

Chapter 2

Isolation of fungi from deep-sea sediments of the Central Indian Basin (CIB) and their identification using molecular approaches.

2.1 Introduction

Deep-sea harbors various kinds of microorganisms comprising bacteria, archaea and fungi, known to play an important role in recycling of nutrients (Snelgrove et al, 1997). Among these, bacteria and archaea have been studied in detail (Stackebrandt et al, 1993; Urakawa et al, 1999; Li et al, 1999a; Takai and Horikoshi, 1999; Delong and Pace, 2001; Sogin et al, 2006; Hongxiang et al, 2008; Luna et al, 2009). Fungi have also been reported in several deep-sea environments (Nagahama et al, 2006), including hydrocasts near hydrothermal plumes from the Mid-Atlantic Ridge near the Azores (Gadanhó and Sampaio, 2005) and in Pacific sea-floor sediments (Nagahama et al, 2001b). Fungi from both of these habitats were dominated by unicellular forms, commonly designated as yeasts. Many of the fungi isolated from the deep ocean have been previously undescribed species. For example, Nagahama and coworkers have described a number of novel species of yeasts from deep-sea sediments (Nagahama et al. 2001a, 2003, 2006). Undescribed species of yeasts were also identified from Atlantic hydrothermal plume waters (Gadanhó and Sampaio, 2005).

Presence of fungi in deep-sea sediments of the Central Indian Basin was reported by direct detection and immuno-fluorescence techniques (Damare et al, 2006). Fungi were also isolated from these deep-sea sediments using various isolation techniques. These cultures showed increase in biomass and germination of their spores under simulated deep-sea conditions of elevated hydrostatic pressure and low temperature (Damare, 2006). Numerous new compounds and antibiotics have also been discovered from fungi isolated from marine organisms (see Raghukumar, 2008). Also, some plant associated fungi have been reported to produce esters, alcohols, and small molecular weight acids (Mitchell et al, 2008). Considering the role of fungi as a source of useful bioactive compounds (Bhadury et al, 2006; Paramaporn et al, 2010; Konishi et al, 2010) and their involvements in various biogeochemical cycles, the diversity of fungi from deep-sea sediments needs to be examined.

2.1.1 Methods for Collection of water and sediment samples

Fungi in deep-sea waters may be collected using routine oceanographic samplers such as Niskin or Nansen bottles or a Rosette or ZoBell sampler (Fig. 2.1a). These samplers do not maintain *in-situ* hydrostatic pressure. First water sampler, used to collect water sample for isolation of piezophilic bacteria which was able to retain deep-sea pressure was reported by Jannasch et al. (1973). In general, mycelial fungi draw nutrients from organic matter in the sediment and produce hyphae, which spread out and grow in between the sediment particles (Raghukumar, 1990). Therefore it is more appropriate to look for fungi in the sediments as they are not well adapted as free living forms. Several types of sampling devices for collection of sediment samples have been used successfully by mycologists.

A deep-sea workstation was developed under the Deep-Star program at JAMSTEC, Japan, for retrieving and culturing deep-sea organisms under simulated deep-sea conditions. This system also contains a pressure-retaining sediment sampler, which is able to obtain sediment sample while maintaining ambient pressure and low temperature after sampling at the deep-sea bottom (Yanagibayashi et al, 1999). For routine microbiological sampling of deep-sea sediments, multiple corers, long gravity corers or box corers may be used (Fig 2.1b). Box corers may be used efficiently for more or less flat oceanic floors for sampling. The box corer is lowered on a ship's trawl wire till it penetrates the bottom. As the corer is pulled out of the seabed, the top and bottom of the sample box are closed. The advantage of a box corer is that it collects a sample which is generally ≥ 20 cm in length which encompasses the bulk of the vertical distribution of deep-sea organisms.

Deep-sea sediments can also be collected using research submarines. A research submarine is comparatively bigger in size and can carry a pilot and one or two scientists in a pressure sphere about 2 m in diameter.



a. Rossette Sampler



b. USNEL Box Corer



c. Sediment sample in box corer



d. A single sediment core with sample



e. Collecting sediment sub-cores in the lab

Fig. 2.1 Water and sediment sampling methods during cruises #ABP26 and #ABP38 from the Central Indian Basin.

Surrounding the sphere is equipment for life support, propulsion, ascent and descent, and equipments for scientific purposes such as manipulator arms, cameras and specialized payload in a carrying basket (Heirtzler and Grassle, 1976).

Another method for sampling is remotely operated vehicles (ROVs) which are self propelled instrument packages. Some operate at the end of a cable that provides power and hosts a two-way communications link; others are untethered, carrying their own power and recording images and data. The instrument package consists of a propulsion unit, sensors (particularly television), and, in some cases, manipulator arms. These are designed either to fly or crawl, e.g. the Remote Underwater Manipulator (Thiel and Hessler, 1974) over sea bottom surface and are more suitable for seabed sampling and experimentation.

2.1.2 Methods for studying microbial communities

Microbial communities play pivotal role in various biogeochemical cycles in different habitats (Molin and Molin, 1997; Trevors, 1998; Wall and Virginia, 1999). The major problem in correct estimation of microbes at a particular habitat is sampling and analyzing comparatively very small proportion of total area. In addition, due to spatial heterogeneity of the species or the habitat, diversity analysis of the microbes may be underestimated. Other factor for inability of correct estimation of microbial communities at a particular habitat is their low culturability. It has been suggested that at least 99% of bacteria observed under a microscope are not cultured by common laboratory techniques (Borneman et al, 1996; Giller et al, 1997; Pace, 1997; Torsvik et al, 1998; Trevors, 1998). Most of the fungal species also elude culturing in the laboratory (van Elsas et al, 2000).

Species diversity consists of species richness, the total number of species present, species evenness, and the distribution of species (Trevors, 1998; Ovreas, 2000). Some of the methods used for the measurement of cultured microbial diversity are as following:

- a) **Direct plate count method:** In this method the samples containing microbes are spread plated on selective media plates, followed by the counting of the colonies appearing after an incubation period. This method has limitations in terms of selection of appropriate media, pH and incubation temperature, colony-colony inhibition (Trevors, 1998) etc. Sometimes the fast growing forms can overtake slow growing ones, often resulting in biased results (Dix and Webster, 1995). In addition, conidiation may also give false over-estimate of some of the sporulating fungi.
- b) **Community level physiological profiling:** In this method the culturable microorganisms can be identified on the basis of different carbon source utilization patterns. Choi and Dobbs (1999) introduced Biolog plates containing all the known carbon sources for this purpose. For analysis, samples containing microbes are inoculated and monitored over time for their ability to utilize substrates and the speed at which these substrates are utilized. Multivariate analysis is applied to the data and relative differences between soil functional diversity can be assessed.
- c) **Fatty acid methyl ester (FAME) analysis:** Fatty acids make up a relatively constant proportion of the cell biomass and signature fatty acids exist that can differentiate major taxonomic groups within a community. Therefore, a change in the fatty acid profile would represent a change in the microbial population. The fatty acid profiles can be used to study microbial community composition and population changes (Siciliano and Germida, 1998; Kelly et al., 1999). Studies of bacteria have, in general, shown that phenotypic relationships among organisms as indicated by fatty acid composition are in agreement with phylogenetic associations based on DNA and rRNA homology (Nichols et al, 1986; Sasser and Smith, 1987; Guckert et al, 1991).

Individual species and strains of bacteria have been shown to have characteristic fatty acid profiles and are now being identified on this basis (Mayberry et al, 1982; Sasser and Fieldhouse, 1984; Veys et al, 1989). This method can also be used for the identification of yeasts (Gunasekaran and Hugh, 1980; Tredoux et al, 1987; Marumo and Aoki, 1990). Stahl and Klug. (1996) reported different biomarkers for different phyla of fungi which can be used for their identification. For example, fungi belonging to Oomycota were found to differentially express 20:5 fatty acids which were absent in fungi from other phyla. Zygomycota were characterized by production of higher number of fatty acids than Dikaryotic forms i.e. Ascomycota and Basidiomycota. Dikaryotic forms never produce 18:3 fatty acids which are major fatty acids for Zygomycetes. There was no difference in fatty acid profiles between Ascomycota and Basidiomycota. Sterile forms of fungi were found to produce 22:0 fatty acids, which was not found in any other taxon, and relatively high amounts of 18:0(3OH) fatty acid and 20:4 fatty acid, which were produced only in small amounts or not at all by other fungi.

- a) **Molecular based techniques:** Polymerase Chain Reaction (PCR) targeting the 16S rDNA has been used extensively to study the identification and diversity of prokaryotes (Pace, 1996, 1997, 1999). Among eukaryotes, 18S and internal transcribed spacer (ITS) regions of SSU rDNA are increasingly used to study fungal communities (Fig 2.2). However, the available databases are not as extensive as for prokaryotes (Prosser, 2002). In addition, the D1/D2 domain as well as other regions of Large Sub Unit (LSU) of 28S rDNA of eukaryotes can also be used for identification and diversity analysis (Pang and Mitchell, 2005; Gadanho and Sampaio, 2005; Burgaud et al, 2010). Restriction fragment length polymorphism (RFLP) for the above amplified products can also be applied for the diversity analysis on the basis of DNA polymorphisms. RFLP is the variation in DNA fragment

banding patterns of electrophoresed restriction digests of DNA from different individuals of a species. The reason for the different banding patterns may be the presence of a restriction enzyme cleavage site at one place in the genome in one individual and the absence of that specific site in another individual. Therefore, on the basis of these specific banding patterns after cleavage with a particular restriction enzyme different strains can be identified or compared.

