

Chapter 3

Fungal community analysis
from deep-sea sediments of CIB
using culture-independent
approach

3.1 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). Little is known about fungal contribution towards these microbial communities. Being a significant part of the marine ecosystems either as saprobes, pathogens or symbionts, the actual diversity assessment of fungal communities is crucial from ecological point of view. There are several reports on diverse forms of fungi from terrestrial environment (Smit et al, 1999; O'Brien et al, 2005; Gadd et al, 2007). The problems associated with determining fungal diversity accurately have been highlighted in a number of reviews dealing with terrestrial systems (Kowalchuk, 1999; Bridge and Spooner, 2001; Horton and Bruns, 2001; Anderson and Cairney, 2004). Similar limitations also apply to the identification, isolation and quantification of fungi from marine environments, with the additional complication of distinguishing between transitory and native forms.

Diversity assessment of fungi from marine environment using culture-dependent approaches has been applied in previous studies (Damare et al, 2006; Wang et al, 2008). These methods can suffer from spore-related identification problems, as well as exhibiting biases in isolate recovery. Such biases result from unknown culturing conditions, competitive interference and over-growth by other fungi (Hyde et al, 2000; Panebianco et al, 2002). The situation is further aggravated by the presence of sterile mycelial forms that cannot be identified using traditional methods. Many reports have highlighted these and other problems associated with traditional mycological approaches when studying interference competition (Panebianco et al, 2002), the delimitation of geographical distributions (Steinke and Jones, 1993; Volkmann-Kohlmeyer and Kohlmeyer, 1993; Schmit and Shearer, 2004), the definition of host specificity or substratum preference (Steinke and Jones, 1993; Tan et al, 1995; Petersen and Koch, 1997; Schmit and Shearer, 2004), and succession patterns of marine fungi (Booth, 1979; Vrijmoed et al, 1986; Tan et al, 1989; Sarma and Hyde, 2001).

As an alternative, culture-independent techniques (Amann et al, 1995) provide ways for fungal identification and for monitoring communities. These methods are entirely molecular and as such they provide a potential link between ecological processes and the organisms involved. Diversity assessments by using molecular approaches offers the possibility of defining fungal communities accurately, studying fungal-host interactions directly in the environment and identifying ecologically active groups (Edgcomb et al, 2010).

3.1.1 Methods for studying fungal diversity by culture-independent approaches

Various molecular methods can be applied for the assessment of fungal communities directly from environmental samples. In particular, comparison of PCR-amplified ribosomal RNA genes sequences in conjunction with screening strategies such as restriction profile analysis and/or nucleic acid hybridization analysis permits rapid assessment of diversity (Fernandez et al, 1998). A variety of molecular techniques are available to separate PCR-amplified fungal genotypes from environmental sources (Fig 3.1). In order to apply these techniques for diversity assessment, DNA or RNA can be extracted from environmental samples (soils, sediments and water) using a variety of purpose-designed kits (Yeats et al, 1998; Griffiths et al, 2000). These extracted nucleic acids are of mixed origin, complex, comprising DNA or RNA from bacteria, animals, plants, fungi and other microeukaryotes. The efficiency with which the nucleic acids are extracted depends upon the species present, the environmental substrata sampled, as well as the method used.

Obtaining PCR amplifications from environmental sequences often depends upon the purity of the environmental nucleic acids. During extraction PCR-inhibitory components, such as humic acids, polysaccharides and tannins can be co-precipitated with the DNA and RNA. Removal of these impurities may be achieved by dilution or the inclusion of selective detergents, such as cetyltrimethylammonium bromide (CTAB) for the removal of

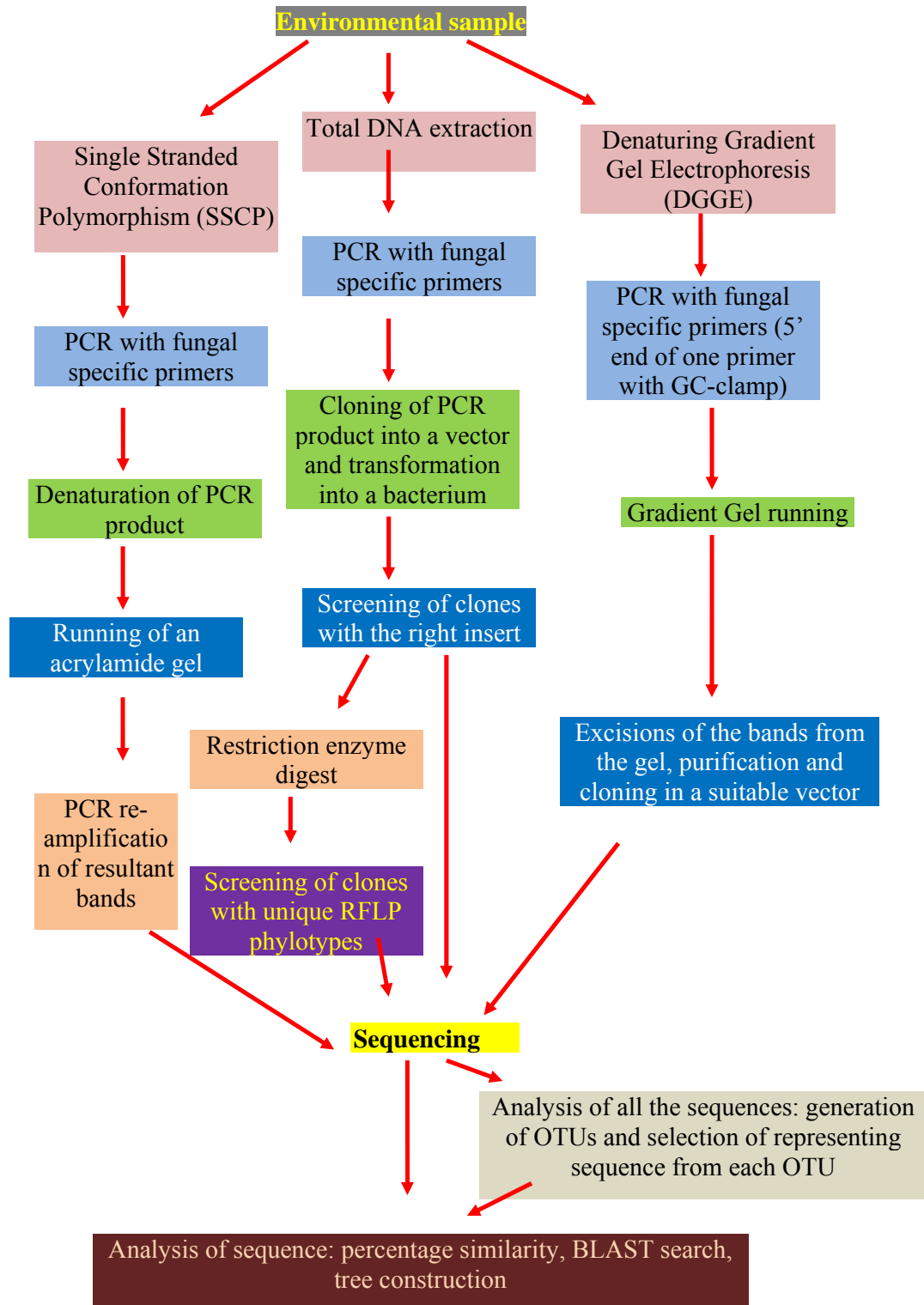


Fig 3.1 Schematic diagram showing different culture-independent approaches used for fungal diversity analysis from environmental samples (modified from Pang and Mitchell, 2005)

polysaccharides, or polyvinylpolypyrrolidone (PVPP), which binds polyphenols, in the extraction buffer. Brief description of some of the molecular methods used for fungal diversity assessment from environmental DNA sample is as follows:

- a) Amplified ribosomal DNA restriction analysis: In this technique ribosomal genes are PCR amplified using fungal specific primers. These amplicons are digested with restriction enzymes and run on the agarose gel. Community profiling can be deduced using this technique for an environmental sample ([Schmidt and Moreth, 2000](#); [Gich et al, 2000](#)).
- b) Automated ribosomal intergenic spacer analysis (ARISA): A fluorescent primer is used for PCR amplification which amplifies both the internal transcribed spacer (ITS) regions along with 5.8S rRNA gene. After running in an automated DNA sequencer, fragments of different sizes show different peaks and thus different species can be identified ([Ranjard et al, 2001](#)).
- c) Denaturing (temperature) gradient gel electrophoresis (DGGE/TGGE): Target gene is amplified by PCR with one primer attached to a GC-clamp. The products (200–650 bp) are run in a polyacrylamide gel with increasing denaturant gradient (DGGE) or temperature gradient (TGGE). The double helix of the PCR products melts in the gel and stops migrating depending on the nucleotide sequence. Therefore, different sequences (species) with identical lengths stop at different positions in the gel. The pattern can be used to reflect community structure. Individual bands can be sequenced and analyzed phylogenetically ([Kowalchuk et al, 1997](#); [Daniell et al, 2001](#); [Anderson et al, 2003b](#); [Zuccaro et al, 2003](#); [Green et al, 2004](#)).
- d) Gene cloning: Target gene is PCR amplified using specific primers. Amplified products are directly cloned in a suitable vector. The clones are sequenced and analyzed phylogenetically ([Birch et al, 1993](#); [Viaud et al, 2000](#)).

- e) Single strand conformation polymorphism (SSCP): In this technique the primer used for amplification is phosphorylated at 5' end. Target gene is amplified and the products are digested to destroy one of the strands. Resultant single stranded products are run in a non-denaturing acrylamide gel. Individual bands can be sequenced and analyzed phylogenetically which provides information about the community structure (Simon et al, 1993; Jansa et al, 2003; Nielsen et al, 2004).
- f) Terminal restriction fragment length polymorphism (T-RFLP): Target gene is amplified by PCR with one fluorescent primer. The amplicons are then digested with restriction enzymes. After sequencing one peak appears for one organism as only the terminal bands with the fluorescent primer are detected (Nikolcheva et al, 2003; Edwards and Turco, 2005).

3.1.2 Primers used for amplification of fungal genotypes from environmental samples

The amplification or detection of fungal sequences, particularly rare ones, from mixed-origin samples requires that the primers exhibit fungal specificity. Many primer combinations have not been fully validated against other organisms, and some, particularly the nuclear SSU primers, can also amplify sequences from a variety of plants, animals and other microeukaryotes (Lord et al, 2003; Anderson et al, 2003a; Zuccaro et al, 2003). The nuclear LSU and ITS primers appear to be more fungal specific, although, as these have not been fully validated against other organisms, it is possible that they might allow cross-kingdom amplifications. To rectify these problems, a nested or semi-nested PCR approach may be used where the first set of primers exhibits a broad host-template annealing range while the second set is more fungal-specific (Kjøller and Rosendahl, 2000, Anderson and Cairney, 2004).

