated from 0.5 g of the sediment sample using the Q-Bio gene Soil DNA extraction kit (MP Biomedicals, OH, US) according to

the manufacturer's instructions. The sediment DNA samples were pooled into two parts of 0–15 and 15–40 cm for each of the two cores. Negative control contained no sediment or fungal mycelia during DNA extractions.

PCR amplification of fungal and sediment DNA and construction of clone libraries

Partial region of 18S rDNA of fungal isolates obtained in pure culture was PCR amplified by using primers NS1 and NS4 (~1100 bp) (White et al. 1990). Fresh PCR products were purified by using gel extraction kit (Sigma, Genosys, USA).

Sediment DNA samples were amplified using fungal specific ITS1F/ITS4 and universal 18S rDNA NS1/NS2 primer set (Singh et al. 2011a). 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× 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 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 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. Clones containing positive insert were further processed for plasmid isolation and purification using Millipore plasmid preparation kit (Millipore, USA). Sequencing of the PCR products from fungal isolates and 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 (3,730 DNA Analyzer, Applied Biosystems, USA). A total of eight environmental clone libraries (2 sediment cores x 2 depths each x 2 primer sets each) were constructed and 48 clones were screened from each of these libraries.

Phylogenetic analyses

Forward and reverse sequences were assembled using Chromas Pro version 1.34 (Technelysium Pvt. Ltd, Tewantia, Queensland, Australia). Sequences obtained with ITS and 18S rRNA gene primers were analyzed separately. All the sequences from environmental libraries were checked using Check_Chimera utility (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. Clones 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 version 1.4.1 (Schloss et al. 2009). The phylotypes were represented as OTU_01 to OTU_18, obtained with universal 18S rRNA gene primer pairs and OTU_19 to OTU_46 (Bold fonts) retrieved with fungal-specific ITS primer pair sets. A representative sequence from each OTU was queried against NCBI-GenBank BLASTN search (Altschul et al. 1990). Multiple alignments were carried out for all the sequences along with their closest match in Clustal W (Thompson et al. 1994). Gaps and ambiguously aligned sequences were removed from further analyses. Phylogenetic analyses were conducted using distance setting (Maximum parsimony) in MEGA 4 (Kumar et al. 2008) with 1,000 bootstrap replicates. Phylogenetic trees were constructed with sequences obtained with ITS and 18S rRNA gene primer sets individually. The statistical analyses for fungal isolates were carried out in Microsoft Excel program and PRIMER 5 software for calculation of diversity indices. All the sequences were deposited in NCBI-GenBank under the accession numbers **JF497103** to **JF497121** for fungal species isolated by conventional culture methods and **JF497122** to **JF497167** for representative sequences obtained by culture-independent method

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 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 10^7 - 10^9 cells g^{-1} dry sediment. Organic carbon ranged from 0.3-0.4 mg g^{-1} dry sediment (Sharma 2010).

Diversity and species richness of fungi by culture-dependent approach

A total of 19 cultures belonging to 12 distinct fungal genera were isolated. Fungi were recovered by particle plating and pressure incubation methods only, the later yielding higher number of isolates. Cultures were isolated from all the depths of the sediment cores including 35-40 cmbsf. Best media for isolation were found to be CDA and PDA. Majority of the fungi belonged to Ascomycota, wherein no single species dominated. It included members of filamentous fungi such as *Aspergillus* sp., *Eurotium* sp., *Cladosporium* sp., *Pleospora* sp., *Chaetomium* sp., *Ascotricha* sp., *Penicillium* sp., and *Sagenomella* sp. (Table 1). Two ascomycetous culture sequences clustered with black yeast *Hortaea* sp. (Fig. 1). Most of these fungal sequences showed >98% similarity with the sequences of their closest relative species. However two ascomycetous fungal genotypes, NIOCC#F55 and NIOCC #Y14 showed only 95 and 97 % similarity with the existing sequences in the NCBI database. Out of the five basidiomycetous fungi, four were affiliated to *Cerrena* sp. and one to *Trametes* sp (Table 1). Fungal species distribution varied among different depths of the sediments cores. The Shannon-Wiener diversity index and species richness were, highest, being 1.0 and 1.8 respectively at 20-25 cm depth.

Diversity of fungi by culture-independent approach

A total of 384 clones were sequenced from the 8 clone libraries. Of these, 67 (~ 35%) and 55 clones (~ 29%) obtained with ITS and 18S rRNA gene primer sets respectively were chimeric and therefore removed from the subsequent analysis. From the remaining clones, a total of 18 and 28 OTUs were obtained with 18S and ITS rRNA gene primer sets respectively (Table 2). The results are presented as OTU_01 to OTU_18 obtained with universal 18S rRNA gene primer pair, and **OTU_19 to OTU_46** with fungal-specific ITS rRNA gene primer pair. Out of the total 46 OTUs, 42 OTUs represented distinct fungal species belonging to Ascomycota and Basidiomycota, the majority being ascomycetous fungi (Table 2).