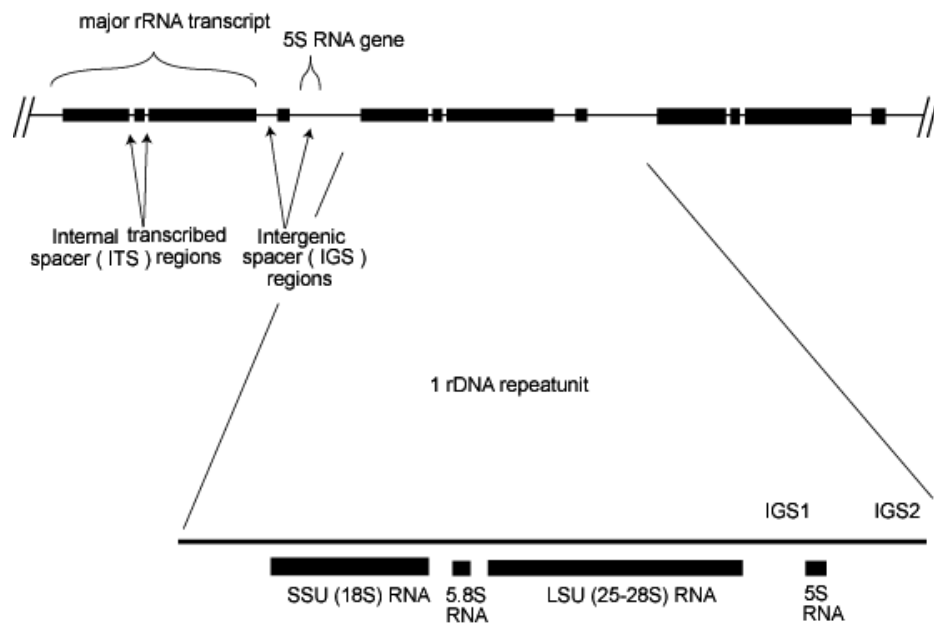


Fig 2.2 SSU rDNA region of eukaryotes displaying locations of 18S and ITS regions

Modified from: (<http://www.biology.duke.edu/fungi/mycolab/primers.htm>)

2.1.3 Fungal diversity from deep-sea habitats using culture-dependent approach

Recently some reports have described presence of fungi in deep-sea habitats using culture- dependent approaches. Several fungi isolated from various marine habitats using culture based technology, however, have also been found to be taxonomically closely related to species from genera that are well known from terrestrial environments, such as *Acremonium*, *Aspergillus*,

Penicillium and *Trichoderma* (Raghukumar et al, 1992; Holler et al, 2000; Wang et al, 2008; Jones et al, 2009). Diversity of culturable marine fungi was reported from deep-sea hydrothermal vents (Burgaud et al, 2009). Their physiological characterization revealed them to be more or less adapted to deep-sea conditions. Burgaud et al. (2010), isolated yeast species belonging to genera, *Rhodotorula*, *Rhodosporidium*, *Candida*, *Debaryomyces* and *Cryptococcus* from hydrothermal vent animals and identified them by analysis of 26 rRNA gene sequences. Fungi were isolated in the earlier reports from the deep-sea sediments of the Central Indian Basin from different sites with geographical locations of ~10-16°S and 73-77°E (Raghukumar et al, 2004; Damare et al, 2006). The sampling sites in the present study were also from the Central Indian Basin with almost the same geographic locations reported in previous studies (Table 2.1). Earlier studies have reported the identification of fungi only on the basis of classical taxonomic method, which may give biased results about actual fungal diversity in this region (Raghukumar et al, 2004; Damare et al, 2006). The present study was carried out in the same location of CIB where the culturable fungi were identified by molecular techniques.

Objectives

Isolation of fungi from deep-sea sediment samples of the Central Indian Basin using various techniques and their identification by amplification and sequencing of their 18S and ITS regions of SSU rDNA.

2.2 Materials and Methods

2.2.1 Collection of Sediment samples

Sediment samples were collected from an average depth of ~ 5000 m from different locations of the Central Indian Basin (Fig. 2.3). Twenty box core samples were collected during cruise # ABP26 in December 2006 and 2

during # ABP38 in September 2009 (10-16.5°S and 72-77°E) (Table 2.1 and 2.2) on board the Russian research vessel Akademik Boris Petrov (ABP). These sediment cores were collected with an USNEL-type box corer of 50 cm³ size (Fig 2.1b). Sediments thus collected were mostly undisturbed and compact. Sub-cores of sediments were collected from a box corer 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 35-40 cm depth were cut from the above sediment and directly introduced into sterile plastic bags to avoid any aerial contamination (Figs. 2.1 d and e). The bags were closed and carried to the microbiology laboratory on board for further processing.

2.2.2 Isolation of fungi

An aliquot of sediment from the central part of the subsection was removed with an alcohol-sterilized spatula and placed in sterile vials for isolation (Raghukumar et al, 2004). In order to isolate maximum number of fungi, four different media were used during cruise # ABP26 and they were 1) Malt Extract Agar (MEA), 2) Czapek Dox Agar (CDA), 3) Corn Meal Agar (CMA) and 4) Malt Extract Broth (MEB) (Damare et al, 2006). The above media were used at 1/5 strength to simulate low nutrient conditions of deep-sea sediments. During cruise # ABP38, two more media i.e., Sabaraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) (Appendix 6.1a-f) were also used in addition to the above four media. They were prepared in seawater and supplemented with Penicillin (40,000 units in 100 ml medium) and streptomycin (0.1 g in 100 ml medium) to inhibit bacterial growth. Isolation of fungi from sediments was done by four different methods:

1) Particle plating method of Bills and Polishook (1994): Approximately 1 g of sediment was sieved through 2 meshes of 200 and 100 microns using sterile seawater. Particles which passed through 200 microns and remained on 100-micron mesh were spread plated on various media plates (1:5 times diluted).

2) Dilution plating: Approximately 0.1 g of sediment was suspended in 9 ml of sterile seawater and vortexed for 1 min. An aliquot of 100 μ l was spread plated on various media plates.

3) Pressure Incubation: Approximately 0.1 g of sediment was suspended in 4 ml of Malt Extract Broth (1:5 times diluted and amended with antibiotics) and sealed in plastic bags. These bags were incubated in pressure vessels at a pressure of 30 MPa and at a temperature of 5°C for 30 days. After incubation bags were opened and 100 μ l was spread plated on media plates.

4) Enrichment method. Approximately 0.1 g of sediment was directly suspended in 5 ml of Malt Extract Broth media (1:5 times diluted and amended with antibiotics) and incubated at 5°C for 30 days.

All the media plates were incubated at 5°C for 30 days. Air-borne fungi present in the ship laboratory and deck, contaminating the culture plates were monitored by exposing various media plates for 10 min on the deck of the research vessel where the cores were received, the microbiology laboratory on board the research vessel and the inoculation hood. This was repeated during every sampling station.

After isolation, fungal cultures were maintained on MEA slants at 5°C. The isolates were originally numbered as NIOCC (National Institute of Culture Collection). The sporulating fungi were deposited in a recognized microbial culture repository, Microbial Type Culture Collection (MTCC) at Chandigarh, India, under the accession number MTCC.

2.2.3 Isolation of DNA from the cultures

Fungi isolated by various techniques were identified by amplification and sequencing of their 18S and ITS region of SSU rDNA. DNA was isolated from all the filamentous fungi and yeast for the above purpose. The fungi were grown in MEB for 4-5 days for DNA isolation. Yeasts were grown in yeast extract peptone and dextrose (YPD) (Appendix 6.1g) medium and shaken at 170 rpm for 3-4 days. Mycelia and cells were harvested, lyophilized and crushed in a mortar and pestle to fine powder. Isolation of DNA was carried

out following the modified form of standard procedure (Stoeck and Epstein, 2003).