Some of the primers used commonly for PCR amplification of fungal sequences from environmental samples have been described by Pang and Mitchell. (2005). The primers described by White et al. (1990) were designed

to be generic, whereas other primer combinations were constructed for use in particular environments or with specific molecular techniques, such as denaturing gradient electrophoresis (DGE). Zuccaro and Mitchell. (2005) listed the nested combinations and the molecular conditions used to separate the mixed PCR products. The primer combinations routinely used for amplification of ITS-5.8S rDNA region as described by Gardes and Bruns. (1993) and Larena et al. (1999) were designed to preferentially amplify basidiomycetous and ascomycetous sequences respectively from plant material, particularly roots. The primers (ITS1-F, ITS4-A and ITS4-B) have also been successfully adopted for use with techniques such as ARDRA, DGE and T-RFLP (Klamer et al, 2002; Anderson et al, 2003b). Martin and Rygielwicz. (2005) described novel primer combinations for this region that co-amplified sequences from the Dikaryomycota, thereby allowing a simultaneous assessment of basidiomycetes and ascomycetes. Other primers targeting the ITS-5.8S rDNA regions from the Zygomycota and Oomycota have also been published (Nikolcheva and Barlocher, 2004).

3.1.3 Fungal diversity from deep-sea habitats using culture-independent approaches

Recently, culture-independent molecular analyses have demonstrated the diversity and ecological importance of fungi in various marine environments (Bass et al, 2007; Edgcomb et al, 2010). In particular, there has been increasing interest in the diversity of fungi from extreme marine environments. A few recent studies have reported fungal 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. 2010; Nagano et al, 2010). 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 reported from methane hydrate bearing deep-sea

sediments collected from water depths down to ~3,000 m in South China (Lai et al, 2007). Using fungal specific primers for construction of 18S rDNA gene libraries, Bass et al. (2007) 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 (Lopez-Garcia et al, 2003).

Furthermore, the use of different primers has been shown to impact evaluation of fungal diversity from different habitats. 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. (2010) found fungal forms in majority from marine deep-sea subsurface by using eukaryotic specific primers. 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). While studying microeukaryotic diversity in an acidic iron river, Gadanho and Sampaio. (2006) 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, environmental gene libraries were constructed with universal and fungal-specific primer sets to assess the fungal diversity in the deep-sea sediments of the Central Indian Basin (CIB) in the present study. Such primers have been known to amplify a variety of fungal sequences from mixed environmental samples (O'Brien et al, 2005). Furthermore, fungal community composition analysis was carried out for distantly located stations as well as two depths at each of these stations. In addition, as fungal recovery from environmental samples have been reported to show primer bias (Jebaraj et al, 2010), a multiple-primer approach was used to study fungal diversity for sediment sample of a single location. The efficiency of primers used for this study was evaluated for amplification of fungal as well as other eukaryotic

sequences from mixed environmental samples by targeting ITS and 18S rDNA regions. The primer set used for ITS region was fungal specific, whereas, out of the three 18S rDNA primer sets used, two were eukaryotic. The reason for this was to widely cover the fungal diversity from a single sediment sample. Three different analyses, carried out with different primer sets with samples of two cruises are as following.

1. The sediment samples from three stations i.e. **SVBC-04 (station A), SVBC-31 (station B) and SVBC-37 (station C)** of **Cruise #ABP26** were amplified with three primer sets i.e. a, b and c each. Where primer set **a) ITS1F/ITS4, b) ITS1/ITS4 and c) NS1/NS2**. A total of 18 environmental gene libraries were constructed during this analysis.
2. The sediment sample from one station i.e. **SVBC-33** of **Cruise #ABP26** was amplified with four primer sets. The primer sets were **1. ITS1F/ITS4, 2) NS1/NS2 3) Euk18S-42F/Euk18S-1492RE and 4) Euk18S-555F/Euk18S-1269R**. A total of 4 environmental gene libraries were constructed during this analysis.
3. The sediment samples from two stations i.e. **IVBC- 18C and IVBC-20A** of **Cruise #ABP38** were amplified with two primer sets i.e. **ITS1F/ITS4 and NS1/NS2**. A total of 8 environmental gene libraries were constructed during this analysis ([Tables 3.1 and 3.2](#)).

Objectives: The objectives were to evaluate fungal diversity from deep-sea sediment samples of the Central Indian Basin using culture-independent approach. For environmental gene library construction, fungal specific as well as universal primers were used targeting 18S and ITS regions of SSU rDNA

and comparison was made between samples of different locations as well as different depths of the same location.

3.2 Materials and Methods

3.2.1 Sampling methods

Sediment samples were collected during 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), SVBC-37 (station C), and SVBC-33 of the Central Indian Basin at depths of ~5,000 m (Table 3.1). During the cruise #ABP-38, sediment samples were collected from 2 locations of the CIB in September 2009 (Table 3.2). The sampling procedure described by Raghukumar et al. (2004) and Damare et al. (2006) 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 an alcohol-sterilized 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 (A, B and C) in order to minimize the number of clone libraries. Thus, finally a total of six DNA samples were processed for these stations. For the Station number SVBC-33, the DNA samples from all the subsections were pooled together as one sample. During cruise #ABP-38 also the DNA samples were pooled into two parts of 0–15 and 15–30 cm for each of the two cores and processed as four samples. 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.

3.2.2 DNA extraction, environmental PCR and clone library analyses

For amplification of fungal genotypes from environmental sediment samples using specific primers, DNA was isolated from each core as described in materials and methods. DNA was isolated from 0.5 g of the sediment sample from each subsection using the Q-Bio gene Soil DNA extraction kit (MP Biomedicals, OH, US) according to the manufacturer's instructions. DNA samples were pooled for each station described in materials and methods. DNA samples from the three stations (A, B and C) were amplified using fungal-specific ITS1F/ITS4 (Gardes and Bruns, 1993), “primer pair a” as well as universal ITS1/ITS4, “primer pair b”, and universal 18S rDNA NS1/NS2 primer set (White et al, 1990), “primer pair c”. For the station SVBC-33, DNA sample 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 (Tables 3.1 and 3.3). DNA samples isolated during the cruise #ABP38 were amplified using

Table 3.1 Details of sediment collection sites from CIB during cruise # ABP26.

Station number	Touch Positions		Water depth (m)	Primer pair used
	Lat (°S)	Long (°E)		
SVBC-04 (Station A)	13 00.000	76 29.950	5377	ITS1F/ITS4, ITS1/ITS4 and NS1/NS2
SVBC-31 (Station B)	12 00.000	74 59.900	5153	
SVBC-37 (Station C)	16 06.891	75 24.828	3992	
SVBC-33	12 59.980	74 59.960	5265	ITS1F/ITS4, NS1/NS2, Euk18S-42F/Euk18S-1492RE and Euk18S-555F/Euk18S-1269R

Table 3.2 Details of sediment collection sites from CIB during cruise # ABP38.

Station Number	Touch Positions		Date	Primer set used
	Lat (°S)	Long (°E)		
IVBC-18C	12 59.747	74 30.150	23.09.09	ITS1F/ITS4 and NS1/NS2
IVBC-20A	11 59.900	75 29.900	30.09.09	

fungal specific ITS1F/ITS4 and universal 18S rDNA NS1/NS2 primer set (Tables 3.2 and 3.3).

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 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 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 (5'-GTTTCCAGTCACGAC-3') and M13 reverse (5'-CAGGAAACAGCTATGACC-3') 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 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 18 environmental gene libraries were constructed from the DNA samples of three stations (A, B and C), each with two depths and three primer pairs (3×2×3) during cruise #ABP26. Four environmental gene libraries were constructed from the DNA sample from station SVBC-33 during cruise #ABP26 and eight during the cruise #ABP38. A total of 48 clones were screened from each library.

3.2.3 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 analyzed separately. All the sequences were checked with Ribosomal Database Project (Cole et al, 2004) 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 (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). For the 3 stations (A, B and C) of the cruise #ABP 26, 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. Out of the 18 environmental gene libraries, for these stations, two showing biased amplification of a single sequence type due to PCR artifact, were eliminated from the further analyses. For the station SVBC-33, the OTUs are represented as OTU_01 to OTU_08 (blue fonts) obtained with fungal-specific ITS primer pair, OTU_9 and OTU_10 (pink

fonts) retrieved with universal 18S rDNA primer pairs, OTU_11 to OTU_20 (green fonts) with eukaryotic primer sets 42F/Euk18S-1492RE and OTU_21 to OTU_27 (red fonts) with eukaryotic primer sets Euk18S-555F/Euk18S-1269R. For the environmental gene libraries prepared during cruise #ABP38, the OTU details are shown as OTU_01 to OTU_18 (pink fonts) obtained with universal 18S rDNA primer pairs and OTU_19 to OTU_46 (blue 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).

Table 3.3 Details of the primers used for construction of environmental libraries.

Primer name	Sequences	Reference
ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-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

Multiple alignments were done for all the sequences along with their closest match in ClustalW (Thompson et al, 1994). Gaps and ambiguously aligned sequences were removed from further analyses. A phylogenetic analysis was conducted using distance setting (Maximum Parsimony) in MEGA 4.1 (Kumar et al, 2008) with 1,000 bootstrap replicates. Phylogenetic trees were constructed with sequences obtained with ITS and 18S rDNA primer sets individually for each 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. Community composition was plotted as relative abundance of taxonomic

subclasses at different stations and depths. Representative sequences of each OTU were deposited in NCBI-GenBank under the accession numbers GU370716 to GU370774 for the 3 stations (A, B and C) of the cruise #ABP 26 (Table 3.4). For station SVBC-33, accession numbers for the OTUs are shown in Table 3.5.

3.3 Results:

3.3.1 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. The seafloor of the CIB is characterized by the presence of homogenous, soft to slightly compact, dark brown sediments with yellowish sediments at deeper depths. The station SVBC-37 was in a seamount area and the sediment texture was calcareous. All the other stations were characterized by the presence of siliceous sediments (Sharma, 2008, 2010).

3.3.2 Environmental 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 768 clones were sequenced from the 16 environmental libraries **for the 3 stations (A, B and C) of the cruise #ABP26**. 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.