The primer pair NS1/NS2 amplified 10 different phylotypes of Ascomycota belonging to three classes, Eurotiomycetes, Saccharomycetes and Dothideomycetes. Four basidiomycetous phylotypes amplified belonged to the classes Agaricomycetes, Exobasidiomycetes and Agaricostilbomycetes. The

basidiomycete phylotype OTU_18 showed 98 % similarity to *Cerrena unicolor* (Table 2). Among other sequences, the most common forms were uncultured fungal clones from marine environment that are deposited in the NCBI database (Table 2). OTU_01, OTU_02, OTU_04 and OTU_11 showed $\leq 97\%$ similarity with their closest relatives in the existing NCBI database. The 18S rRNA gene primer set amplified four OTUs affiliating with eukaryotic sequences out of which two affiliated with Alveolata ($\leq 86\%$) and two with Viridiplantae with 99 % similarity (Table 2, Fig. 2).

The primer pair ITS1F/ITS4 amplified 16 fungal phylotypes of the phylum Ascomycota belonging to four different classes, Sordariomycetes, Eurotiomycetes, Saccharomycetes and Dothideomycetes (Table 2). Whereas among Basidiomycota, twelve phylotypes belonging to seven different classes as shown in Table 2. OTU_24, OTU_27, OTU_35, OTU_39 and OTU_46 were found to show $\leq 97\%$ similarity with their closest relatives. One phylotype, OTU_44 clustered with the *Hortaea* sp. with 98 % similarity (Table 2, Fig. 3).

Discussion

Diversity of cultured fungi

Out of the two sediment cores used for isolation, nineteen fungal cultures were recovered which is comparatively higher than the previous studies from the same sampling area where 28 cultures were isolated from twenty sediment cores (Singh et al. 2010). A possible explanation for this disparity in result may be due to the use of two more media, PDA and SDA for isolation in the present study. A total of six and two fungi were isolated using PDA and SDA media respectively with various isolation techniques.

All the fungal genotypes obtained during this cruise showed $>98\%$ similarity with the existing fungal sequences in the NCBI database except NIOCC#F55 and NIOCC#Y14. These sequences showed a similarity of $<97\%$ with their closest match suggesting their possibility of being novel phylotypes of *Aspergillus* and *Hortaea* sp. respectively (Table 1, Fig. 1). A multigene analysis combined with detailed morphological and ultra structural studies will aid in deciding their novelty. The isolation of *Sagenomella*, *Aspergillus*, *Penicillium* and *Cladosporium* spp. during the previous (Singh et al. 2010) as well as the present study suggests their frequent occurrence and greater distribution in these sediments. *Sagenomella chlamydospora* was reported as a pathogen in terrestrial environments (Gene et al. 2003)

suggesting the possible role of *Sagenomella* as pathogen of deep-sea organisms. These fungi except *Sagenomella* have also been reported to be present in deep-sea sediments by others (Damare et al. 2006; Burgaud et al. 2009). These geofungi may have a role in deep-sea environments in spite of not being indigenous. Occurrence of terrestrial fungi in deep-sea sediments from the Chagos Trench in the Indian Ocean at 5000 m depth was reported (Raghukumar et al. 2004), wherein, *Aspergillus* sp., a mitosporic fungus was isolated as a dominant form from 450 cmbsf. Spores of *Aspergillus sydowii* from these sediments germinated and grew at elevated hydrostatic pressure and low temperature. It has been hypothesized that air-borne spores or those carried with terrestrial run off water might eventually sink to the deep-sea surficial sediments, undergo natural selection mechanisms with time and acquire capabilities to grow and multiply in the presence of suitable nutrient sources (Raghukumar et al. 2004).

Two of the isolates (NIOCC#F68, NIOCC#Y14) showed similarity to *Hortaea werneckii*. This black yeast-like fungus (Fig. 4) is characterized as halophilic or extremely halotolerant in various studies (Gunde-Cimerman et al. 2000; Kogej et al. 2005). Two isolates of black yeast, *Hortaea werneckii* were obtained by Le Calvez et al. (2009) from deep-sea hydrothermal ecosystems. *Nigrospora oryzae* (NIOCC#F51) was isolated from marine sponge (Ding et al. 2011). Several of the marine sponge-derived fungi were reported to produce useful antimicrobial compounds and thus playing an important ecological role in chemical defense in sponges (Bugni & Ireland 2004).

We are aware that the international commission on fungal barcoding has proposed ITS region as the prime fungal barcode for species identification. However, our earlier work showed that microscopic identification of sporulating cultures matched either with 18S or ITS rRNA gene sequences but seldom with both (Singh et al. 2010). Besides, D'Souza et al. (2009) and Verma et al. (2010) also could identify biotechnologically useful mangrove fungi accurately with 18S rRNA gene sequencing. Moreover insufficient data base for ITS sequences (Anderson et al. 2003; Zachow et al. 2009) may limit the diversity assessment. Therefore, to identify fungi obtained in cultures we used 18S gene sequencing and for environmental samples we used both ITS as well as 18S rRNA gene sequencing.

Diversity of fungi by culture-independent approach

A total of 18 and 28 OTUs were recovered from 192 clones (2 cores x 2 depths x 48 clones) obtained with 18S and ITS rRNA gene primer sets each, resulting in frequency of 9.3 and 14.5 % respectively.