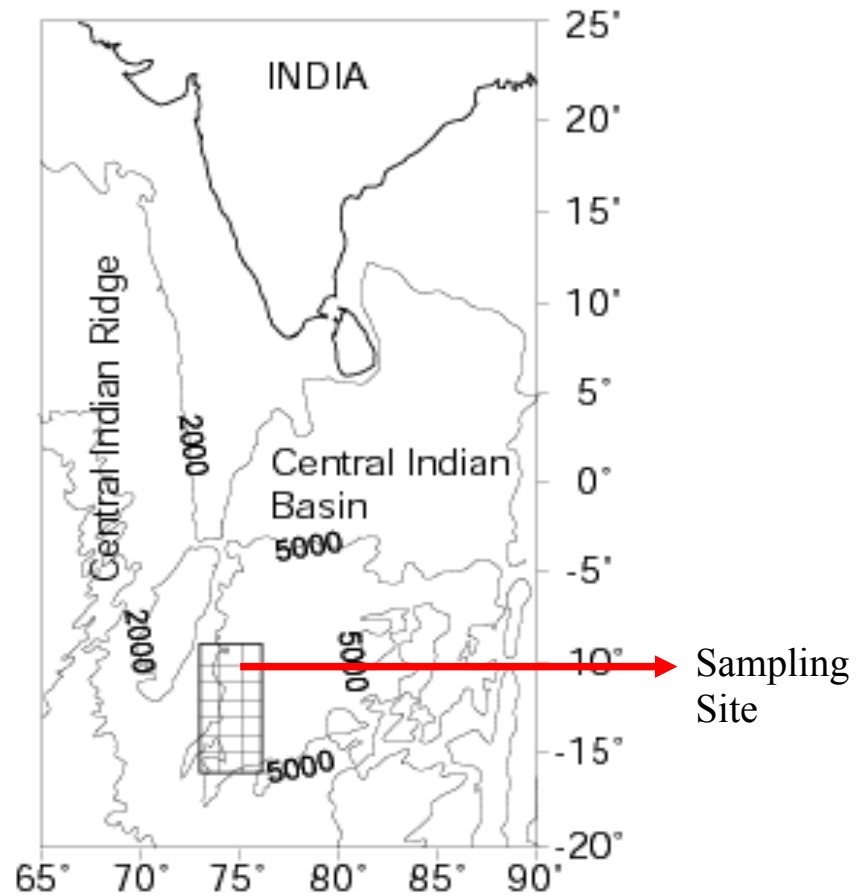


Fig. 2.3 Sampling sites (within inset) in the Central Indian Basin

The above samples were incubated at 65°C for two hours in a high salt extraction buffer containing 100 mM Tris-HCl buffer with 8.0 pH containing 100 mM Na₂EDTA, 100 mM NaPO₄, 1.5 M NaCl, 1% cetyltrimethylammonium bromide, 20% Sodium Dodecyl Sulphate, Proteinase K, 100 µg ml⁻¹ for obtaining fungal DNA. A low salt extraction buffer containing 10 mM Tris-HCl with pH 8.0, 10 mM Na₂EDTA pH 8.0, 0.5 M NaCl, 1% cetyltrimethylammonium bromide, 20% Sodium Dodecyl Sulphate and Proteinase K, 100 µg ml⁻¹ was used for isolating yeast DNA.

Table 2.1 Details of sediment collection sites from CIB during cruise # ABP26.

Sr. No.	Station No.	Date	Touch Positions		Water depth (m)
			Lat (°S)	Long (°E)	
1	SVBC-03	12.12.06	12 00.000	76 29.900	5373
2	SVBC-04	15.12.06	13 00.000	76 29.950	5377
3	SVBC-13	21.12.06	14 59.919	75 29.849	4894
4	SVBC-18	19.12.06	12 59.747	74 30.150	5122
5	SVBC-19	16.12.06	13 00.070	75 29.900	5104
6	SVBC-20	13.12.06	11 59.900	75 29.900	5223
7	SVBC-25	10.12.06	11 00.000	75 29.800	5303
8	SVBC-26	09.12.06	09 59.848	75 30.085	5339
9	SVBC-27	12.12.06	11 30.130	75 59.600	5274
10	SVBC-28	11.12.06	11 30.000	75 30.000	5263
11	SVBC-29	14.12.06	12 29.900	74 59.900	5174
12	SVBC-30	14.12.06	12 30.056	75 29.983	5189
13	SVBC-31	13.12.06	12 00.000	74 59.900	5153
14	SVBC-32	15.12.06	12 29.853	75 59.950	5309
15	SVBC-33	17.12.06	12 59.980	74 59.960	5265
16	SVBC-34	19.12.06	13 30.033	75 29.845	5219
17	SVBC-35	21.12.06	14 30.081	75 29.792	5211
18	SVBC-36	23.12.06	16 01.887	75 28.770	5042
19	SVBC-37	23.12.06	16 06.891	75 24.828	3992
20	SVBC-38	24.12.06	15 57.590	75 31.950	5642

Table 2.2 Details of sediment collection sites from CIB during cruise # ABP38.

Sr. No.	Station No.	Date	Touch Positions		Water depth (m)
			Lat (°S)	Long (°E)	
1	IVBC-18C	23.09.09	12 59.747	74 30.150	5122
2	IVBC-20A	30.09.09	11 59.900	75 29.900	5223

2.2.4 PCR amplification of 18S and ITS regions of rDNA

For amplification of 18S and ITS regions of the fungal SSU rDNA, specific primers were used. Partial region of 18S rDNA was PCR amplified by using primers, NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') of ~1100 bp. Full length of ITS region of ~600 bp was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al, 1990). The conditions for PCR included an initial hot start incubation (5 min at 94°C) followed by 34 cycles (denaturation at 94° C for 30 sec, annealing at 55° C for 30 sec and extension at 72° C for 1 min) and a final extension at 72°C for 5 min.

2.2.5 Sequencing and phylogenetic analyses

Fresh PCR products were purified by using gel extraction kit (Sigma, Genosys, USA) and sequenced at National Centre for Cell Sciences, Pune, India, using the Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) according to the manufacturer's protocol and analyzed in a DNA Analyzer (3730 DNA Analyzer, Applied Biosystems, USA). Sequence data were edited using Chromas Pro version 1.34. For tentative identification, fungal 18S and ITS rDNA sequences were compared with NCBI (National Centre for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>)

database. Fungal rDNA -18S and ITS sequences in this study and the matched sequences from GenBank were edited and aligned using CLUSTAL-X, version 1.81 (Thompson et al, 1997). The aligned sequences were imported into DAMBE 4.5.47 (Xia and Xie, 2001). Neighbour-joining (NJ) trees were created using pairwise genetic distances using MEGA 3.1 (kumar et al, 2008). The quality of the branching patterns for NJ was assessed by bootstrap resampling of the data sets with 1,000 replications.

2.2.7 Statistical analyses

The statistical analyses were carried out in Microsoft Excel program and PRIMER 5 software for calculation of diversity indices.

2.3 Results

2.3.1 Physical parameters of the sampling site

The sediment samples collected for the present study were occasionally characterized by the presence of polymetallic nodules. The nodule abundance was found to be $\sim 0.4 \text{ Kg m}^{-2}$. Moderate brown sediments dominated the core tops followed by yellow-brown clayish sediments. Intercalations and mottling were seen in most of the sediment cores. Majority of the sediment cores were siliceous in texture. One core (BC-37) was fully calcareous in composition. The approximate salinity, temperature and pH were found to be 35 PSU (practical salinity units), 3°C and 7.0 respectively. Among other parameters, the total proteins, carbohydrates and lipids were approximately 1.5, 1.0 and 1.0 mg g^{-1} sediment respectively. Total bacterial counts 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, 2008, 2010).

2.3.2 Isolation of cultures using different techniques

A total of 28 and 19 cultures were isolated during cruises #ABP26 and #ABP38 respectively with majority being the filamentous fungi. Pressure Incubation method for isolation yielded recovery of maximum number of culturable forms (Tables 2.3 and 2.4). The percentage frequency of occurrence of fungi obtained by particle plating and pressure incubation techniques was higher during cruise #ABP38 than the cruise #ABP26. Only these two methods yielded recovery of fungal cultures during cruise #ABP38. In contrast fungi could be obtained by all the four isolation techniques during the cruise #ABP26 (Tables 2.3 and 2.4).

The number of fungal isolates recovered during cruise #ABP26 was maximum with dilution plating technique whereas pressure incubation was found to be the best during #ABP38 (Tables 2.5 and 2.6). Cultures could be isolated from all the depths of the sediment cores including 35-40 cm depth (Table 2.6). Best media for isolation was found to be MEA during cruise #ABP26 whereas both CDA and PDA were found to be good during #ABP38 (Tables 2.5 and 2.6).

Table 2.3 Details of fungi isolated during cruise #ABP26

	Particle plating	Dilution plating	Pressure incubation at 30 MPa/5°C	Direct incubation in MEB at 5°C
Total no. of sediment samples used	116	188	39	153
No. of fungi isolated by using all the four media	7	12	6	3
% frequency of occurrence	6	6	15	2

Table 2.4 Details of fungi isolated during cruise #ABP38

	Particle plating	Dilution plating	Pressure incubation at 30 MPa/5°C	Direct incubation in MEB at 5°C
Total no. of sediment samples used	19	19	19	19
No. of fungi isolated by using all the six media	6	0	13	0
% frequency of occurrence	31.6	0	68.4	0

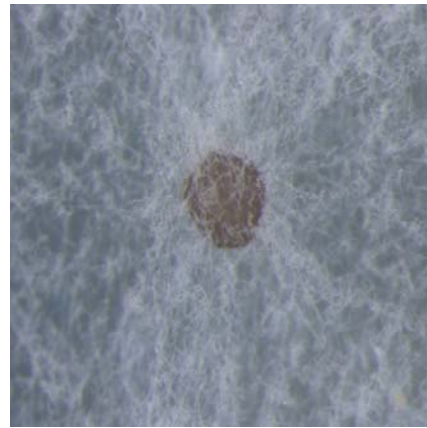
2.3.3 Diversity of fungi during cruise #ABP26

A total of 28 cultures were isolated during cruise #ABP26 including 16 filamentous fungi and 12 yeast isolates. The cultures were identified by classical taxonomic method as well as by amplification and sequencing of their 18S and ITS regions of SSU rDNA (Table 2.7). Only sporulating forms could be identified by classical taxonomic method. The identification of sporulating cultures carried out by classical morphological taxonomy matched with molecular identification based on either 18S or ITS sequences and only seldom with both these sequences (Table 2.7). The 18S and ITS sequences of all the cultures were deposited in GenBank and accession numbers obtained (Table 2.7). Most of the fungi belonged to the phylum Ascomycota and based on 18S sequences clustered into 7 subgroups, namely *Aspergillus* sp., *Sagenomella* sp. (Fig 2.4), *Exophiala* sp. (Fig 2.5), *Capronia* sp., *Cladosporium* sp., *Acremonium* sp. and *Tritirachium* sp. (Fig. 2.6).