Table 3.4 Phylogenetic affiliation of the Fungal OTUs obtained with different primer sets for the stations A, B and C of the cruise #ABP26 (Sequences showing <97 % identity are represented in bold)

OTUs with Fungal Specific ITS primers, ITS1F/ITS4 (a)	OTUs with Universal ITS primers, ITS1/ITS4 (b)	OTUs with Universal 18S rDNA primers, NS1/NS2 (c)	Closest identified relative (GenBank Accession no.)	% Identity	Phylum	Source of isolation of closest identified relative
OTU_02	OTU_11	-	<i>Sagenomella</i> sp. (FJ357782)	99	Ascomycota	Deep sea sediment of CIB
-	OTU_21	-	<i>Dothideomycete</i> sp. (EU680535)	99		Moth exoskeleton
-	OTU_15	OTU_23	<i>Aspergillus penicillioides</i> (DQ985959)	97		Lacaziosis patient
-	-	OTU_24	<i>Aspergillus restrictus</i> (AB008407)	99		Soil
OTU_01	OTU_10	-----	<i>Hortaea</i> sp. F 47 (FJ755827)	95		Marine sponge
OTU_04	OTU_13	-	<i>Stenella musicola</i> (EU514294)	99		<i>Musa acuminata</i>
OTU_05	OTU_14	-	<i>Candida</i> sp. F15 (FJ755821)	100		Coastal waters of china
-	OTU_16	OTU_27	<i>Aspergillus restrictus</i> (AB002079)	94		Not known
-	OTU_19*	-	<i>Debaryomyces yamadae</i> (AB054266)	98		Not known
OTU_06*	-	-	<i>Pichia jadinii</i> (EU568927)	99		Pilot-scale Municipal compost
OTU_07*	-	-	<i>Nodulisporium</i> sp. (EF600033)	98		Stroma of <i>Epichloe typhina</i>
-	-	OTU_25	<i>Aspergillus niger</i> (GQ228449)	96		Soil from Kalabagh iron ore deposit
-	-	OTU_28	<i>Candida glucosophila</i> (AB013519)	97		Not known
-	-	OTU_29*	<i>Aspergillus fumigatus</i> (GQ169424)	99		Fossa cheese
-	-	OTU_30*	<i>Aspergillus</i> sp. (FJ864683)	99		Root of <i>Angelica sinensis</i>
-	-	OTU_32	<i>Phoma herbarum</i> (AY293777)	99		Not known

Table 3.4 contd.

OTUs with Fungal Specific ITS primers, ITS1F/ITS4 (a)	OTUs with Universal ITS primers, ITS1/ITS4 (b)	OTUs with Universal 18S rDNA primers, NS1/NS2 (c)	Closest identified relative (GenBank Accession no.)	% Identity	Phylum	Source of isolation of closest identified relative
-	-	OTU_35	<i>Aspergillus unguis</i> (EF067336)	99	Ascomycota	Culture collection
-	-	OTU_37*	<i>Ulospora bilgramii</i> (DQ384071)	98		Dung
-	-	OTU_39*	<i>Capnodium coffeae</i> (DQ247808)	93		Maize rhizosphere Soil
-	OTU_22*	-	<i>Bionectriaceae</i> sp. (FJ821507)	97		Basidioma of <i>Phellinus gilvus</i>
OTU_08*	-	-	fungal sp. ARIZ L365 (FJ612791)	99		Tree seed
-	-	OTU_26	Uncultured fungal clone (GQ120138)	98		Arabian Sea sediment
-	-	OTU_31*	Uncultured fungal clone (AB074653)	98		Arabian Sea sediment
-	-	OTU_33	Uncultured fungal clone (AJ515945)	97		Maize rhizosphere Soil
-	OTU_20*	-	<i>Candida parapsilosis</i> (GQ395610)	99		Feeding production sample
-	OTU_17	-	<i>Malassezia pachydermatis</i> (DQ457640)	96		Not known
OTU_03	OTU_12	-	<i>Trichosporon asahii</i> (AB369919)	99	Basidiomycota	Skin of patient
-	-	OTU_34	<i>Wallemia sebi</i> (AY741380)	97		Hypersaline water of salterns
OTU_09*	-	-	<i>Rhodosporidium sphaerocarpum</i> (EF643587)	100		Deep-sea of pacific ocean
-	-	OTU_38	fungal sp. FCAS11 (GQ120154)	97		Arabian Sea sediment
-	-	OTU_36	Uncultured fungal clone (AJ515927)	99		Contaminated aquifer sediment
-	OTU_18	-	Uncultured fungal clone (FJ889114)	94		Hawaiian coastal waters

Singletons are represented with *

Table 3.5 Phylogenetic affiliations of the OTUs obtained with different primer sets for station SVBC-33 of the cruise #ABP26 (Sequences showing <97 % identity are represented in bold)

Fungal OTU obtained			Closest identified relative					% Identity
OTU no.	Primer set used	Accession no.	Taxon	Phylum	Class	Accession no.	Source of isolation	
OTU_01	ITS1F/ ITS4	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*		GU370754	<i>Aphyllphorales</i> sp. LM82	Basidiomycota	Agaricomycetes	EF060457	Sea water	98
OTU_06*		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/ NS2	HM572252	Uncultured <i>Malassezia</i> clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	99
OTU_10		HM572251	Uncultured <i>Aspergillus</i> clone	Ascomycota	Eurotiomycetes	FJ393417	Gut of HBV infected patients	99
OTU_11	18S-42F/ Univ1492 RE	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>Aphyllphorales</i> sp. LM82	Basidiomycota	Agaricomycetes	EF060457	Sea water	98
OTU_15*		GU370761	Unidentified fungal clone S3	Basidiomycota	Wallemiomycetes	FJ820491	Air sample	79

Table 3.5 contd.

Fungal OTU obtained			Closest identified relative					% Identity
OTU no.	Primer set used	Accession no.	Taxon	Phylum	Class	Accession no.	Source of isolation	
OTU_16*	18S-42F/ Univ1492RE	GU370762	<i>Nectria mauritiicola</i>	Ascomycota	Sordariomycetes	AJ557830	Not known	99
OTU_17		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	EK555F/ EK1269R	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		HM587247	<i>Aspergillus restrictus</i>	Ascomycota	Eurotiomycetes	AB008407	Not known	99
OTU_24*		GU370771	<i>Wallemia sebi</i>	Basidiomycota	Wallemiomycetes	AY741380	Hypersaline water of saltern	98
OTU_25*		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

Singletons are represented with *

The other 511 clones (~66.5%) were bacterial, eukaryotic, or chimeric in nature and thus were excluded from the study. These 39 fungal OTUs comprised 32 distinct fungal taxa (Table 3.4).

For the station SVBC-33, a total of 48 and 144 clones from ITS and 18S primer pairs respectively were sequenced from the environmental libraries. This resulted in 27 fungal OTUs, eight with ITS and nineteen with 18S rDNA primer pairs 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 3.5).

None of the primers amplified sequences affiliating with eukaryotes, other than fungi. Out of 48 and 144 total clones obtained from 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 obtained, mostly belonged to the phyla Ascomycota and Basidiomycota (Table 3.5). 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 3.5). The remaining OTUs were present ≥ 2 times in different libraries.

During the cruise #ABP38, a total of 192 clones were sequenced for each primer pair. Out of these clones, 67 (~ 35%) and 55 clones (~ 29%) were found to be chimeric with ITS and 18S rDNA primer sets respectively and were removed from the subsequent analysis. A total of 18 and 28 OTUs were obtained from the resultant clones obtained with 18S rDNA and ITS primer sets (Table 3.6). The results are presented as **OTU_01 to OTU_18** obtained with universal 18S rDNA primer pairs, NS1/NS2 and **OTU_19 to OTU_46** with fungal -specific ITS primer pair (Table 3.6). All the 46 OTUs were found to be affiliating with distinct fungal species.

Table 3.6 Phylogenetic affiliations of the OTUs obtained with different primer sets from environmental libraries prepared with the samples during cruise #ABP38 (Sequences showing <97 % identity are represented in bold)

OTU no.	Primer set used	Closest identified relative					% Identity
		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 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

Table 3.6 contd.

OTU no.	Primer set used	Closest identified relative					% Identity
		Taxon	Phylum	Class	Accession no.	Source of isolation	
OTU_15	NS1/ NS2	<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*		<i>Cerrena unicolor</i>	Basidiomycota	Agaricomycetes	AY850007	Culture Collection	98
OTU_19	ITS1F/ ITS4	<i>Sterigmatomyces</i> sp.	Basidiomycota	Agaricostilbomycetes	FJ755830	Marine sponge	99
OTU_20		<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

Table 3.6 contd.

OTU no.	Primer set used	Closest identified relative					% Identity
		Taxon	Phylum	Class	Accession no.	Source of isolation	
OTU_31	ITS1F/ ITS4	<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 Enhalus	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*		<i>Corynespora cassicola</i>	Ascomycota	Dothideomycetes	AY238606	Cucumber	99
OTU_46*		<i>Aspergillus</i> sp.	Ascomycota	Eurotiomycetes	EU139858	Culture Collection	95

Singletons are represented with *

The fungal OTUs obtained belonged to Ascomycota and Basidiomycota phyla. In addition, the 18S rDNA primer set amplified four OTUs affiliating with eukaryotic sequences also (Table 3.6).

3.3.3 Analyses of Fungal Diversity Obtained with Different Primer Sets

For the stations A, B and C of cruise #ABP26, Out of the 39 fungal OTUs, four were exclusively amplified by primer set a, six with primer set b, and 15 by the primer set c (Table 3.4). Ten OTUs were simultaneously recovered with primer pairs a and b (Table 3.4) and four with b and c. The number of singletons, i.e., detected only once in libraries constructed with primer pairs a, b, and c were four, three, and five, respectively (marked with an asterisk in Table 3.4. 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. 3.2 and 3.3). Primer pair a and primer pair b recovered seven and ten OTUs respectively belonging to Ascomycota. Both the ITS primer sets detected Sordariomycetes, Dothideomycetes, and Saccharomycetes (Figs. 3.2 and 3.3). In addition, the primer set b amplified members of Eurotiomycetes as well. The primer pair a and the primer pair b amplified one (OTU_01) and two (OTU_10, OTU_16) new sequence types respectively having similarities less than 97% to the known fungal taxa belonging to ascomycota (Table 3.4). Primer pair a and primer pair b recovered two and three OTUs belonging to Basidiomycota, respectively. This group included members from Tremellomycetes, Microbotryomycetes, and Ustilaginomycetes. Out of these, only Tremellomycetes was the common clade shared by both the primer sets (Fig. 3.3).