These OTUs represented 42 fungal and 4 non-fungal eukaryotic sequences. All the forty two fungal OTUs represented a distinct phylotype indicating a high diversity of fungi in the sampling site. However, mere detection of genes does not mean that all these fungi are active in the deep sea. Future studies should include RNA-derived lineages to identify active members of the community as was reported by [Edgcomb et al. \(2011\)](#). The singletons obtained during this study were 4 and 10 with 18S and ITS rRNA gene primer sets respectively, reflecting a lack of saturation level in selection of clones for analysis.

Among fungi, only Dikaryotic forms were recovered in the present study whereas [O'Brien et al. \(2005\)](#) reported amplification of other phylotypes of fungi using NS1/NS2 primer set. The absence of phylotypes from Zygomycota and Chytridiomycota in the present as well as the previous study by [Singh et al. \(2011a\)](#) suggests that these classes of fungi are not abundant in the deep-sea environment or additional sampling events might be necessary to capture their presence. Several OTUs clustered with the sequences of uncultured fungal clones ([Table 2, Figs. 2 & 3](#)). These sequences were also detected in the earlier studies from the CIB sampling site albeit at different sampling locations ([Singh et al. 2011a](#)) suggesting their abundance in the sediments of CIB. Further a total of four and five OTUs obtained with 18S and ITS rRNA gene primer sets respectively showed <97% similarity with sequences in existing NCBI database suggesting probability of them being novel ([Table 2](#)). The Ascomycete phylotypes, OTU_24, OTU_39 and OTU_46 showed affiliation with the *Aspergillus* sp. with a percentage similarity of ≤ 97 . The phylotype OTU_46, showing 95% similarity with *Aspergillus* sp. was also isolated in culture form (NIOCC#55) from the same site ([Table 1](#)). Such high divergence of this phylotype from the existing terrestrial species may be useful in understanding its adaptation and evolution strategies under extreme conditions of deep-sea. On the other hand, [Zuluaga-Montero et al. \(2010\)](#) demonstrated that the terrestrial and marine isolates of *Aspergillus flavus* did not form a discrete populations, with no clade particular to marine environments. Two of the phylotypes, OTU_18 and OTU_44, affiliated with *Cerrena* and *Hortaea* sp. respectively. These species were isolated in culture form also suggesting their possibility to be the active members of the deep-sea fungal community. However, the NCBI blast searches of the above three fungi showed different accession numbers for the cultures isolated and environmental sequences ([Table 1 & 2](#)). Therefore, the culture isolates and the environmental sequences belonging to the same genera were not identical phylotypes. The phylotype, OTU_08 belonged to *Saccharomyces* species with a percentage similarity of 99 ([Table 2](#)). The recovery of this species is

being reported for the first time from this area in the present study. Several workers have used this species as a model organism for studies on high pressure stress effects at physiological, genomic and proteomic level (Abe & Horikoshi 2000; Fernandes 2005; Domitrovic et al. 2006). The phylotype belonging to *Malassezia* sp. was recovered from methane hydrate-bearing deep-sea sediments (Lai et al. 2007) and marine subsurface (Edgcomb et al. 2011). Such methylotrophic yeasts may play a crucial role in converting methane into more accessible carbon and energy substrates. They are of great interest in physiological studies and industrial applications. *Malassezia* is also a known pathogen and its presence in deep-sea sediments suggests that it may be an opportunistic pathogen of marine mammals (Edgcomb et al. 2011). The Basidiomycete phylotypes, OTU_15 and OTU_19, were closely related to *Sterigmatomyces* sp., a new marine yeast isolated from the Biscayne Bay, Florida, USA (Fell 1966). This phylotype was amplified by 18S as well as ITS primer pairs. In general, both culture-dependent and culture-independent approaches yielded a low number of yeasts from sediments (Table 1 & 2) compared to our earlier reports (Singh et al. 2010, 2011a). Bass et al. (2007) reported yeasts to be dominant forms in deep-sea samples. Nagahama et al. (2003, 2006, 2008) reported three novel yeast species from deep-sea sediments of the Pacific Ocean. Several culturable yeasts belonging to Ascomycota and Basidiomycota were reported from deep-sea hydrothermal vent fauna (Burgaud et al. 2010).

Fungal sequences belonging to some of the classes such as Eurotiomycetes, Sordariomycetes, Dothideomycetes, Agaricomycetes, Saccharomycetes and Wallemiomycetes are abundant in deep-sea sediments of CIB. They may play an important role in the deep-sea ecosystems of the CIB. Cultivating these terrestrial fungi identified in the clone libraries to determine their adaptive strategies under such extreme conditions of pressure and temperature is our future goal.

Damare et al. (2008) demonstrated that 90 % spores of several deep-sea isolates of *Aspergillus* lost viability within 16-17 days of incubation at 5°C/ 0.1 MPa. On the other hand mycelial fragments showed growth and biomass production under elevated pressure at 5°C. These results have implications on differential effects of low temperatures and elevated hydrostatic pressures on long-term survival and further growth of spores and mycelial fragments. These factors may in turn affect the fungal diversity recovered from deep-sea sediments.