Based on ITS sequences they grouped into 6 clusters showing maximum similarity to *Aspergillus* sp., uncultured member of Hypocreaceae family, *Exophiala* sp., an uncultured soil fungus clone DQ682584.1, *Hypocreales* and *Trichothecium* sp. (Fig. 2.7). The two basidiomycetes



A. Culture grown on MEA plate



**B. Mycelia observed under
Stereo-microscope**

Fig 2.4 *Sagenomella* sp. (NIOCC#F15, #F23, #F65)



**Fig 2.5 *Exophiala* sp. (NIOCC#F16)
(Culture grown on MEA plate)**

Table 2.5: Distribution of fungi in various depths and effect of different methods and media on isolation during cruise #ABP26

Isolate	Core	Section of the core (cm)	Method of isolation*	Medium used**
NIOCC#F2	BC-33	8-10	DP	CMA
NIOCC#F5	BC-25	0-2	PI	MEA
NIOCC#F6	BC-25	4-6	PI	CMA
NIOCC#F8	BC-20	15-20	PP	MEA
NIOCC#F13	BC-19	10-15	PI	MEA
NIOCC#F15	BC-26	4-6	PP	MEA
NIOCC#F16	BC-29	6-8	PP	CDA
NIOCC#F21	BC-26	25-30	PP	MEA
NIOCC#F23	BC-26	0-2	DP	MEA
NIOCC#F30	BC-37	0-2	PI	CDA
NIOCC#F33	BC-36	30-35	PI	MEA
NIOCC#F46	BC-29	6-8	DP	CMA
NIOCC#F47	BC-38	20-25	DP	CDA
NIOCC#F48	BC-26	25-30	PP	MEA
NIOCC#F50	BC-25	20-25	DP	MEA
NIOCC#PF49	BC-29	4-6	DI	MEB
NIOCC#Y1	BC-29	2-4	DP	MEA
NIOCC#Y2	BC-18	30-35	DP	CDA
NIOCC#Y3	BC-29	10-15	DP	CDA
NIOCC#Y4	BC-37	4-6	PP	MEA
NIOCC#Y5	BC-04	20-25	DP	MEA
NIOCC#Y6	BC-19	20-25	PP	MEA
NIOCC#Y7	BC-31	2-4	DP	MEA
NIOCC#Y8	BC-29	4-6	DP	CMA
NIOCC#Y9	BC-29	0-2	DP	MEA
NIOCC#Y10	BC-36	8-10	PI	MEA
NIOCC#PY12	BC-29	4-6	DI	MEB
NIOCC#PY13	BC-29	8-10	DI	MEB

*PI: Pressure Incubation, PP: Particle Plating, DP: Dilution Plating, DI: Direct Incubation,

**MEB: Malt Extract Broth, MEA: Malt Extract Agar, CDA: Czapek Dox Agar, CMA: Corn Meal Agar

(NIOCC#F21 and #F33) belonged to *Tilletiopsis* sp. as identified by 18S as well as ITS sequencing (Figs. 2.6, 2.7 and Table 2.7). Species of filamentous fungi *Sagenomella*, *Exophiala*, *Capronia*, and *Tilletiopsis* are being reported for the first time from the deep-sea sediments. The majority of the yeast isolates belonged to the phylum Basidiomycota (Figs. 2.8 and 2.9) and only

one isolate, NIOCC # Y8 belonged to Ascomycota identified both by 18S and ITS sequencing (Fig. 2.9 and Table 2.7). Sequences of 18S as well as ITS gave matching identification of yeasts (Table 2.7). The basidiomycetous yeast belonged to the genera *Sporobolomyces* sp., *Sporidiobolus*, *Rhodospiridium*, *Rhodotorula* and *Cryptococcus* sp. A 100% consensus in identification was seen between 18S and ITS sequences of the isolates NIOCC#Y2, Y3, Y6, Y7 and PY12 up to generic level (Table 2.7).

Table 2.6: Distribution of fungi in various depths and effect of different methods and media on isolation during cruise #ABP38

Isolate	Core	Section of the core (cm)	Method of isolation*	Medium used**
NIOCC#F51	BC-18C	8-10	PI	MEA
NIOCC#F52	BC-18C	10-15	PI	CDA
NIOCC#F53	BC-18C	0-2	PI	MEA
NIOCC#F54	BC-18C	8-10	PI	PDA
NIOCC#F55	BC-18C	4-6	PI	CDA
NIOCC#F56	BC-18C	10-15	PI	MEA
NIOCC#F57	BC-18C	20-25	PI	PDA
NIOCC#F58	BC-18C	2-4	PI	PDA
NIOCC#F59	BC-18C	0-2	PI	MEA
NIOCC#F60	BC-18C	30-35	PI	PDA
NIOCC#F61	BC-18C	20-25	PI	SDA
NIOCC#F62	BC-20A	20-25	PP	CMA
NIOCC#F63	BC-18C	35-40	PP	PDA
NIOCC#F64	BC-18C	4-6	PP	CDA
NIOCC#F65	BC-18C	6-8	PP	MEA
NIOCC#F66	BC-20A	4-6	PI	MEA
NIOCC#F67	BC-20A	15-20	PI	SDA
NIOCC#F68	BC-20A	15-20	PP	CMA
NIOCC#Y14	BC-20A	6-8	PP	PDA

*PI: Pressure Incubation, PP: Particle Plating,

**MEA: Malt Extract Agar, CDA: Czapek Dox Agar, SDA: Sabourauds Dextrose Agar, PDA: Potato Dextrose Agar, CMA: Corn Meal Agar

2.3.4 Diversity of fungi during cruise #ABP38

Nineteen fungi were isolated during cruise #ABP38 resulting in a total of 12 distinct fungal genera (Table 2.8).

Table 2.7 Identification of fungi by morphological and molecular approaches and their accession numbers isolated during the cruise #ABP26

Fungal Isolate			Identification		
Designation	Accession number		Microscopic	18S (Closest identified relative)	ITS (Closest identified relative)
	18S	ITS			
NIOCC#F2	FJ357792	EU729705	<i>Penicillium</i> sp.	<i>Penicillium</i> sp.	<i>Penicillium citreonigrum</i>
NIOCC#F5	EU723484	EU729711	<i>Cladosporium</i> sp.	Pezizomycotina sp.	<i>Cladosporium cladosporioides</i>
NIOCC#F6 (MTCC9336)	EU723485	EU729718	<i>Aspergillus</i> sp.	Uncultured marine eukaryote clone U112H09	<i>Aspergillus</i> sp.
NIOCC#F8	EU723486	EU729712	<i>Cladosporium</i> sp.	Pezizomycotina sp.	<i>Cladosporium cladosporioides</i>
NIOCC#F13 (MTCC9333)	EU723487	EU729719	<i>Cladosporium</i> sp.	<i>Cladosporium</i> sp.	<i>Cladosporium</i> sp.
NIOCC#F15	EU723488	FJ357782	<i>Sagenomella</i> sp.	<i>Sagenomella sclerotialis</i> sp.	Uncultured fungus clone
NIOCC#F16 (MTCC9332)	EU723489	FJ357783	<i>Rhinoctadiella</i> sp.	Ascomycete MV_26C	<i>Exophiala spinifera</i>
NIOCC#F21 (MTCC9339)	EU723490	FJ357784	Sporulating, unidentified sp.	<i>Tilletiopsis albescens</i>	<i>Tilletiopsis albescens</i>
NIOCC#F23	EU723491	FJ357785	<i>Sagenomella</i> sp.	<i>Sagenomella sclerotialis</i> sp.	Uncultured fungus clone
NIOCC#F30 (MTCC9337)	EU723492	EU729707	<i>Acremonium</i> sp.	Ascomycete MV_25C	Hypocreales sp.
NIOCC#F33	EU723493	EU729706	Sporulating, unidentified sp.	<i>Tilletiopsis albescens</i>	<i>Tilletiopsis albescens</i>
NIOCC#F46 (MTCC9334)	EU723494	EU729708	<i>Exophiala</i> sp.	Ascomycete MV_26C	<i>Exophiala xenobiotica</i>
NIOCC#F47	EU723495	EU729709	<i>Aspergillus</i> sp.	<i>Aspergillus restrictus</i>	<i>Aspergillus caesiellus</i>
NIOCC#F48 (MTCC9341)	EU723496	FJ357786	<i>Sagenomella</i> sp.	<i>Sagenomella sclerotialis</i>	<i>Phialemonium dimorphosporum</i>
NIOCC#F49 (MTCC9340)	EU723497	EU729713	<i>Acremonium</i> sp.	<i>Acremonium</i> sp.	<i>Trichothecium roseum</i>
NIOCC#F50	EU723498	EU729710	Non -sporulating sp.	<i>Capronia coronata</i>	<i>Exophiala dermatitidis</i>

Table 2.7 contd.