Primer pair b amplified two new phylotypes 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 3.4). Out of these eight OTUs, three were new phylotypes. Five fungal taxa were simultaneously recovered with universal and fungal specific ITS primer sets (Table 3.4). A major part of the sequences recovered with universal 18S rDNA primer set NS1/NS2 (primer pair c) belonged to Ascomycota (Fig. 3.4). The number of OTUs belonging to Ascomycota and Basidiomycota were 14 and 3, respectively. Out of these, three sequences showing less than 97% identity 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. 3.4). 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. 3.4). Only two fungal taxa namely *Aspergillus penicillioides* and *Aspergillus restrictus* were simultaneously amplified with universal ITS and 18S rDNA primers (Table 3.4).

For the Station SVBC-33, the fungal specific primer set ITS1F/ITS4 amplified eight OTUs belonging to seven different classes of Ascomycota and Basidiomycota. Majority of the sequences affiliated with the unidentified and uncultured fungal clones (Table 3.5). Wherein OTU_02 affiliated with an unidentified clone from the class Wallemiomycetes with 79% identity.

Universal 18S rDNA primer pair NS1/NS2 amplified sequences matching two phylotypes i.e. uncultured *Malassezia* and *Aspergillus* clone with 99% similarity belonging to the class Exobasidiomycetes and Eurotiomycetes respectively (Table 3.5).

The eukaryotic primer set 18S-42F/Univ1492RE, recovered sequences belonging to ten different phylotypes and these belonged to six different classes of the phyla Ascomycota and Basidiomycota (Table 3.5). 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.

The other eukaryotic primer set EK555F/EK1269R, amplified seven fungal phylotypes belonging to four different classes. OTU_21 and OTU_22

showing low similarity values of 96 and 97% respectively with the sequences existing in the data base. They 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 3.5). This eukaryotic primer set amplified a total of three sequences including OTU_26 besides the above two, showing < 97% identity with existing sequences in the database and thus may be new.

For the gene libraries prepared during **the cruise #ABP38**, both ITS and 18S rDNA primer sets amplified sequences affiliating with Ascomycota as majority (Table 3.6). Primer pair NS1/NS2 amplified two sequences clustering with Alveolata and 2 matching with Viridiplantae among eukaryotes (Fig 3.5). Among Ascomycetes, 10 different phlotypes were recovered belonging to three classes i.e. Eurotiomycetes, Saccharomycetes and Dothideomycetes. Four basidiomycetous phlotypes were found to show similarity with classes Agaricomycetes, Exobasidiomycetes and Agaricostilbomycetes. The basidiomycete phlotype OTU_18 belonged to *Cerrena unicolor* with a percentage identity of 98. Among other sequences most common forms were uncultured fungal clones, reported from marine environment in previous studies (Table 3.6). OTU_01, OTU_02, OTU_04 and OTU_11 showed $\leq 97\%$ identity with their closest relative in the existing database.

The primer pair ITS1F/ITS4 also amplified sequences belonging to Ascomycota and Basidiomycota groups. Among Ascomycota, 16 fungal phlotypes were recovered affiliating with four different classes i.e. Sordariomycetes, Eurotiomycetes, Saccharomycetes and Dothideomycetes. Whereas among Basidiomycota, twelve phlotypes were amplified belonging to seven different classes as shown in Table 3.6. OTU_24, OTU_27, OTU_35, OTU_39 and OTU_46 were found to show $\leq 97\%$ identity with their closest relative. One phlotype, OTU_44 clustered with the *Hortaea* sp. with a percentage identity of 98 (Fig 3.6).

3.3.4 Fungal Diversity at Different Stations

Fungal taxa were more or less unevenly distributed at different stations (Table 3.7). Some of these OTUs were found exclusively in station A, B, or C of the cruise #ABP26. 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*, *A. penicillioides*, *Stenella musicola*, and *Candida* sp. occurred in high frequency (Table 3.7).

3.3.5 Distribution of Different Subclasses and Species Richness

Seven and five major subclasses of fungi were recovered with ITS and 18S rDNA primers, respectively from the stations A, B and C of the cruise #ABP26 (Fig. 3.7). 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. 3.2, 3.3 and 3.4). Members of Eurotiomycetes were present in all the stations. Members of Sordariomycetes and Dothideomycetes were found to be the major forms in stations 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.

The proportional distribution (shown in percentage) of fungal taxonomic groups varied in different clone libraries prepared for the station SVBC-33 (Figs. 3.8 and 3.9). *Trichosporon asahii* (32 %) formed a major portion of sequences amplified by the ITS primer set (Fig. 3.8).

Table 3.7 Distribution of closest identified fungal taxa in different stations.

OTU no.	Closest identified relative (GenBank Accession no.)	Number of clones recovered			
		Station A	Station B	Station C	Total
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

Table 3.7 contd.

OTU no.	Closest relative (GenBank Accession no.)	Number of clones recovered			
		Station A	Station B	Station C	Total
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>Rhodosporidium 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)

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. 3.9a). The four phylotypes, *Aphylllophorales* sp., *Nectria mauritiicola*, unidentified isolate f4Fc56 and unidentified fungal clone S3 amplified by the ITS1F/ITS4 primer set were amplified by 18S-42F/Univ1492RE as well (Fig. 3.9b). Maximum number of distinct fungal sequences was obtained with eukaryotic primer set, 18S-42F/Univ1492RE, out of which *Wallemia* sp. (28%) contributed to the major proportion (Fig. 3.9b). A majority of the sequences amplified by these two primer sets belonged to Basidiomycota. The two sequences amplified by the primer pair NS1/NS2 were amplified by EK555F/EK1269R as well (Fig. 3.9a and 3.9c). There were no common OTUs shared by the primer sets 18S-42F/Univ1492RE and EK555F/EK1269R. The major phylotypes amplified by the primer set EK555F/EK1269R belonged to the class Eurotiomycetes of the phyla Ascomycota (Fig. 3.9c).

3.4 Discussion

3.4.1 Diversity with Multiple Primers

Primer sets for the amplification of two different segments of rRNA gene were chosen in the present study 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 (O'Brien et al, 2005). Therefore, to overcome this problem and get a better diversity estimate, both of these segments were amplified 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 (O'Brien et al, 2005; Jebaraj et al, 2010).

3.4.2 Fungal diversity for the stations A, B and C of cruise #ABP26

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 3.4). Jebaraj et al. (2010) 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 (Bass et al, 2007).

In the previous chapter 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 during cruise #ABP26. 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 3.4). 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. (2005) using the same primer sets. In contrast to our results, O'Brien et al. (2005) recovered sequences belonging to Chytridiomycota and Zygomycota with these primer pairs. Lai et al. (2007) 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, six were affiliated to Ascomycota and two to Basidiomycota. Thus, these new sequences add to the existing database for probable novel fungal signatures.

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. 3.7). 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 (Levin et al, 2001). Substrate heterogeneity was shown to play an important role in structuring nematode diversity in deep-sea sediments (Vanreusel et al, 2010). 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 (Bass et al, 2007). 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 (Sogin et al, 2006).

Out of 39 OTUs, 12 OTUs showed affiliation with sequences already reported from marine sources (Table 3.4), thus adding support to the view that these were possibly typically marine. Out of these 12, two OTUs representing *Sagenomella* sp. and the yeast *Rhodospiridium* sp. were recovered in culture from the CIB in the earlier chapter using culture-dependent approach. 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 (Kuncic et al, 2010). Its sequence was detected in a marine sponge from Hawaii (Gao et al, 2008) and was also isolated in culturable form from different species of marine sponges of the South China Sea (Liu et al, 2010), suggesting its wide-spread occurrence in marine environment. Cantrell et al. (2006) 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 on the 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*, *Rhodospidium*) includes mycoparasites, phytopathogens, and putative saprotrophs with a diversity of micromorphological characters. Many species contain organelles, termed colacosomes, which are associated with mycoparasitism (Bauer and Oberwinkler, 1991). The recovery of other pathogenic yeasts like *Candida* and *Debaryomyces*, 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 (Moreira and Lopez-Garcia, 2003; Van Dover et al, 2007). These results are in accordance with earlier observation that oceans are incubators and conveyor belt for pathogenic microorganisms (Harvell et al, 2002).

A large proportion (~ 69%) of fungal sequences recovered in high frequency (Table 3.7) matched with terrestrial taxa, supporting the earlier hypothesis (Raghukumar et al, 2004; Damare et al, 2006, 2008) 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 (Damare et al, 2006; Burgaud et al, 2009) and culture-independent methods from various marine habitats (Bass et al, 2007; Lai et al, 2007; Jebaraj et al, 2010). Therefore, the ecological role and adaptations of these ubiquitous terrestrial forms in such extreme conditions need to be further investigated. Takishita et al. (2006) and Shao and Sun et al. (2007) 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 as well as culture-independent approach (Table 3.4) 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 antifungal properties (Paz et al, 2010)

and anti-tumor and anti-bacterial activities (Liu et al, 2010), suggesting that terrestrial taxa found in the marine environment could be a rich source of new biologically active natural products (Liu et al, 2010).

3.4.2 Fungal diversity for the station SVBC-33 of cruise #ABP26

The diversity study was carried out using a single core of deep-sea sediment from the Central Indian Basin by using four different sets of primers. Previous studies on fungal diversity from the same area in three distantly located (Stations A, B and C) stations did not show a rich fungal diversity and was perhaps due to primer bias 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 diversity obtained from one sediment core of the station SVBC-33 in the present study with four primer pairs was higher with a total of 8 and 19 fungal OTUs recovered from 48 and 144 clones obtained with ITS and 18S primer sets (relative frequency, 17 and 13 % respectively) than in the previous study. A total of 20 distinct fungal sequences were obtained. In the previous study from the same site (Stations A, B and C), using three primer pairs from three sediment cores and two depths, a total of 9 and 17 fungal OTUs from 240 and 288 clones (relative frequency= 4 and 6 % respectively) were obtained with fungal specific ITS and universal 18S rDNA primer pairs respectively from 16 environmental libraries. Therefore, a multiple-primer approach appears to be better for assessment of fungal diversity. The primers used in the present study have been reported to amplify diverse forms of eukaryotic lineages from mixed environmental samples from various habitats (Lopez-García et al, 2003; Takishita et al, 2005; Stoeck et al, 2006; Edgcomb et al, 2010). However, the absence of eukaryotic sequences other than fungi 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 known to amplify fungal 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 or by culture-independent approach suggests their absence or low abundance in sediments of the sampling sites. 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 of clone libraries is required.