Several fungi that appeared in culture-dependent approach showed no signatures in the environmental DNA-derived lineages. They are not likely to be contaminants as control checks carried out at every stage of operation did not show their presence. Only a fraction of the deep-sea sediments

were used for culturing or amplification which may underestimate the real diversity. Culturable fungi were obtained from CIB from depths down to 30 cmbsf in the present study. The sedimentation rates here are reported to be ~1 cm per 1000 years (Gupta 1999). This suggests that the fungi found at these depths were buried at least ~30,000 years before the present. Do they survive as dormant forms or are they actively growing and contributing to the biogeochemical cycles in the deep-sea needs to be explored. RNA-based clone libraries as generated by Edgcomb et al. (2011) and metagenomics (Wegley et al. 2007) may help in understanding this problem.

Four sequences belonging to non-fungal eukaryotic phylotypes using the 18S rRNA gene primer set were amplified (Table 2, Fig. 2). Two of them belonged to Alveolata whereas the other two affiliated with Viridiplantae. These sequences were not recovered during earlier studies from the CIB even after amplification with eukaryotic primers (Singh et al. 2011b). The sampling locations selected for analyses during the previous studies were different from the sites sampled in the present study. Therefore, the recovery of non-fungal eukaryotic sequences during the present study and their absence in the earlier studies may be attributed to the spatial variation in distribution patterns. Edgcomb et al. (2011) and Lopez-Garcia et al. (2001) have reported amplification of non-fungal eukaryotic sequences from deep-sea marine environments by using eukaryotic primer sets. One of the phylotypes in the present study, OTU_05, clustered with an uncultured marine alveolate clone, NA1-4G9 (EF526792) (Behnke et al. 2010), which was recovered from an O₂/H₂S gradient in a Norwegian fjord. The percentage similarity of the OTU_05 with the above alveolate clone was found to be 86 (Table 2), suggesting it to be a novel species. The recovery of such marine eukaryotic signatures may provide insight into the evolutionary history of protists (Steenkamp et al. 2006).

In conclusion, this study revealed a greater diversity of fungal signatures in deep-sea sediments of the CIB by combining culture-dependent and culture-independent approaches than earlier reports using single approach (Singh et al. 2010, 2011a). Isolates showing low similarity with the sequences in the existing NCBI database and novel marine isolates of the terrestrial species are some of the highlights of the present study. These isolates may be screened for novel bioactive compounds. In addition, use of improved techniques for culturing the uncultured will lead towards unraveling the hidden fungal diversity.

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Legends to the figures

Fig. 1. Maximum parsimony (MP) phylogenetic tree for deep-sea fungi obtained by culture-dependent approach based on 18S rDNA gene sequences using universal primers, NS1/NS4.. Topology was built using Mega 4 from a ClustalW 1.83 alignment for ~1100 bp long sequences. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates.

Fig. 2. Maximum parsimony (MP) phylogenetic tree for the fungal OTUs based on 18S rDNA gene sequences using universal primers, NS1/NS2. Topology was built using Mega 4 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. MP phylogenetic tree for the fungal OTUs based on ITS gene sequences using fungal specific primers, ITS1F/ITS4. Topology was built using Mega 4 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. *Hortaea* sp. in a culture plate on a solid medium.

Table 1. Phylogenetic affiliations of culturable fungi identified on the basis of 18S rDNA gene sequences (Sequences showing <97 % similarity values are represented in bold)

| Isolate ID | Closest Identified relative (Accession number) | Phylum of closest identified relative | Source of isolation of the closest relative | % identity |
|------------------|---|---------------------------------------|---|------------|
| NIOCC#F51 | <i>Nigrospora oryzae</i> (AB220233) | Ascomycota | Not known | 99 |
| NIOCC#F52 | <i>Cladosporium</i> sp. (GU322367) | Ascomycota | Soil | 99 |
| NIOCC#F53 | <i>Trametes versicolor</i> (AY336751) | Basidiomycota | Not known | 99 |
| NIOCC#F54 | <i>Chaetomium elatum</i> (M83257) | Ascomycota | Not known | 99 |
| NIOCC#F55 | <i>Aspergillus versicolor</i> (AB002064) | Ascomycota | Not known | 95 |
| NIOCC#F56 | <i>Ascotricha lusitanica</i> (AB048282) | Ascomycota | Leaf | 99 |
| NIOCC#F57 | <i>Pleospora herbarum</i> (GU238232) | Ascomycota | Not known | 98 |
| NIOCC#F58 | <i>Cladosporium</i> sp. (GU322367) | Ascomycota | Soil | 99 |
| NIOCC#F59 | <i>Eurotium herbariorum</i> (AF548072) | Ascomycota | Air | 99 |
| NIOCC#F60 | <i>Cerrena</i> sp. NIOCC#2a (FJ010210) | Basidiomycota | Mangrove wood | 99 |
| NIOCC#F61 | <i>Cerrena</i> sp. NIOCC#2a (FJ010210) | Basidiomycota | Mangrove wood | 99 |
| NIOCC#F62 | <i>Penicillium griseofulvum</i> (FJ717697) | Ascomycota | Atmosphere of Porto | 100 |
| NIOCC#F63 | <i>Penicillium griseofulvum</i> (FJ717697) | Ascomycota | Atmosphere of Porto | 99 |
| NIOCC#F64 | <i>Aspergillus versicolor</i> (GU227343) | Ascomycota | Not known | 99 |
| NIOCC#F65 | <i>Sagenomella</i> sp. (EU140822) | Ascomycota | Not known | 99 |
| NIOCC#F66 | <i>Cerrena</i> sp. NIOCC#2a (FJ010210) | Basidiomycota | Mangrove wood | 99 |
| NIOCC#F67 | <i>Cerrena</i> sp. NIOCC#2a (FJ010210) | Basidiomycota | Mangrove wood | 99 |
| NIOCC#F68 | <i>Hortaea werneckii</i> (GU296153) | Ascomycota | Culture collection | 99 |
| NIOCC#Y14 | <i>Hortaea werneckii</i> (GU296153) | Ascomycota | Culture collection | 97 |