Fungal isolate			Identification		
Designation	Accession number		Microscopic	18S (Closest identified relative)	ITS (Closest identified relative)
	18S	ITS			
NIOCC#Y1	EU723499	FJ357787	Yeast sp.	<i>Rhodotorula cassicola</i>	<i>Sporobolomyces</i> sp.
NIOCC#Y2	EU723500	FJ357788	Yeast sp.	<i>Sporidiobolus johnsonii</i>	<i>Sporidiobolus salmonicolor</i>
NIOCC#Y3	EU723501	EU729715	Yeast sp.	<i>Rhodospiridium toruloides</i>	<i>Rhodospiridium toruloides</i>
NIOCC#Y4	EU723502	EU729716	Yeast sp.	<i>Sporidiobolus johnsonii</i>	<i>Sporobolomyces</i> sp.
NIOCC#Y5	EU723503	FJ357793	Yeast sp.	<i>Sporidiobolus johnsonii</i>	<i>Rhodotorula</i> sp.
NIOCC#Y6	EU723504	EU72972	Yeast sp.	<i>Sporidiobolus johnsonii</i>	<i>Sporidiobolus</i> sp.
NIOCC#Y7	EU723505	FJ357789	Yeast sp.	<i>Rhodotorula cassicola</i>	<i>Rhodotorula calyptogenae</i>
NIOCC#Y8	EU723506	EU729717	Black Yeast sp.	<i>Coniosporium perforans</i>	<i>Sarcinomyces petricola</i>
NIOCC#Y9	EU723507	FJ357790	Filamentous Yeast sp.	<i>Graphiola cylindrica</i>	<i>Tilletiopsis oryzaicola</i>
NIOCC#Y10	EU723508	EU729721	Yeast sp.	<i>Rhodotorula mucilaginoso</i>	<i>Cryptococcus albidosimilis</i>
NIOCC#PY12	EU723509	FJ357791	Yeast sp.	<i>Cryptococcus vishniacii</i>	<i>Cryptococcus albidosimilis</i>
NIOCC#PY13	EU723510	EU729714	Yeast sp.	<i>Cryptococcus vishniacii</i>	<i>Rhodotorula mucilaginoso</i>

MTCC-Microbial Type Culture Collection, Chandigarh, NIOCC= National Institute of Oceanography Culture Collection

Table 2.8 Phylogenetic affiliations of fungal cultures isolated during cruise #ABP38 on the basis of 18S sequences

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

NIOCC= National Institute of Oceanography Culture Collection

The identification of these isolates was done on the basis of 18S rDNA region amplification and sequencing. Majority of the forms belonged to Ascomycota. Among these ascomycetous forms, no single species was found to be dominating. It included members from *Aspergillus* sp., *Eurotium* sp., *Cladosporium* sp., *Pleospora* sp., *Chaetomium* sp., *Ascotricha* sp., *Penicillium* sp., *Sagenomella* sp. among filamentous fungi (Fig. 2.10). Two ascomycetous culture sequences were found to cluster with black yeast i.e. *Hortaea* sp. Most of these fungal sequences showed >98% similarity with the sequences of their closest relative species. However two ascomycetous fungal genotypes i.e. NIOCC#F55 and #Y14 showed only 95 and 97 % homology with the existing sequences of the database.

Among basidiomycota, two fungal genera belonging to *Cerrena* and *Trametes* sp. were identified (Table 2.8). Out of 5 basidiomycetous fungi, four showed similarity with *Cerrena* sp. with a percentage similarity of 99. The number of cultures isolated during this cruise was comparatively higher than the previous cruise in spite of less number of samples and also belonged to more diverse fungal species. The isolation of *Cerrena* and *Trametes* sp. is being reported for the first time from the deep-sea sediments of the Central Indian Basin. Some of the fungi such as *Aspergillus*, *Cladosporium*, *Penicillium* and *Sagenomella* sp. were isolated during both the cruises. Whereas other fungi such as *Nigrospora*, *Trametes*, *Chaetomium*, *Ascotricha*, *Pleospora*, *Eurotium*, *Cerrena* and *Hortaea* sp. were isolated exclusively during the cruise #ABP38.

2.3.5 Species distribution and richness at different depths

Fungal species distribution varied among different depths of the sediments cores. The Shannon-Wiener diversity indices values were higher during cruise #ABP26 than the cruise #ABP38 (Tables 2.9 and 2.10). The values were found to be different for different depths, highest being 1.8 for depth 4-6 cm in cruise #ABP26 (Table 2.9). During cruise #ABP38 the highest Shannon-Wiener diversity indices value was 1.0 for the depth 20-25 cm (Table 2.10).

Table 2.9 Species distribution at different depths in the sediment core during the cruise #ABP26

Genera (Represented with closest identified relative)	Depths (cm)										
	0-2	2-4	4-6	6-8	8-10	10-15	15-20	20-25	25-30	30-35	35-40
Total species isolate	4	1	6	1	3	2	1	3	2	2	0
Total sediment sections used	20	20	20	20	20	20	20	20	17	8	4
<i>Penicillium</i> sp. (NIOCC#F2)	-	-	-	-	1	-	-	-	-	-	-
<i>Cladosporium</i> sp. (NIOCC#F5, F8, F13)	1	-	-	-	-	1	1	-	-	-	-
<i>Aspergillus</i> sp. (NIOCC#F6, F47)	-	-	1	-	-	-	-	1	-	-	-
<i>Sagenomella</i> sp. (NIOCC#F15, F23, F48)	1	-	1	-	-	-	-	-	1	-	-
Ascomycete sp. (NIOCC#F16, F30, F46)	1	-	-	2	-	-	-	-	-	-	-
<i>Tilletiopsis</i> sp. (NIOCC#F21, F33)	-	-	-	-	-	-	-	-	1	1	-
<i>Capronia coronata</i> (NIOCC#F50)	-	-	-	-	-	-	-	1	-	-	-
<i>Acremonium</i> sp. (NIOCC#PF49)	-	-	1	-	-	-	-	-	-	-	-
<i>Sporidiobolus johnsonii</i> (NIOCC#Y2, Y4, Y5, Y6)	-	-	1	-	-	-	-	2	-	1	-
<i>Rhodosporidium toruloides</i> (NIOCC#Y3)	-	-	-	-	-	1	-	-	-	-	-
<i>Rhodotorula</i> sp. (NIOCC#Y1, Y7, Y10)	-	2	-	-	1	-	-	-	-	-	-
<i>Coniosporium perforans</i> (NIOCC#Y8)	-	-	1	-	-	-	-	-	-	-	-
<i>Graphiola cylindrica</i> (NIOCC#Y9)	1	-	-	-	-	-	-	-	-	-	-
<i>Cryptococcus</i> sp. (NIOCC#PY12, PY13)	-	-	1	-	1	-	-	-	-	-	-
Shannon index (log e)	1.4	0	1.8	0	1.1	0.7	0	1	0.7	0.7	0
Pielou's evenness	1	-	1	-	1	1	-	0.9	1	1	-
Species richness	2.2	0	2.8	0	1.8	1.4	-	1.4	1.4	1.4	-

Table 2.10 Species distribution at different depths in the sediment core during the cruise #ABP38

Genera (Represented with closest identified relative)	Depth (cm)										
	0-2	2-4	4-6	6-8	8-10	10-15	15-20	20-25	25-30	30-35	35-40
Total species isolate	2	1	3	2	2	2	2	3	0	1	1
Total sediment sections used	2	2	2	2	2	2	2	2	1	1	1
<i>Nigrospora</i> sp. (NIOCC#F51)	-	-	-	-	1	-	-	-	-	-	-
<i>Cladosporium</i> sp. (NIOCC#F52, F58)	-	1	-	-	-	1	-	-	-	-	-
<i>Trametes</i> sp. (NIOCC#F53)	1	-	-	-	-	-	-	-	-	-	-
<i>Chaetomium</i> sp. (NIOCC#F54)	-	-	-	-	1	-	-	-	-	-	-
<i>Aspergillus</i> sp. (NIOCC#F55, F64)	-	-	2	-	-	-	-	-	-	-	-
<i>Ascotricha</i> sp. (NIOCC#F56)	-	-	-	-	-	1	-	-	-	-	-
<i>Pleospora</i> sp. (NIOCC#F57)	-	-	-	-	-	-	-	1	-	-	-
<i>Eurotium</i> sp. (NIOCC#F59)	1	-	-	-	-	-	-	-	-	-	-
<i>Cerrena</i> sp. (NIOCC#F60, F61, F66, F67)	-	-	1	-	-	-	1	1	-	1	-
<i>Penicillium</i> sp. (NIOCC#F62, F63)	-	-	-	-	-	-	-	1	-	-	1
<i>Sagenomella</i> sp. (NIOCC#F65)	-	-	-	1	-	-	-	-	-	-	-
<i>Hortaea</i> sp. (NIOCC#F68, Y14)	-	-	-	1	-	-	1	-	-	-	-
Shannon index (log e)	0.7	0	0.6	0.7	0.7	0.7	0.7	1	0	0	0
Pielou's evenness	1	-	0.9	1	1	1	1	1	-	-	-
Species richness	1.4	-	0.9	1.4	1.4	1.4	1.4	1.8	-	-	-

Species richness was also higher during cruise #ABP26 with a maximum value of 2.8 at 4-6 cm depth than the cruise #ABP38, with a maximum value of 1.8 at 20-25 cm depth (Tables 2.9 and 2.10).