Fungal specific primer set ITS1F/ITS4 amplified a sequence (OTU_04), showing high similarity and one novel sequence, showing low similarity with uncultured or unidentified form of the class Wallemiomycetes. One sequence (OTU_24) amplified with EK555F/EK1269R showed high similarity with *Wallemia sebi* which has been isolated from hypersaline waters of saltern and is known to be very efficient halophilic and osmophilic species (Kuncic 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. 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 have been found to be dominating 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. 3.9c). These species have been reported as potential pathogenic forms of animals (Lai et al, 2007). In addition, *Aspergillus* species has been reported to play important role in denitrification process from anaerobic marine sediments

off Goa (Jebaraj et al, 2010). This further suggests the 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 sequences (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 as reported in the previous chapter. Apart from this, OTU_07 and OTU_19 (*Rhodotorula* sp.) and OTU_23 and OTU_26 (*Aspergillus* sp.) were also obtained in culture. There was no overlapping sequence amplified by these two eukaryotic primer sets which suggests that each primer pair amplified 18S rRNA gene sequences not recoverable with other primer pairs.

None of the fungal phylotypes obtained from the sampling site SVBC-33 matched with the phylotypes obtained from the other three cores from the CIB during the same sampling period reported in the earlier study (Stations A, B and C) except *Trichosporon asahii* amplified with ITS primer pair, suggesting 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 used in the present study is being reported 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) has been reported and is known to consist of species which are plant pathogen (Begerow et al, 2006). In addition, *Rhodotorula* sp. from Cystobasidiomycetes subphylum is reported as mycoparasite (Bauer and Oberwinkler, 1991).

Proportional distribution of fungal sequences recovered varied for different primer sets (Figs. 3.8 and 3.9). Diverse types of sequences were recovered with eukaryotic primer set 18S-42F/Univ1492RE. This suggests the efficiency of this primer set to amplify diverse forms which is in contrast to the previous studies (López-García et al, 2003; Stoeck et al, 2006) where only a

few fungal OTUs were obtained. In addition, there was hardly any overlap of sequences between different primer sets resulting in detection of higher diversity of fungal forms by multiple-primer approach (Fig 3.9).

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 of mostly 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 from suboxic sediments of the Arabian Sea, off Goa by using multiple primer approach.

3.4.2 Fungal diversity observed during the cruise #ABP38

A total of 18 and 28 fungal OTUs recovered from 192 clones each obtained with 18S rDNA and ITS primer sets (relative frequency, 9.3 and 14.5 % respectively). These results are comparable to two studies with the samples, 1. Stations A, B, C and 2. SVBC-33 (Table 3.1) of the cruise #ABP26. The OTUs from the samples of the cruise #ABP38 resulted in a total of 46 distinct fungal species which is quite high in spite of few number of clones screened. This may be due to the substrate, spatial or temporal heterogeneity existing in the deep-sea environment affecting diversity patterns (Sogin et al, 2006; Vanreusel et al, 2010). The singletons obtained during this study were 4 and 10 with 18S rDNA and ITS primer sets respectively, reflecting a lack of saturation level in sampling for clones.

Amplification of four sequences belonging to eukaryotic phylotypes by 18S rDNA primer set was a striking feature of the present study (Table 3.6). Two of them belonged to Alveolata whereas other two affiliated with Viridiplantae. These sequences were not recovered from the sample of the station SVBC-33 of cruise #ABP26 even after amplification with eukaryotic primers. The sites selected for analyses during cruise #ABP26 i.e. 1. Stations

A, B, C and 2. SVBC-33 (Table 3.1) had different geographical locations than the sites during cruise #ABP38 (Table 3.2). Therefore, the recovery of eukaryotic sequences during cruise #ABP38 and their absence in the samples of #ABP26 may be attributed to the spatial variation in distribution patterns. Edgcomb et al. (2010) reported amplification of similar eukaryotic sequences from subsurface marine environments by using eukaryotic primers. Several other studies have also reported recovery of these eukaryotic phylotypes from different habitats such as Cariaco Basin (Stoeck et al, 2003) the Mid-Atlantic Ridge (Lopez-Garcia et al, 2003) the Guaymas Basin (Edgcomb et al, 2002) and the coastal and equatorial Pacific Ocean (Moonvander Staay et al, 2001). In addition, Lopez-Garcia et al. (2001) reported the amazing variety of alveolates to be the dominating forms among deep-sea plankton. One of the phylotypes in the present study, OTU_05, was detected to cluster with uncultured marine eukaryote clone (Behnke et al, 2010), which was recovered from an O(2)/H(2)S gradient in a Norwegian fjord. The percentage similarity was found to be 86 (Table 3.6), suggesting it to be probable novel species of such alveolate. The recovery of such marine eukaryotic signatures may provide insight into the ecological evolutionary history of protists (Cavalier-Smith, 1998; Moreira and Lopez-Garcia, 2002; Steenkamp et al, 2006).

In addition, the amplification of eukaryotic sequences using 18S rDNA primer set NS1/NS2 has been reported by O'Brien et al. (2005). This primer set amplified eukaryotic sequences from the stations of cruise #ABP38 in the present study also suggesting their efficiency towards amplification of eukaryotic sequences from mixed environmental samples. However, among fungi only Dikaryotic forms could be recovered in the present study whereas O'Brien et al. (2005) reported amplification of other phylotypes of fungi also using NS1/NS2 primer set. The absence of other forms like Zygomycetes and Chytridiomycetes from all the studies conducted with the samples of the CIB suggests their non abundant nature in these environments.

Among other OTUs, most of them clustered with sequences of uncultured fungal clones (Table 3.6). These sequences were also detected in the environmental libraries prepared with samples of stations A, B and C of cruise #ABP26. In spite of sampling from a different site during cruise #ABP38, the recovery of the similar sequences reveals the abundant nature of these fungal signatures in the sediments of CIB. Further a total of four and five OTUs obtained with 18S rDNA and ITS primer sets respectively showed <97% similarity with sequences of existing database suggesting their probability of being novel (Table 3.6). The Ascomycete phylotypes, OTU_24, OTU_39 and OTU_46 showed affiliation with the *Aspergillus* sp. with a percentage similarity of ≤ 97 . This species has also been isolated from marine sponge by other workers (Wang et al, 2008; Liu et al, 2010). The phylotype, OTU_46, showing 95% similarity with *Aspergillus* sp. was also isolated in culture form from the same site, as mentioned in the previous chapter (NIOCC#55). Such high divergence of this phylotype from the existing terrestrial species may be employed in understanding its adaptation and evolution strategies under extreme conditions of deep-sea. Likewise, the recovery of few other phylotypes such as OTU_18 and OTU_44, affiliating with *Cerrena* and *Hortaea* sp., which have also been isolated in culture form, from samples of cruise #ABP38 as reported in the previous chapter, strongly support their possibility to be the native forms of deep-sea. *Cerrena* species has been proved to be a potent source for production of lignin degrading enzymes (Michniewicz et al, 2006; D'Souza-Ticlo et al, 2009) in the terrestrial ecosystems. *Hortaea* species is reported to be a halotolerant and halophilic marine fungi in the earlier studies (Kogej et al, 2005).

One Ascomycete phylotype, OTU_08 belonged to *Saccharomyces* species with a percentage similarity of 99 (Table 3.6). The recovery of this species has been reported for the first time from this area in the present study. Several studies have used this species as a model organism for high pressure stress effects at physiological, genomic and proteomic level (Guerzoni et al, 1999; Abe and Horikoshi, 2000; Iwahashi et al, 2001; Arao et al, 2005; Fernandes, 2005; Domitrovic et al, 2006; Sheehan et al, 2007). The

isolation of this species in culture form in future studies from such extreme environments can help in stress related studies for identification of novel genes responsible for adaptation mechanism.

Other phylotypes recovered during this cruise were also detected in previous studies from station SVBC-33 of CIB during cruise #ABP26. For example phylotype belonging to *Malassezia* sp., was amplified from samples of both the cruises (Table 3.8). The sequences affiliating with *Malassezia* were also recovered from methane hydrate bearing deep-sea sediments (Lai et al, 2007). The Basidiomycete phylotypes, OTU_19 and OTU_15, amplified with both the primer sets were closely related to *Sterigmatomyces* sp., a yeast isolated from the marine areas at Biscayne Bay, Florida, USA (Fell, 1966).

Remaining subgroups amplified from the samples of this cruise were same (Table 3.6) as obtained from two analyses i.e. 1. Stations A, B, C and 2. SVBC-33 of cruise #ABP26 (Tables 3.1, 3.4 and 3.5). From these observations, it is suggested that 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. Also, recovery of marine fungal sequences from this environment suggests that they may not be anthropogenic contaminant and have an ecological role in the deep-sea ecosystems of the CIB. However most of the phylotypes matching with terrestrial species such as *Resinicium*, *Gibberella*, *Stenella*, *Neurospora* and *Corynespora* sp. were also detected suggesting their transport and gradual adaptation under extreme conditions of deep-sea. Attempts can be made to cultivate these terrestrial fungi identified in the clone libraries to determine their adaptive strategies under such extreme conditions of pressure and temperature.

Table 3.8 Fungal phylotypes represented with closest identified relatives recovered during the cruises, #ABP26 and #ABP38 using culture-dependent and culture-independent approaches based on 18S rDNA sequences (Fungal isolate/clone obtained only once i.e. singleton is marked with *)