Table 2. Phylogenetic affiliations of the OTUs obtained with two primer sets from two depths of two sediment cores . (Sequences showing <97 % similarity values are represented in bold)

| OTU no. | Primer set used | Closest identified relative | | | | | % Similarity |
|---------|-----------------|--|---------------|----------------------|---------------|----------------------|--------------|
| | | Taxon | Phylum | Class | Accession no. | Source of isolation | |
| OTU_01 | NS1/NS2 | Uncultured soil ascomycete | Ascomycota | Eurotiomycetes | AJ515945 | Soil | 97 |
| OTU_02* | | Uncultured fungus clone | Ascomycota | Eurotiomycetes | GU370739 | Marine sediment | 95 |
| OTU_03 | | Uncultured <i>Aspergillus</i> clone | Ascomycota | Eurotiomycetes | FJ393417 | Gut | 99 |
| OTU_04 | | <i>Phialosimplex caninus</i> | Ascomycota | Eurotiomycetes | GQ169310 | Culture Collection | 97 |
| OTU_05 | | Uncultured marine non-fungal eukaryote clone | Alveolata | Dinophyceae | EF526792 | Marine environment | 86 |
| OTU_06 | | Eimeriidae environmental clone | Alveolata | Apicomplexa | EF024655 | Rhizosphere | 87 |
| OTU_07 | | <i>Angelica gigas</i> | Viridiplantae | Apiaceae | DQ647697 | Culture Collection | 99 |
| OTU_08 | | <i>Saccharomyces</i> sp. | Ascomycota | Saccharomycetes | DQ345287 | Culture Collection | 99 |
| OTU_09 | | Uncultured marine fungus clone | Ascomycota | Saccharomycetes | GQ120138 | Arabian sea sediment | 99 |
| OTU_10 | | Uncultured soil ascomycete | Ascomycota | Sordariomycetes | AJ515930 | Soil | 100 |
| OTU_11 | | Uncultured fungus clone | Ascomycota | Eurotiomycetes | GU370729 | Marine sediment | 95 |
| OTU_12 | | <i>Pycnoporus</i> sp. | Basidiomycota | Agaricomycetes | GU182936 | Kraurotic chump | 100 |
| OTU_13 | | <i>Nicotiana tabacum</i> | Viridiplantae | Solanaceae | AY079155 | Culture Collection | 99 |
| OTU_14 | | Uncultured <i>Malassezia</i> clone | Basidiomycota | Exobasidiomycetes | FJ393445 | Gut | 99 |
| OTU_15 | | <i>Sterigmatomyces halophilus</i> | Basidiomycota | Agaricostilbomycetes | DQ092916 | Culture Collection | 100 |
| OTU_16* | | Uncultured soil ascomycete | Ascomycota | Dothideomycetes | AJ515936 | Soil | 99 |
| OTU_17* | | <i>Dothideomycete</i> sp. | Ascomycota | Dothideomycetes | AY275186 | Culture Collection | 99 |