2.4 Discussion

2.4.1 Distribution of fungi in deep-sea sediments

The fungi isolated mostly belonged to Ascomycota and Basidiomycota. Ascomycetes dominated among the filamentous fungi whereas basidiomycetes were dominant among the yeast (unicellular fungi). Bass et al. (2007) observed that yeast sequences dominated in deep-sea sediments of the Pacific Ocean at 1,500–4,000 m depth. On the other hand, in the present study as well as that of Damare et al. (2006) filamentous fungi were the dominant culturable fungi. It is possible that deep-sea yeasts may require more specialized media for culturing. The ITS and 18S sequences gave matching results for identification for only some of the non-sporulating and sporulating mycelia cultures (Table 2.7). On the other hand, there was a 100% consensus between these two techniques while identifying most of the yeast cultures (Table 2.7).

These results indicate that identification up to species level should be based on more than two techniques. Using a few other fungal specific primers as reported by Pang and Mitchell (2005), this problem could be resolved. The presence of fast growing sporulating forms such as *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Curvularia* sp., *Acremonium* sp., and *Fusarium* sp., have been reported as culturable forms (Roth et al, 1964; Raghukumar et al, 1992; Damare et al, 2006) and their molecular sequences have been detected in the oceanic environment (Bass et al, 2007). We are reporting for the first time the presence of *Sagenomella* sp., *Capronia coronata*, *Exophiala* sp., *Tilletiopsis* sp., and *Cerrena* sp. from deep-sea sediments. Several of the cultured fungi in our studies showed phylogenetic similarity to fungi reported in marine environment. A *Capronia*-like fungus (order *Chaetothyriales*), isolated from deep-sea hydrothermal vent elicited a host immune response in mussels and was associated with tissue deterioration

(Burgaud et al, 2009). The 18S sequences of the filamentous fungi NIOCC#F5 and #F8 showed 99-100% homology to Pezizomycotina isolate EU594360, originally reported to have been isolated from a marine sponge *Haliclona simulans* (Table 2.11). The 18S sequences of the isolate NIOCC#F16 and #F30 showed homology to the isolates obtained from deep hydrothermal ecosystem of Mid-Atlantic Ridge and the isolate NIOCC#F13 to *Cladosporium* sp., reported from hypersaline environment (Table 2.11). The ITS sequences of the isolates NIOCC#F5, #F15, #F16, #Y5, and #Y7 showed homology to the cultures either from deep-sea or marine environment (Table 2.12).

2.4.2 Diversity of fungi during cruise #ABP26

The low recovery of culturable fungi from deep-sea sediments was the most striking feature of the sampling carried out during the cruise #ABP26. A total of 28 fungi, inclusive of filamentous and unicellular yeasts were recovered from 496 sediment samples (5.6% frequency) by methods such as particle plating, dilution plating, pressure incubation and low temperature incubation and four different culture media. At the same geographical positions i.e. 10–15°S and 73-76°E of the Indian Ocean Damare et al. (2006) isolated a total of 181 fungi from 672 sediment samples collected during three cruises from 2001–2003 (9% frequency). Almost similar methods and culture media for isolation of fungi were used by Damare et al. (2006). Such a difference in recovery of fungi may indicate large temporal variation or possibility of extremely patchy distribution of fungi in deep-sea sediments with pockets of high fungal population. To isolate more diverse forms of culturable fungi from deep-sea sediments, optimization of media and nutrient conditions may be applied in future. Turner (1973) reported sinking waterlogged wood originating from offshore during monsoon runoffs creating “islands of wood” in the deep sea. Such persistent but shifting islands of wood might bring in saprophytic species, serve as dispersal centres and contribute to habitat diversity, niche specialization and enrichment. Similar route for several mussels in the deep-sea vents has been hypothesized (Distel et al, 2000).

Patchy distribution of fungi seen in the Central Indian Basin appears to be similar to the scenario of high bacterial population associated with “whale fall” in deep sea (Smith and Baco, 2003). The sinking particulate organic matter from the dynamic surface waters might also be contributing to this vast temporal difference in the almost static deep-sea sediments.

Table 2.11 Phylogenetic affiliations of fungi and yeast isolated during cruise #ABP26 on the basis of 18S sequences

Isolate ID	Closest identified relative, phylum (GenBank Accession no.)	% identity	Source of isolation of Closest relative
(NIOCC#F2)	<i>Penicillium phialosporum</i> , Ascomycota (AF245266)	84	Not known
(NIOCC#F5)	Pezizomycotina , Ascomycota (EU594360)	100	<i>Haliclona simulans</i> (a marine sponge)
(NIOCC#F6)	Uncultured marine eukaryote clone UII2H09, Ascomycota (EU446322)	99	“Anoxic deep hypersaline L'Atalante Basin, eastern Mediterranean Sea”
(NIOCC#F8)	Pezizomycotina sp., Ascomycota (EU594360)	99	<i>Haliclona simulans</i> (a marine sponge)
(NIOCC#F13)	<i>Cladosporium</i> sp. EXF228, Ascomycota (DQ780930)	99	Hypersaline environments
(NIOCC#F15)	<i>Sagenomella sclerotialis</i> , Ascomycota (AB024592)	99	Not known
(NIOCC#F16)	Ascomycete sp. MV_26C, Ascomycota (EF638702)	100	Mid Atlantic Ridge in deep hydrothermal ecosystem
(NIOCC#F21)	<i>Tilletiopsis albescens</i> , Basidiomycota (D83188)	99	Not known
(NIOCC#F23)	<i>Sagenomella sclerotialis</i> , Ascomycota (AB024592)	99	Not known
(NIOCC#F30)	Ascomycete sp. MV_25C, Ascomycota (EF638701)	100	Mid Atlantic Ridge in deep hydrothermal ecosystem
(NIOCC#F33)	<i>Tilletiopsis albescens</i> , Basidiomycota (D83188)	100	Not known

Table 2.11 contd.

Isolate ID	Closest identified relative, phylum (GenBank Accession no.)	% identity	Source of isolation of Closest relative
(NIOCC#F46)	Ascomycete sp. MV_26C, Ascomycota (EF638702)	100	Mid Atlantic Ridge in deep hydrothermal ecosystem
(NIOCC#F47)	<i>Aspergillus restrictus</i> , Ascomycota (AB008407)	100	Culture collection
(NIOCC#F48)	<i>Sagenomella sclerotialis</i> , Ascomycota (AB024592)	99	Culture collection
(NIOCC#F50)	<i>Capronia coronata</i> , Ascomycota (AJ232939)	99	Not known
(NIOCC#PF49)	<i>Acremonium</i> sp., Ascomycota (AY392130)	99	Onychomyces
(NIOCC#Y1)	<i>Rhodotorula cassicola</i> , Basidiomycota (AB055191)	99	Thailand
(NIOCC#Y2)	<i>Sporidiobolus johnsonii</i> , Basidiomycota (L22261)	100	Dead pustule of <i>Phragmidium rubi-idaei</i> , on leaf of <i>Rubus</i>
(NIOCC#Y3)	<i>Rhodospidium toruloides</i> , Basidiomycota (DQ832192)	100	Wood pulp from Coniferae, Sweden
(NIOCC#Y4)	<i>Sporidiobolus johnsonii</i> , Basidiomycota (L22261)	100	Dead pustule of <i>Phragmidium rubi-idaei</i> , on leaf of <i>Rubus</i>
(NIOCC#Y5)	<i>Sporidiobolus johnsonii</i> , Basidiomycota (L22261)	100	Dead pustule of <i>Phragmidium rubi-idaei</i> , on leaf of <i>Rubus</i>
(NIOCC#Y6)	<i>Sporidiobolus johnsonii</i> , Basidiomycota (L22261)	100	Dead pustule of <i>Phragmidium rubi-idaei</i> , on leaf of <i>Rubus</i>
(NIOCC#Y7)	<i>Rhodotorula cassicola</i> , Basidiomycota (AB055191)	99	Thailand
(NIOCC#Y8)	<i>Coniosporium perforans</i> , Ascomycota (EF137365)	100	Rocks
(NIOCC#Y9)	<i>Graphiola cylindrica</i> , Basidiomycota (D63929)	95	Not known
(NIOCC#Y10)	<i>Rhodotorula mucilaginoso</i> , Basidiomycota (EF218987)	100	Not known
(NIOCC#PY12)	<i>Cryptococcus vishniacii</i> , Basidiomycota (AB032657)	99	Culture collection
(NIOCC#PY13)	<i>Cryptococcus vishniacii</i> , Basidiomycota (AB032657)	99	Culture collection

Table 2.12 Phylogenetic affiliations of fungi and yeast isolated during cruise #ABP26 on the basis of ITS sequences

Isolate ID	Closest identified relative, Phylum (GenBank Accession no.)	% identity	Source of isolation of Closest relative
(NIOCC#F2)	<i>Penicillium citreonigrum</i> , Ascomycota (AY373908)	99	Dust
(NIOCC#F5)	<i>Cladosporium cladosporioides</i> , Ascomycota (EU497957)	100	Deep-sea
(NIOCC#F6)	<i>Aspergillus</i> sp., Ascomycota (EF672304)	99	<i>Coffea arabica</i>
(NIOCC#F8)	<i>Cladosporium cladosporioides</i> , Ascomycota (EF136373)	99	Rice wine, wheat Qu
(NIOCC#F13)	<i>Cladosporium</i> sp., Ascomycota (EF105367)	99	Toxic mold <i>Stachybotrys chartarum</i>
(NIOCC#F15)	Uncultured fungus clone, Eukaryota (DQ279836)	91	Deep-sea marine sediments in South China Sea
(NIOCC#F16)	<i>Exophiala spinifera</i> , Ascomycota (AM176734)	99	Sediment from deep sea of Pacific Ocean
(NIOCC#F21)	<i>Tilletiopsis albescens</i> , Basidiomycota (AB025695)	98	Culture collection, Tokyo
(NIOCC#F23)	Doubtful, Ascomycota	-	-
(NIOCC#F30)	Hypocreales sp., Ascomycota (DQ682584)	100	Coffee plants with the fungal entomopathogen <i>Beauveria bassiana</i>
(NIOCC#F33)	<i>Tilletiopsis albescens</i> , Basidiomycota (AB025695)	98	Culture collection, Tokyo
(NIOCC#F46)	<i>Exophiala xenobiotica</i> , Ascomycota (EF025407)	99	Fungus Testing Laboratory, Texas, US
(NIOCC#F47)	<i>Aspergillus caesiellus</i> , Ascomycota (AY373865)	100	Not known
(NIOCC#F48)	<i>Phialemonium dimorphosporum</i> , Ascomycota (DQ403199)	99	Lesions excised from <i>Mugil gyrans</i>

Table 2.12 contd.