#ABP26		#ABP38	
Culture-dependent approach	Culture-independent approach	Culture-dependent approach	Culture-independent approach
<i>Penicillium phialosporum</i> *	<i>Aspergillus penicillioides</i> (23 clones)	<i>Nigrospora oryzae</i> *	Uncultured soil ascomycete (3 clones)
Pezizomycotina sp. (2 isolates)	<i>Aspergillus restrictus</i> (15 clones)	<i>Cladosporium</i> sp. (2 isolates)	Uncultured fungus clone (2 clones)
<i>Cladosporium</i> sp.*	<i>Aspergillus niger</i> (13 clones)	<i>Trametes versicolor</i> *	Uncultured <i>Aspergillus</i> clone*
<i>Sagenomella sclerotialis</i> (3 isolates)	<i>Candida glucosophila</i> (5 clones)	<i>Chaetomium elatum</i> *	<i>Phialosimplex caninus</i> *
Ascomycete sp. MV_26C (2 isolates)	Uncultured Ascomycota clone (6 clones)	<i>Aspergillus versicolor</i> (2 isolates)	<i>Saccharomyces</i> sp.*
<i>Tilletiopsis albescens</i> (2 isolates)	<i>Aspergillus unguis</i> (3 clones)	<i>Ascotricha lusitanica</i> *	Uncultured marine fungus clone*
Ascomycete sp. MV_25C*	Uncultured soil basidiomycete (3 clones)	<i>Pleospora herbarum</i> *	<i>Pycnoporus</i> sp.*
<i>Aspergillus restrictus</i> *	fungus sp. FCAS11 (3 clones)	<i>Eurotium herbariorum</i> *	Uncultured <i>Malassezia</i> clone*
<i>Capronia coronata</i> *	<i>Phoma herbarum</i> (2 clones)	<i>Cerrena</i> sp. NIOCC#2a (4 isolates)	<i>Sterigmatomyces halophilus</i> *
<i>Acremonium</i> sp.*	<i>Wallemia sebi</i> (2 clones)	<i>Penicillium griseofulvum</i> (2 isolates)	<i>Dothideomycete</i> sp.*
<i>Rhodotorula cassiicola</i> , (2 isolates)	<i>Aspergillus fumigates</i> *	<i>Sagenomella</i> sp.*	<i>Cerrena unicolor</i> *
<i>Sporidiobolus johnsonii</i> (4 isolates)	<i>Aspergillus</i> sp.*	<i>Hortaea werneckii</i> (2 isolates)	---
<i>Rhodosporidium toruloides</i> *	<i>Ulospora bilgramii</i> *	---	---
<i>Coniosporium perforans</i> *	<i>Capnodium coffeae</i> *	---	---
<i>Graphiola cylindricl</i> *	Uncultured fungus clone*	---	---
<i>Rhodotorula mucilaginoso</i> *	Uncultured <i>Malassezia</i> clone*	---	---
<i>Cryptococcus vishniacii</i> (2 isolates)	Uncultured <i>Aspergillus</i> clone*	---	---

In conclusion, the use of multiple primer approach enabled the recovery of diverse forms of fungal sequences from mixed environmental sediment samples from different locations of the Central Indian Basin. The higher diversity obtained in spite of less number of clones screened for station SVBC-33 of cruise #ABP26 and two stations of cruise #ABP38, suggested the abundance of fungal forms 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 genes in deep-sea conditions. Fungi such as *Aspergillus*, *Penicillium* and *Cerrena*, species obtained by culture-dependent as well as culture-independent approach (Table 3.8) could be used as a model organism to understand pressure tolerance in fungi. The lack of saturation level with all the primer sets used 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 favor 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 (Synnes, 2007). A recently described antibiotic-resistance enzyme from the deep-sea bacterium *Oceanobacillus iheyensis* isolated from 1,050 m depth in the Pacific Ocean

(Toth et al, 2010) 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 2,409 m (Ohta and Hatada, 2006) are some of the examples. Deep-sea sediments may harbor 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.

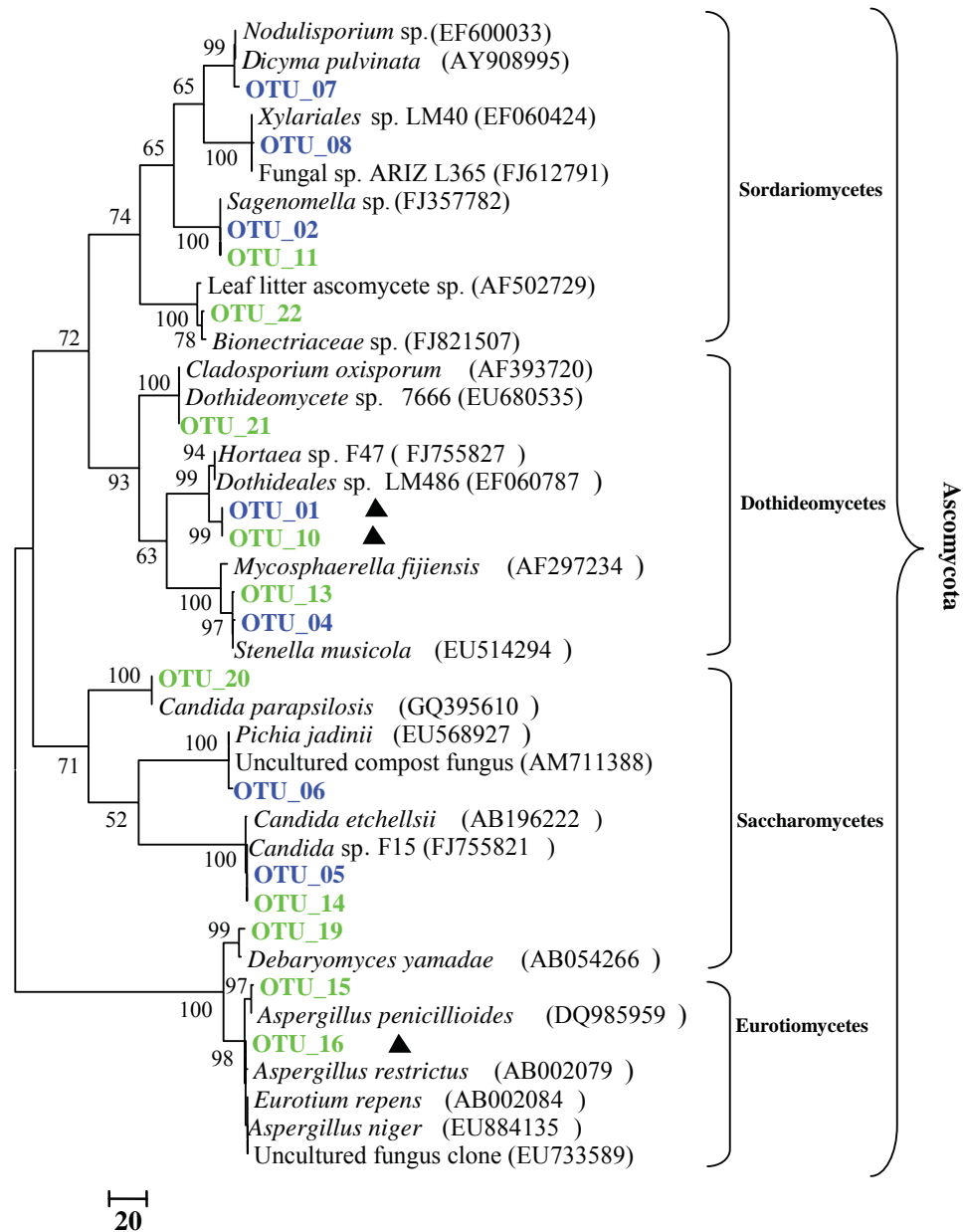


Fig 3.2 Phylogenetic tree based on fungal ITS gene sequences of phylum Ascomycota after amplification with primer set,ITS1F/ITS4 (fungal specific, marked with blue color) and ITS1/ITS4(universal ITS, marked with green color).Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates.New sequence types are marked with triangle. Analysis was done for the stations A, B and C of the cruise #ABP26.

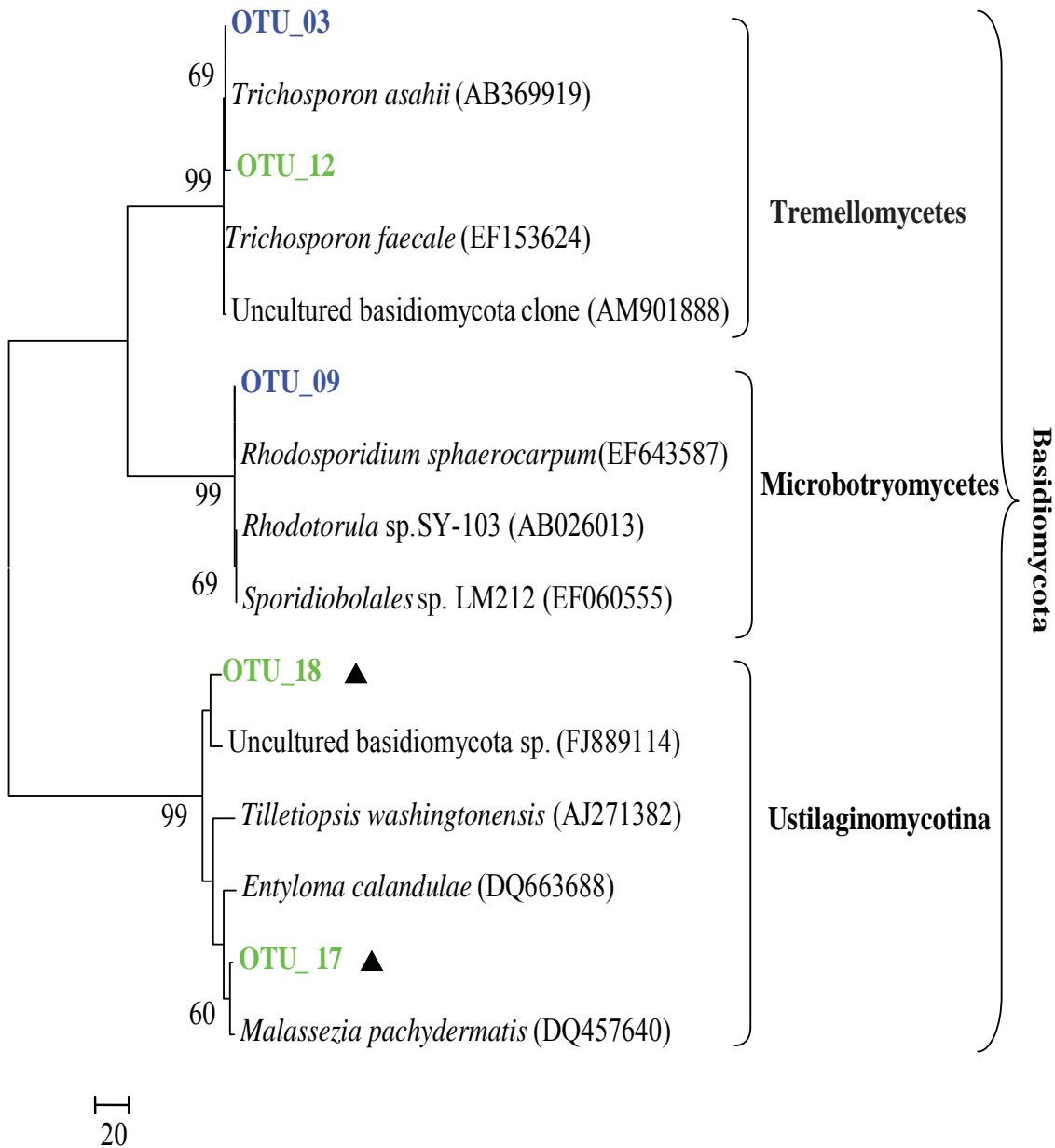


Fig 3.3 Phylogenetic tree based on fungal ITS gene sequences of phylum Basidiomycota after amplification with primer set, **ITS1F/ITS4 (fungal specific, marked with blue color)** and **ITS1/ITS4 (universal ITS, marked with green color)**. Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle. Analysis was done for the stations A, B and C of the cruise #ABP26.