| | | | | | | | |
|---------|---------|------------------------------------|---------------|----------------------|----------|------------------------|-----|
| OTU_18* | NS1/NS2 | <i>Cerrena unicolor</i> | Basidiomycota | Agaricomycetes | AY850007 | Culture Collection | 98 |
| OTU_19 | ITS1F/ | <i>Sterigmatomyces</i> sp. | Basidiomycota | Agaricostilbomycetes | FJ755830 | Marine sponge | 99 |
| OTU_20 | ITS4 | <i>Gibberella moniliformis</i> | Ascomycota | Sordariomycetes | EU717682 | Culture Collection | 99 |
| OTU_21 | | <i>Schizophyllum commune</i> | Basidiomycota | Agaricomycetes | EF155505 | Decaying wood | 99 |
| OTU_22 | | Uncultured fungus clone | Ascomycota | Saccharomycetes | GU370744 | Marine sediment | 99 |
| OTU_23 | | Uncultured fungus clone | Basidiomycota | Agaricomycetes | GU370760 | Marine sediment | 99 |
| OTU_24* | | <i>Aspergillus</i> sp. | Ascomycota | Eurotiomycetes | FJ755817 | Marine sponge | 97 |
| OTU_25 | | <i>Trichosporon asahii</i> | Basidiomycota | Tremellomycetes | AB369919 | Culture Collection | 99 |
| OTU_26 | | Uncultured fungus clone | Ascomycota | Dothideomycetes | GU721670 | Filter dust | 100 |
| OTU_27 | | Uncultured fungus clone | Basidiomycota | Agaricomycetes | GQ999291 | Air filter sample | 97 |
| OTU_28 | | <i>Rhodotorula slooffiae</i> | Basidiomycota | Cystobasidiomycetes | FJ515213 | Sea surface microlayer | 99 |
| OTU_29 | | <i>Rhodotorula</i> sp. | Basidiomycota | Microbotryomycetes | AB025984 | Deep-sea environments | 99 |
| OTU_30 | | Uncultured endophytic fungus clone | Basidiomycota | Wallemiomycetes | FJ524297 | Root endophyte | 99 |
| OTU_31 | | <i>Debaryomyces hansenii</i> | Ascomycota | Saccharomycetes | GQ458025 | Camembert cheese | 99 |
| OTU_32* | | <i>Stenella musicola</i> | Ascomycota | Dothideomycetes | EU514294 | Banana | 99 |
| OTU_33* | | <i>Malassezia slooffiae</i> | Basidiomycota | Exobasidiomycetes | AY743633 | Domestic animals | 99 |
| OTU_34* | | <i>Gibberella moniliformis</i> | Ascomycota | Sordariomycetes | EU717682 | Culture Collection | 100 |
| OTU_35 | | <i>Resinicium friabile</i> | Basidiomycota | Agaricomycetes | DQ826543 | Hardwood | 97 |
| OTU_36* | | <i>Aspergillus</i> sp. | Ascomycota | Eurotiomycetes | EU301661 | Forest soil | 99 |
| OTU_37 | | <i>Rhodotorula mucilaginosa</i> | Basidiomycota | Microbotryomycetes | DQ386306 | Culture Collection | 99 |
| OTU_38 | | <i>Malassezia restricta</i> | Basidiomycota | Exobasidiomycetes | EU400587 | Rust pathogen | 99 |
| OTU_39 | | <i>Aspergillus penicillioides</i> | Ascomycota | Eurotiomycetes | GU017496 | Seagrass | 96 |

| | | | | | | | |
|----------------|---------------|------------------------------|------------|-----------------|----------|--------------------|-----------|
| OTU_40 | | <i>Aspergillus oryzae</i> | Ascomycota | Eurotiomycetes | EU301638 | Forest soil | 99 |
| OTU_41* | | <i>Neurospora</i> sp. | Ascomycota | Sordariomycetes | GU183173 | Rice | 99 |
| OTU_42 | | <i>Penicillium</i> sp. | Ascomycota | Eurotiomycetes | GU566250 | Rhizosphere | 100 |
| OTU_43* | | Uncultured fungus clone | Ascomycota | Eurotiomycetes | FJ820795 | Air sample | 99 |
| OTU_44* | | <i>Hortaea</i> sp. | Ascomycota | Dothideomycetes | FJ755827 | Marine sponge | 98 |
| OTU_45* | ITS1F/ | <i>Corynespora cassicola</i> | Ascomycota | Dothideomycetes | AY238606 | Cucumber | 99 |
| OTU_46* | ITS4 | <i>Aspergillus</i> sp. | Ascomycota | Eurotiomycetes | EU139858 | Culture Collection | 95 |

Singletons are represented with * (OTU numbers amplified with ITS primer sets are represented in bold)