Isolate ID	Closest identified relative, Phylum (GenBank Accession no.)	% identity	Source of isolation of Closest relative
(NIOCC#F50)	<i>Exophiala dermatitidis</i> , Ascomycota (EF568099)	100	Clinical sample
(NIOCC#PF49)	<i>Trichothecium roseum</i> , Ascomycota (EU622273)	100	Culture collection, china
(NIOCC#Y1)	<i>Sporobolomyces</i> sp., Basidiomycota (EU002893)	95	Peduncle endophyte
(NIOCC#Y2)	<i>Sporidiobolus salmonicolor</i> , Basidiomycota (AY015434)	100	Not known
(NIOCC#Y3)	<i>Rhodospodium toruloides</i> , Basidiomycota (AB073266)	99	Not known
(NIOCC#Y4)	<i>Sporobolomyces</i> sp., Basidiomycota (EU002893)	100	Coffee plants
(NIOCC#Y5)	<i>Rhodotorula</i> sp., Basidiomycota (AB025984)	99	Deep-sea environments around the northwest Pacific Ocean
(NIOCC#Y6)	<i>Sporidiobolus</i> sp., Basidiomycota (DQ317366)	100	Soils and historic wood from the Ross Sea Region of Antarctica
(NIOCC#Y7)	<i>Rhodotorula calyptogenae</i> , Basidiomycota (EU669878)	99	Seawater
(NIOCC#Y8)	<i>Sarcinomyces petricola</i> , Ascomycota (AJ244275)	98	Meristematic tissues
(NIOCC#Y9)	<i>Tilletiopsis oryzicola</i> , Basidiomycota (AB045708)	95	Leaves
(NIOCC#Y10)	<i>Rhodotorula mucilaginoso</i> , Basidiomycota (EU781664)	98	Stem from 5 year old tree growing in a subtropical monsoon climate at an elevation of 600-800m
(NIOCC#PY12)	<i>Cryptococcus albidosimilis</i> , Basidiomycota (AF145331)	100	Antarctica

(NIOCC#PY13)	<i>Rhodotorula mucilaginosa</i> , Basidiomycota (EU781664)	99	Stem from 5 year old tree growing in a subtropical monsoon climate at an elevation of 600-800m
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A total of 12 and 8 distinct species of filamentous and unicellular fungi respectively were identified in the present study. Using classical morphology-based identification system [Damare et al. \(2006\)](#) reported only 8 identified filamentous fungi and several unidentified sporulating and non-sporulating fungi. Therefore, although total number of fungi isolated in the present study was lower than that reported by [Damare et al. \(2006\)](#), the species diversity was comparatively higher.

Both sporulating as well as non-sporulating fungi could be identified by molecular-based identification system in the present study. However, insufficient database for ITS sequences also is one of the reasons for reduced diversity assessment ([Anderson et al, 2003a](#); [Zachow et al, 2009](#)). Using 18S and ITS sequences, all the fungi isolated during cruise #ABP26 were identified, sequences and cultures deposited in respective repositories, thus adding to the data base for fungi from extreme environment. Culture-based diversity assessments suffer from biases in isolate recovery, unknown culture conditions, competitive interference and over-growth by other fungi ([Panebianco et al, 2002](#); [Pang and Mitchell, 2005](#)). These factors might have played a role in underestimating fungal diversity in deep-sea sediments in the present study.

2.3.4 Diversity of fungi during cruise #ABP38

During this cruise the fungal isolates were classified only based on sequencing a region within the 18S rRNA gene which is generally considered to yield good comparison with the existing database ([Guarro et al, 1999](#)). Although the taxonomic resolution of 18S rDNA may not always be sufficient to identify fungal species and strains, this gene is conserved enough to allow comparison across a wide range of fungal taxa. The results obtained for the identification of fungal communities during cruise #ABP26 were found to be more reliable using 18S than ITS primers for SSU rDNA. Therefore, only 18S region was selected for the identification of fungi isolated during cruise #ABP38. During this cruise out of the total 76 sediment samples used for isolation, nineteen fungal cultures were recovered. The percentage frequency of isolation is

~25%, which is comparatively higher than the cruise #ABP26. The probable reason for the above may be the use of two more media i.e. PDA and SDA during cruise #ABP38 for isolation methods. A total of six and two fungal cultures were isolated from the PDA and SDA media plates respectively after incubation of sediment samples using various isolation techniques (Table 2.6). However the species richness was found to be lower than that found in cruise #ABP26 (Tables 2.7 and 2.8). The two stations sampled during this cruise were among the 20 stations of the cruise #ABP26 having same geographical locations (Tables 2.1 and 2.2). During cruise #ABP26 only one culture was isolated from each of these two stations (Table 2.5). However, a total of 19 fungi could be isolated from the same stations during cruise #ABP38 suggesting temporal variation playing a significant role on diversity of fungi in these sediments. The isolation of *Sagenomella* sp., *Aspergillus* sp., *Penicillium* sp. and *Cladosporium* sp. during both the cruises suggests their frequent occurrence and larger distribution in these deep-sea sediments. These sporulating species except *Sagenomella* have also been reported to be present in deep-sea sediments in previous studies (Damare et al, 2006; Burgaud et al, 2009). The recovery of *Aspergillus*, *Penicillium* and *Cladosporium* sp. during both the cruises, suggested them to be natural inhabitant of deep-sea. These species may have a role in deep-sea environments in spite of not being indigenous and without exhibiting optimal growth conditions. Occurrence of fungi in deep-sea sediments from the Chagos Trench in the Indian Ocean at 5000 m depth was reported (Raghukumar et al, 2004). In this study, *Aspergillus* sp., a mitosporic fungus was isolated as a dominant form from a 450 cm long sediment core. Spores of *Aspergillus sydowii* from these core samples germinated and grew at elevated hydrostatic pressures and low temperatures. It has been hypothesized that such spores 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). *Sagenomella* species has been reported as a pathogenic fungus in terrestrial environments causing infection

in dogs (Gene et al, 2003) suggesting its possible role as pathogen of deep-sea organisms.

Two of the isolates (NIOCC#F68, #Y14) showing homology to *Hortaea* sp. were isolated. This species has been reported to be potential pathogenic form of mussel from hydrothermal vent site (Van Dover et al, 2007). Also, this black yeast-like fungus has been characterized as halophilic or extremely halotolerant in different studies (Gunde-Cimerman et al, 2000; Kogej et al, 2006). Two interesting fungi isolated during this cruise were *Trametes* (NIOCC#F53) and *Cerrena* sp. (NIOCC#F60, #61, #66 and #67) as these have been reported earlier as potential source of lignin-degrading enzymes (Cullen, 1997; D'Souza-Ticlo et al, 2006; Verma et al, 2010). This is the first report of isolation of these two species from deep-sea sediments. The white-rot fungus *Cerrena unicolor* was found to cause canker rot on two northern hardwood tree species, sugar maple and paper birch (Enebak and Blanchette, 1989). Later this species was found to be an efficient producer of laccase, a well known lignin degrading enzyme (Leonowicz et al, 1997; Rogalski et al, 1999). These two species have a powerful extracellular enzymatic complex, able to de-polymerize this aromatic polymer i.e. lignin into lower molecular weight compounds (Rogalski et al, 1999; Kim et al, 2002; Bajpai, 2004). Degradation of lignin and, more specifically, the regulation of the production of individual ligninolytic enzymes, is a complex phenomenon (Liers et al, 2006) that needs to be studied, in order to find out the correct way to obtain efficient biotechnological applications. The isolation of such ligninolytic species from extreme environment of deep-sea suggests their significant role in ecological cycles.