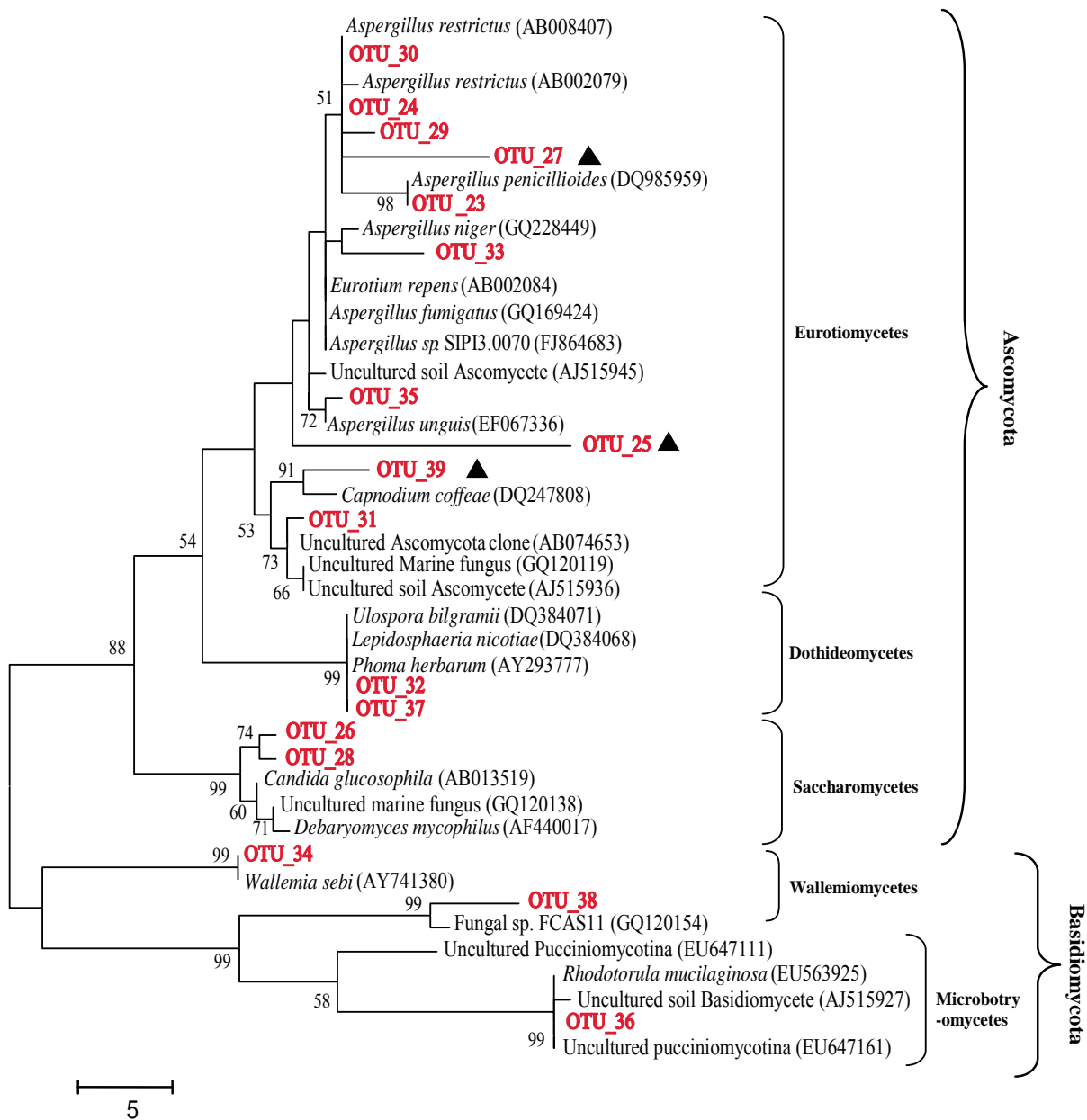


Fig 3.4 Phylogenetic tree based on fungal 18S rDNA gene sequences of phylum Ascomycota and Basidiomycota after amplification with universal 18S rDNA primer set, NS1/NS2 (Universal 18S rDNA, marked with Red color). Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle. Analysis was done for the stations A, B and C of the cruise #ABP26.

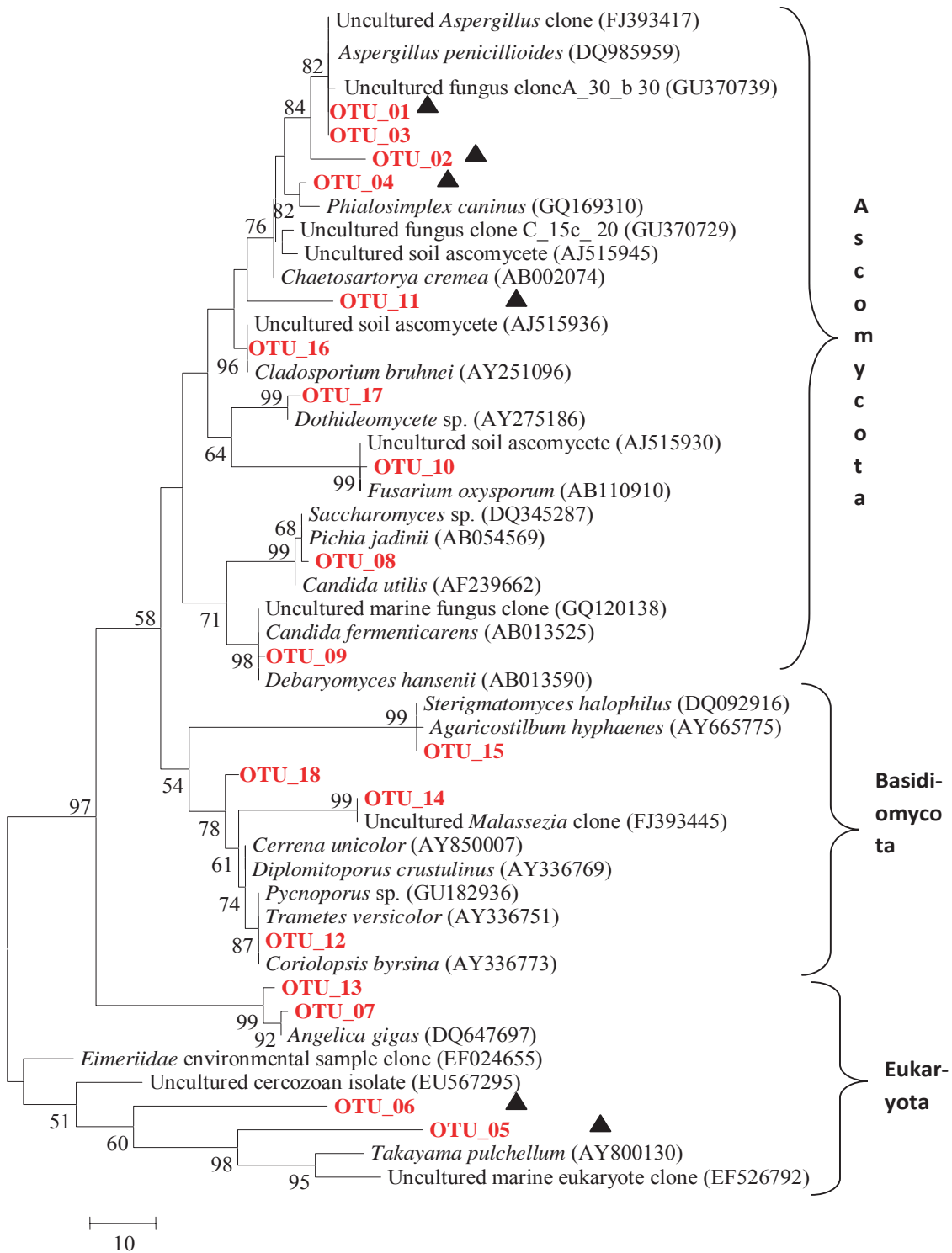


Fig. 3.5 Phylogenetic tree based on fungal 18S rDNA gene sequences of phylum Ascomycota and Basidiomycota after amplification with universal 18S rDNA primer set, NS1/NS2 (Universal 18S rDNA, marked with Red color) for stations of cruise #ABP38. Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle.