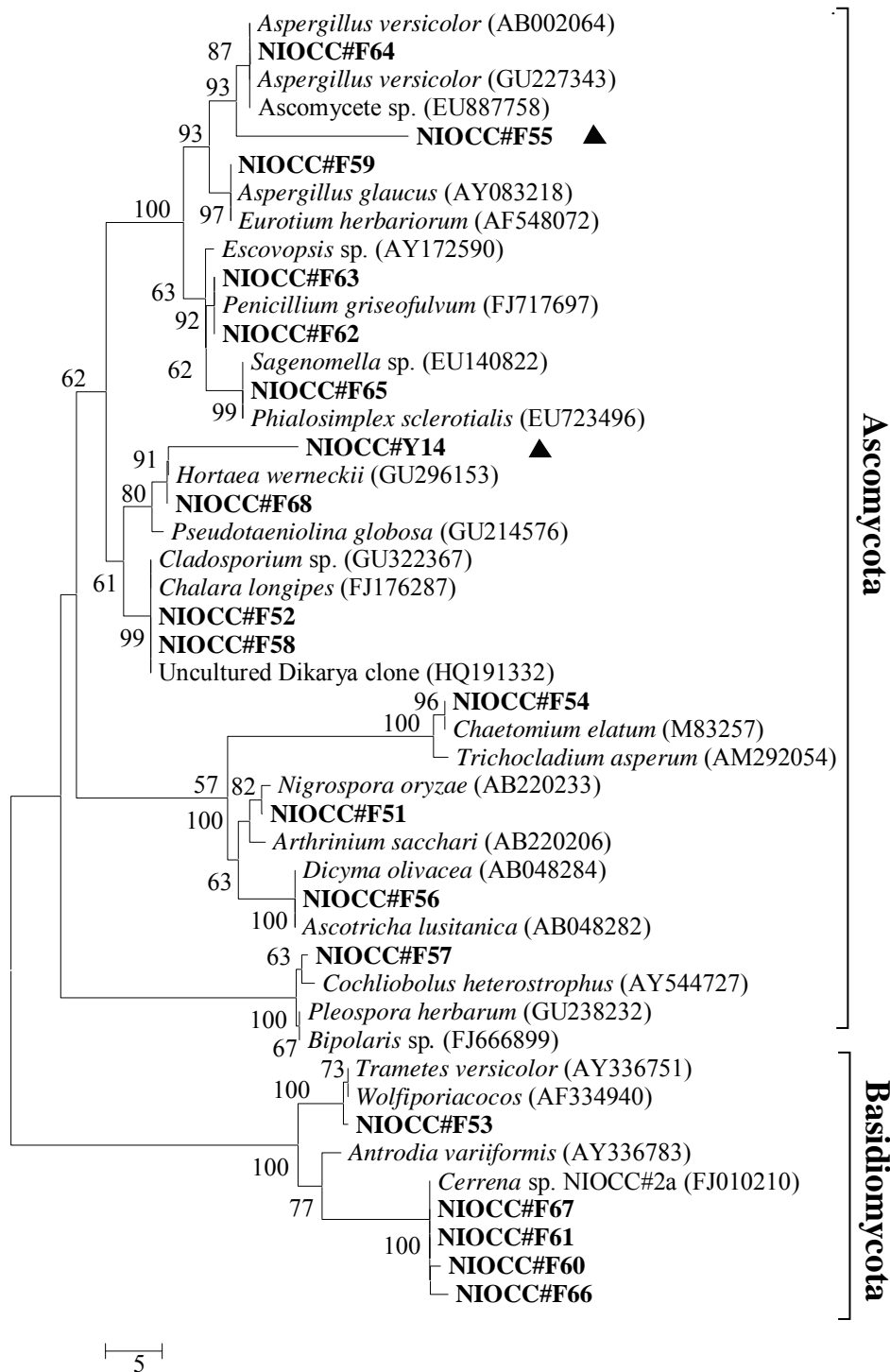


Fig. 1

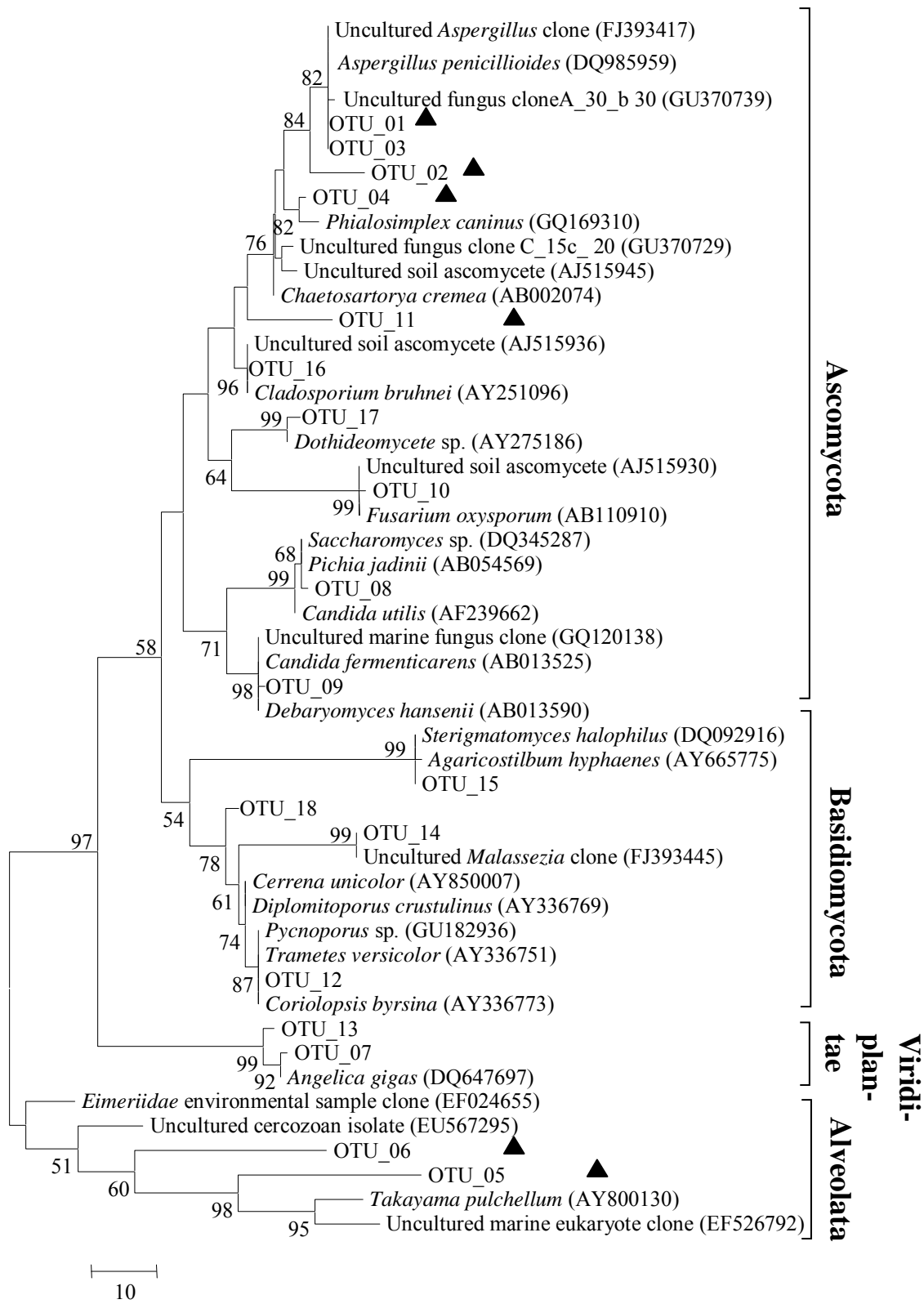


Fig. 2

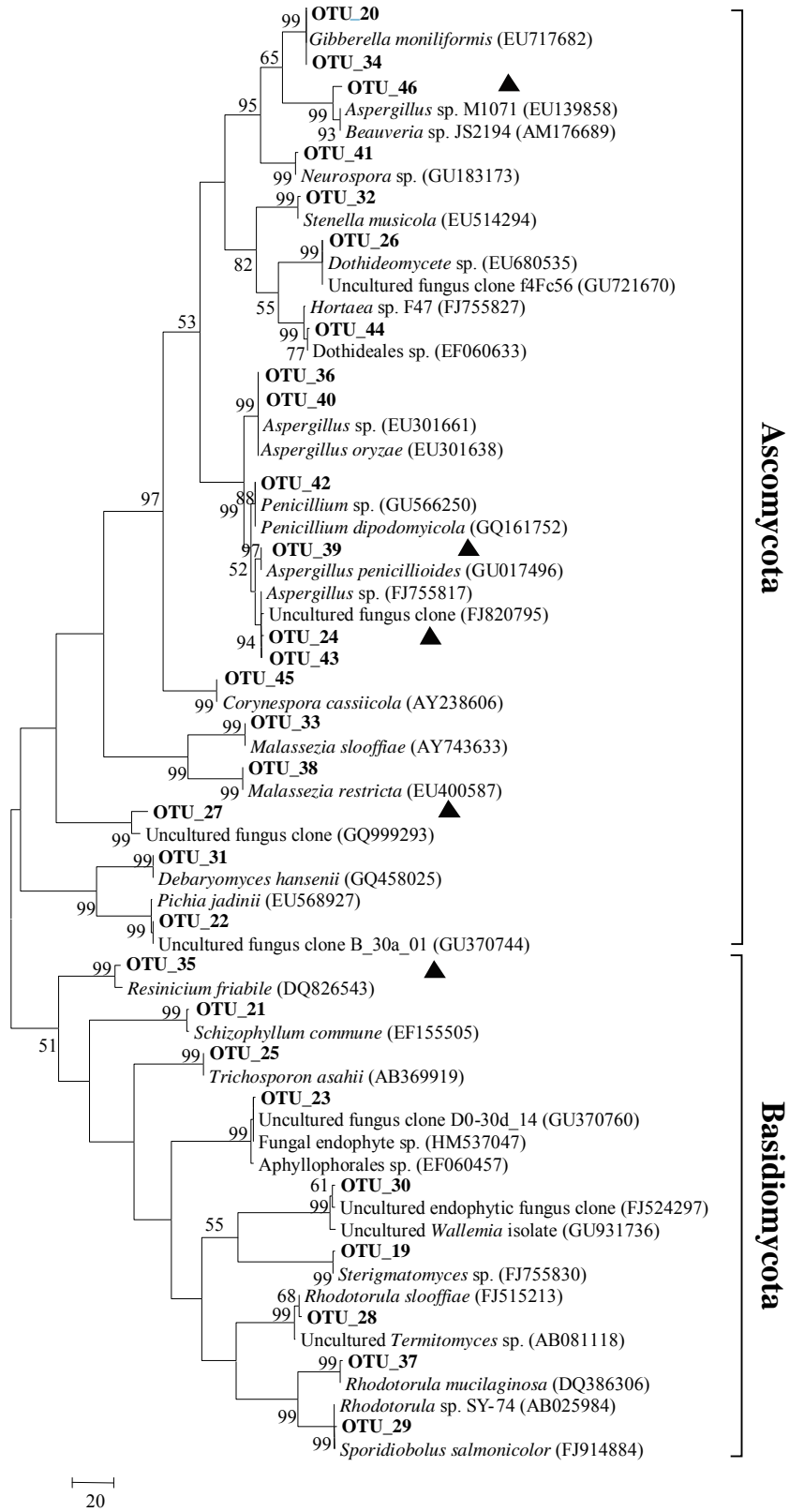


Fig. 3



Fig. 4