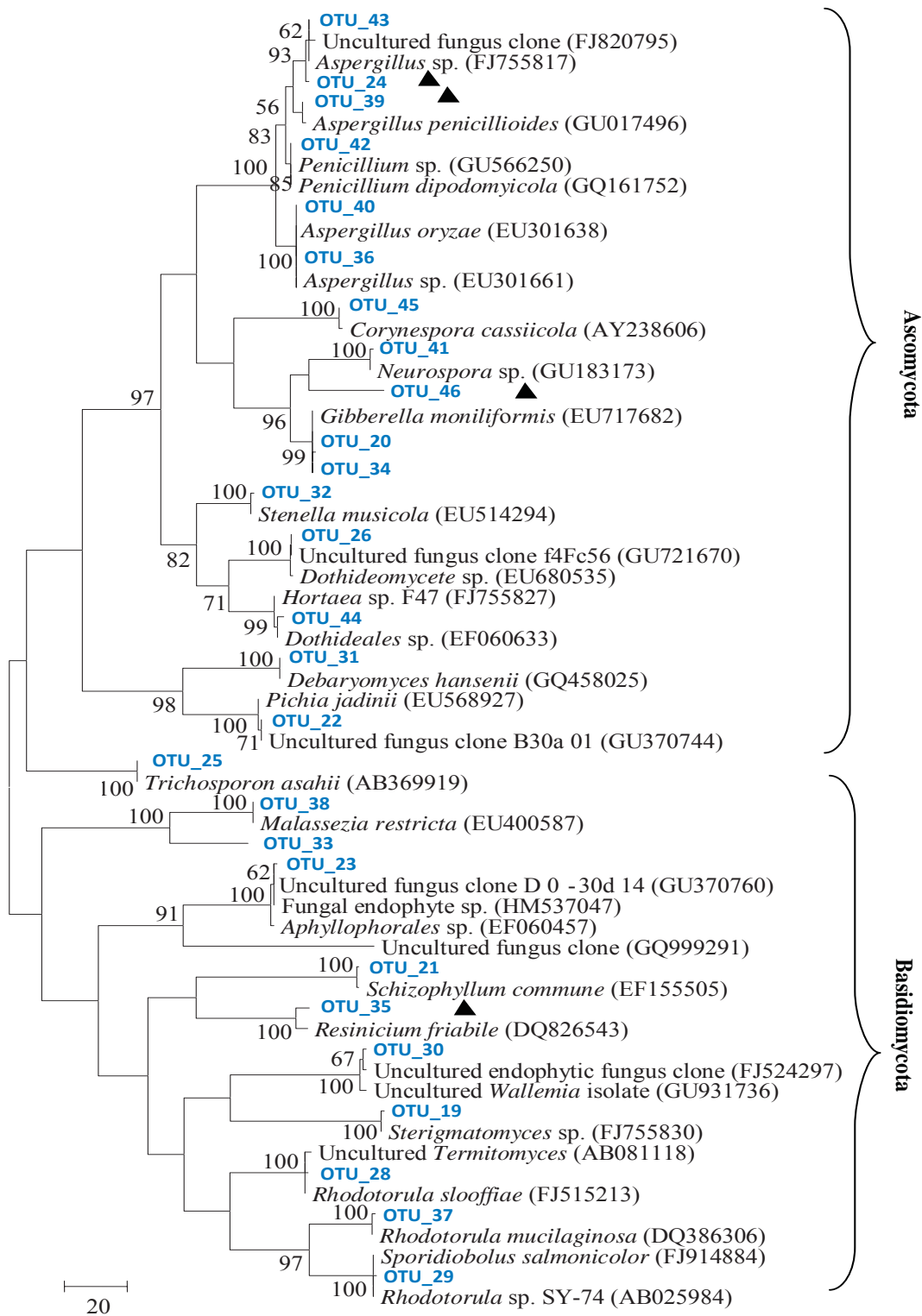


Fig. 3.6 Phylogenetic tree based on fungal ITS gene sequences of phylum Ascomycota and Basidiomycota after amplification with primer set, **ITS1F/ITS4 (fungal specific, marked with blue color)** for stations of cruise #ABP38. Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle. Ambiguously aligned sequences were removed from the analysis.

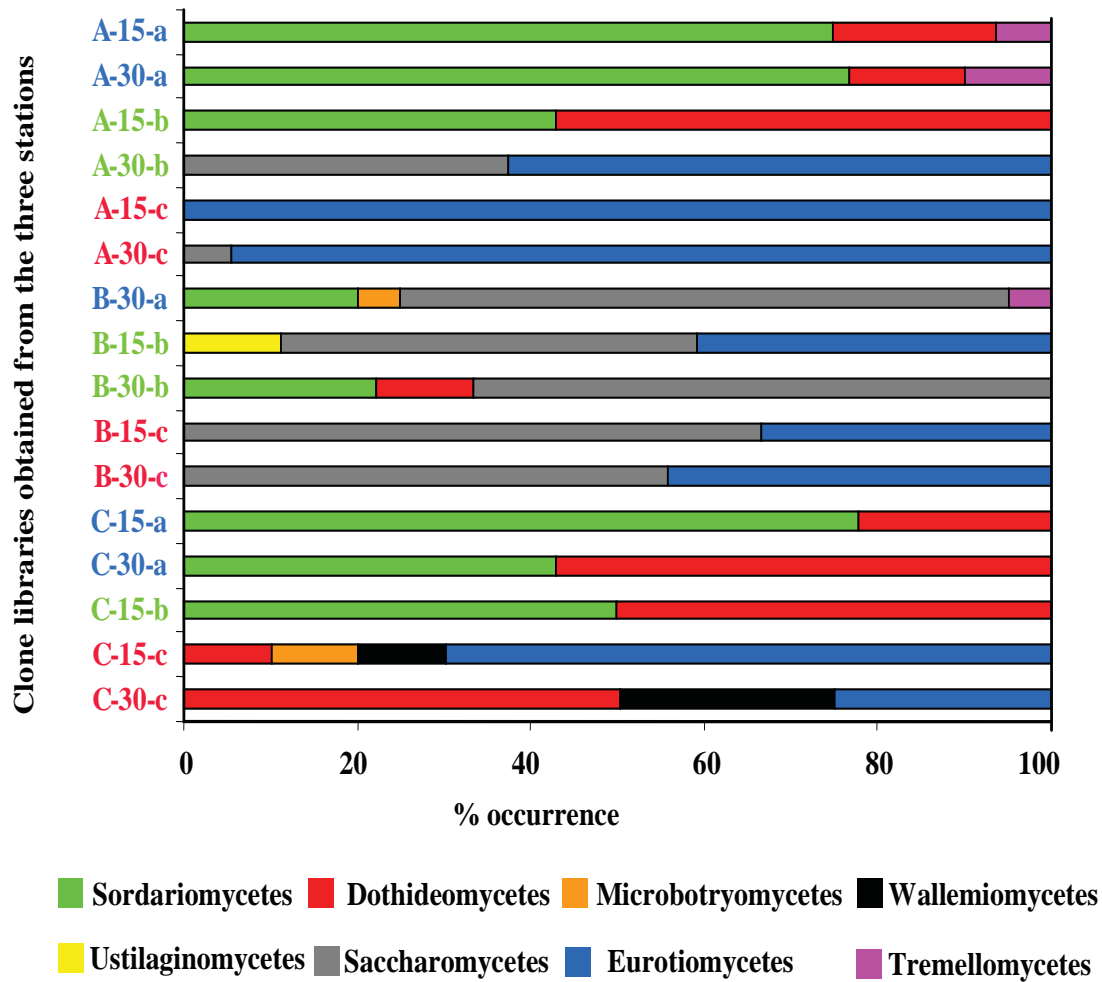


Fig 3.7 Proportional distribution of different taxonomic groups in various clone libraries for stations A, B and C of the cruise #ABP26. Percentage occurrence of sequences of each taxonomic group is indicated on x-axis. Clone libraries are represented on y-axis. The clone libraries are represented in 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.

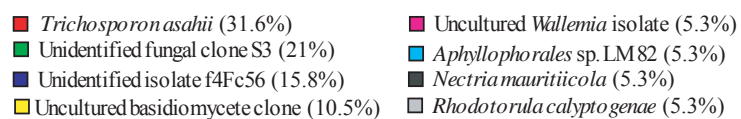


Fig 3.8 Proportional distribution of different fungal taxa in clone library constructed with primer set ITS1F/ITS4 for the station SVBC -33 of the cruise #ABP-26 (Percentage values are shown within brackets).

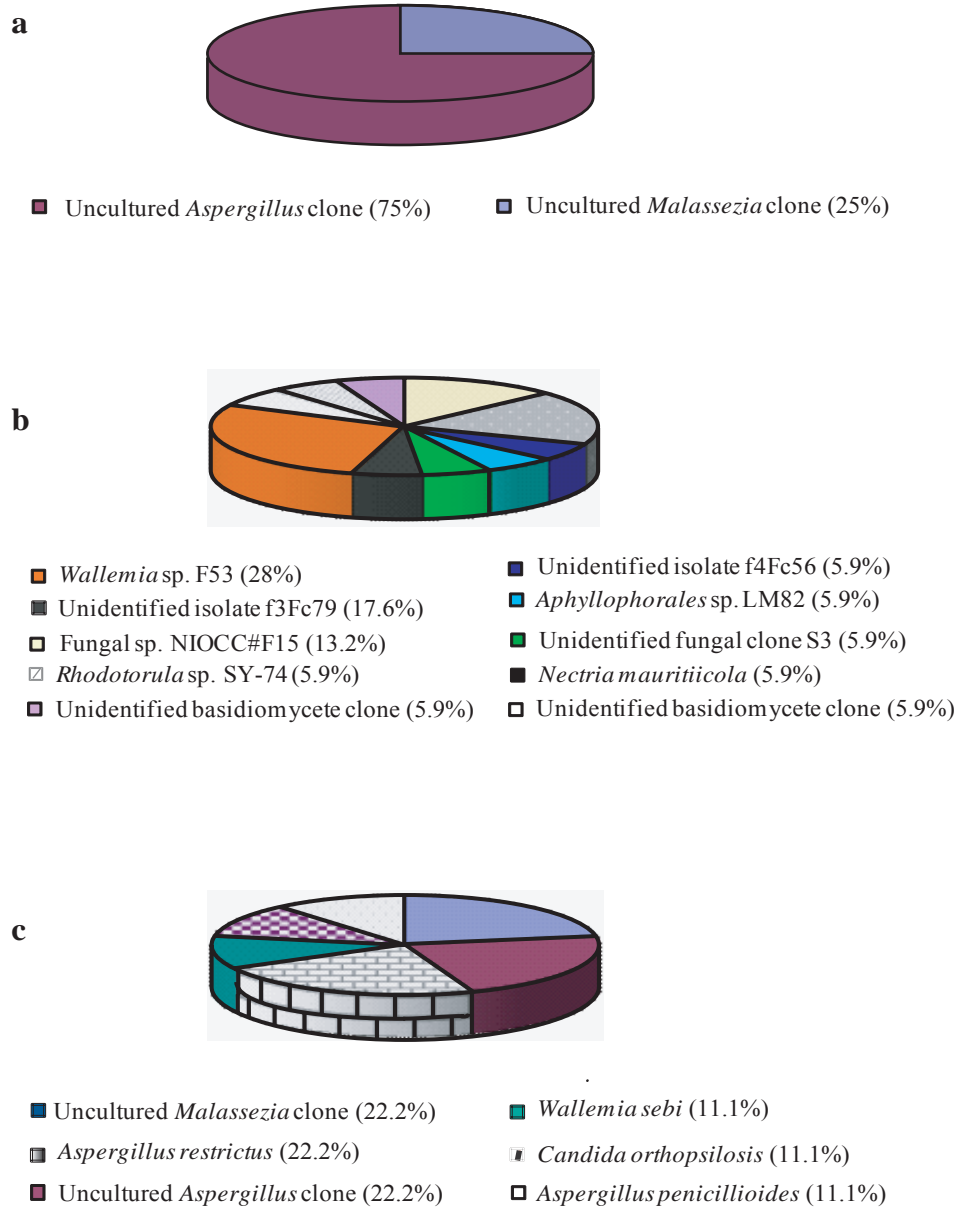


Fig 3.9 Proportional distribution of different fungal taxa in clone libraries constructed with 18S rDNA primer sets for the station SVBC -33 of the cruise #ABP-26. a: primer set NS1/NS2; b: primer set Euk18S-42F/Euk18S-1492RE and c: Euk18S-555F/Euk18S-1269R. Uncultured *Malassezia* clone (22.2%) includes OTU_21 (11.1%) and OTU_27 (11.1%). (Percentage values are shown within brackets).