Two fungi i.e., NIOCC#F51 and NIOCC#F57, identified as *Nigrospora* and *Pleospora* sp. respectively have also been isolated from marine sponge (Ding et al, 2010). These species have been reported to be marine in nature, producing useful antimicrobial compounds and thus playing important ecological role in chemical defense in sponges (Bugni and Ireland 2004). Among other cultures, *Chaetomium* sp. has been reported earlier as terrestrial pathogenic or symbiotic forms, colonizing plants roots (Violi et al,

2007). Isolation of other terrestrial species such as *Eurotium* and *Ascotricha* sp. from deep-sea sediments of the Central Indian Basin suggests their transport and gradual adaptation under such extreme conditions. In general, these two species have been known to exhibit pathogenic properties in terrestrial plants and animals (Valldosera and Guarro, 1988; Roussel et al, 2010; Popov et al, 2010). In addition, *Eurotium* species has also been reported to produce secondary metabolites having useful chemical and biological activities (Slack et al, 2009) suggesting the possibility of this species being an efficient source of marine bioactive compounds.

All the fungal genotypes obtained during this cruise showed >98% similarity with the existing fungal sequences in the database except NIOCC#F55 and #Y14. These sequences showed a similarity of <97 % with their closest match suggesting their possibility of being novel forms of *Aspergillus* and *Hortaea* sp. respectively (Table 2.8). Their physiological characteristics such as GC content, fatty acid profiles, RFLP and Randomly amplified polymorphic DNA (RAPD) patterns needs to be analyzed in order to prove them as novel marine forms of the above species. Deep-sea fungi were found to generate different RFLP patterns compared to their terrestrial counterparts after digesting with similar restriction enzymes (Damare et al, 2006) suggesting them to be different from the terrestrial isolates.. Randomly amplified polymorphic DNA (Williams et al, 1990) is also used to construct RFLP maps that are useful in genetic analysis and may be helpful for population studies at the intra-specific level (Goodwin and Annis, 1991; Guthrie et al, 1992; Ouellet and Seifert, 1993).

The fungal diversity obtained using culture-dependent approach during both the cruises was found to be low. Therefore, in order to isolate hidden diverse forms of fungal cultures, new culture media and methodologies need to be used as is done for bacterial diversity studies. This study is the first report of molecular phylogenetics to assess the diversity of culturable fungi in deep-sea sediments. Molecular identification facilitated in identifying non-sporulating species which would not have been possible by classical taxonomic method. Low diversity of culturable fungi might also be due to the

failure to culture the yet-uncultured fungi, a problem similar to the bacterial diversity in deep-sea environment and many other environments. Direct sequencing of fungal genotypes from environmental samples would improve our assessment of fungal diversity in deep seas.

In the subsequent chapter, diversity of fungi is assessed applying a culture-independent approach using different fungal as well as eukaryotic primers.

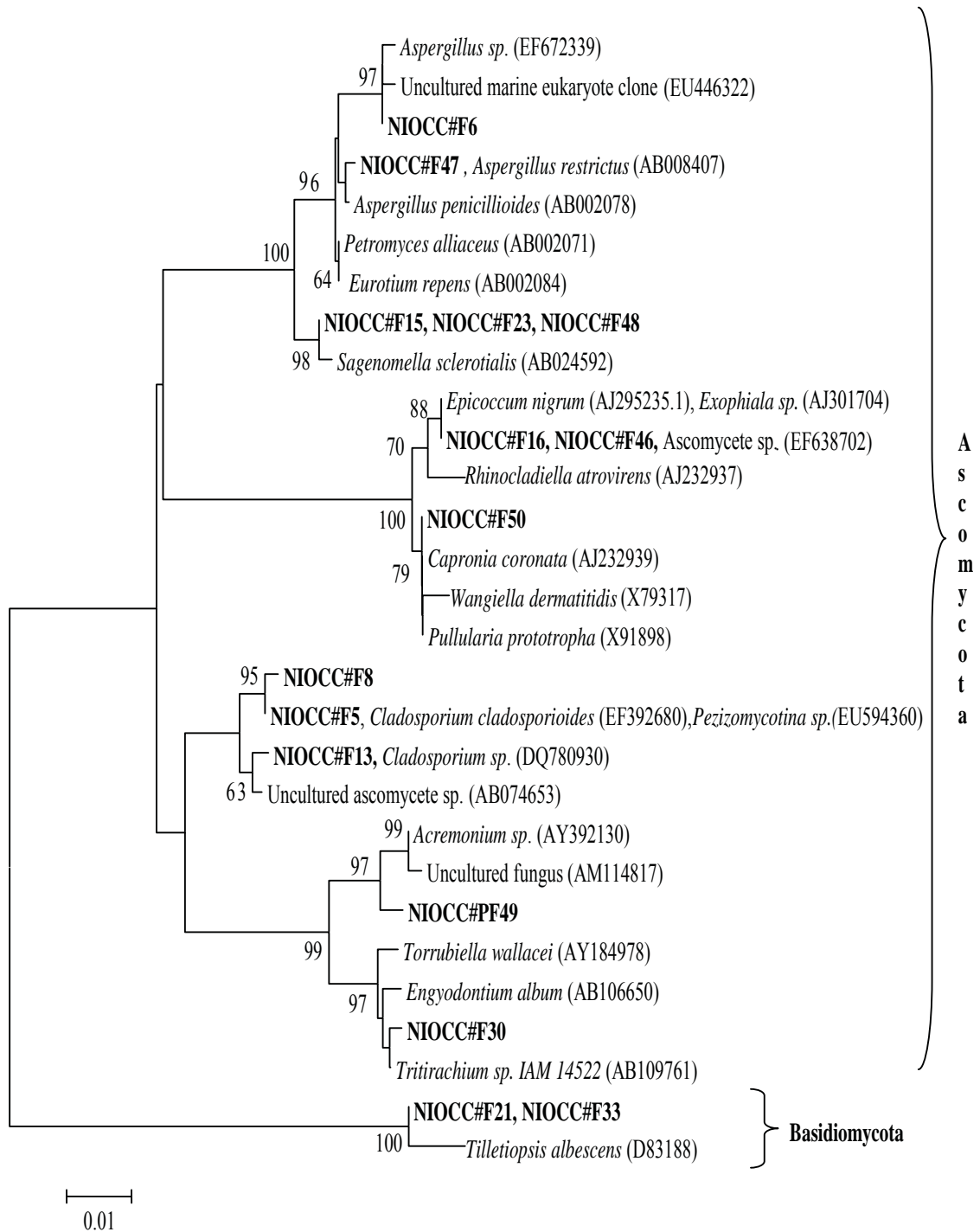


Fig 2.6 Neighbor-joining phylogenetic tree for mycelial fungi isolated during cruise #ABP26 based on rDNA-18S sequences (~1100 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).

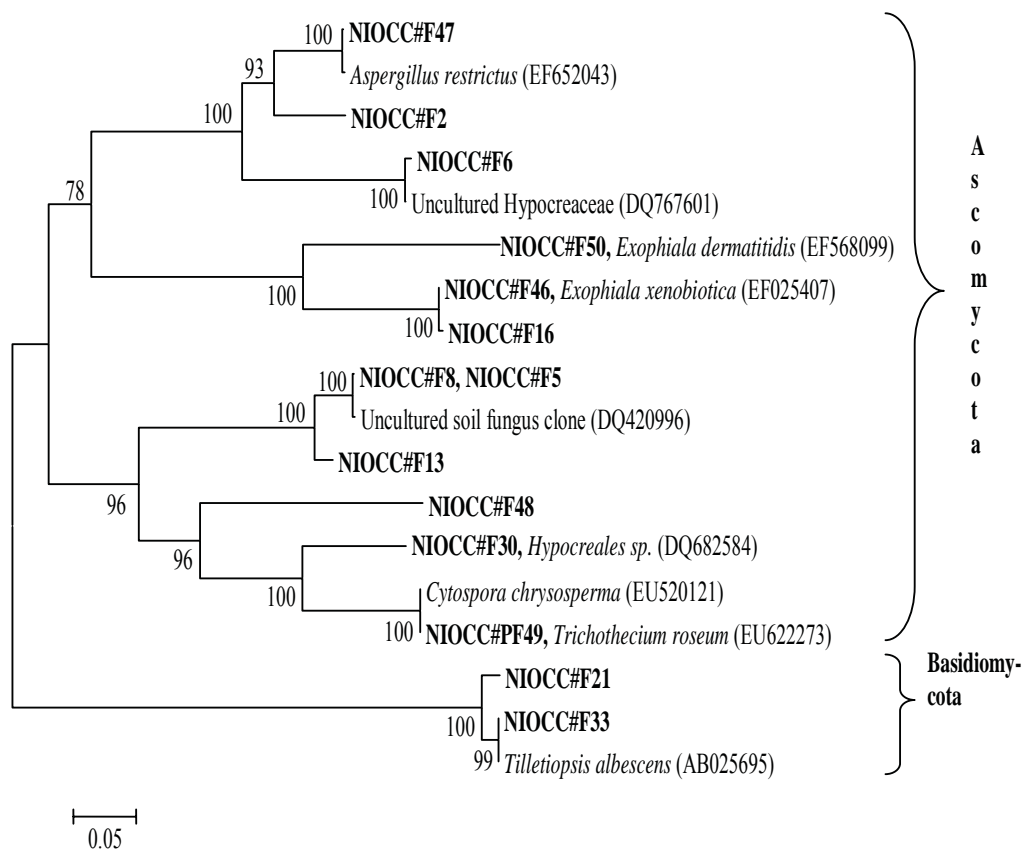


Fig 2.7 Neighbor-joining phylogenetic tree for mycelial fungi isolated during cruise #ABP26 based on rDNA-ITS sequences (~600 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).

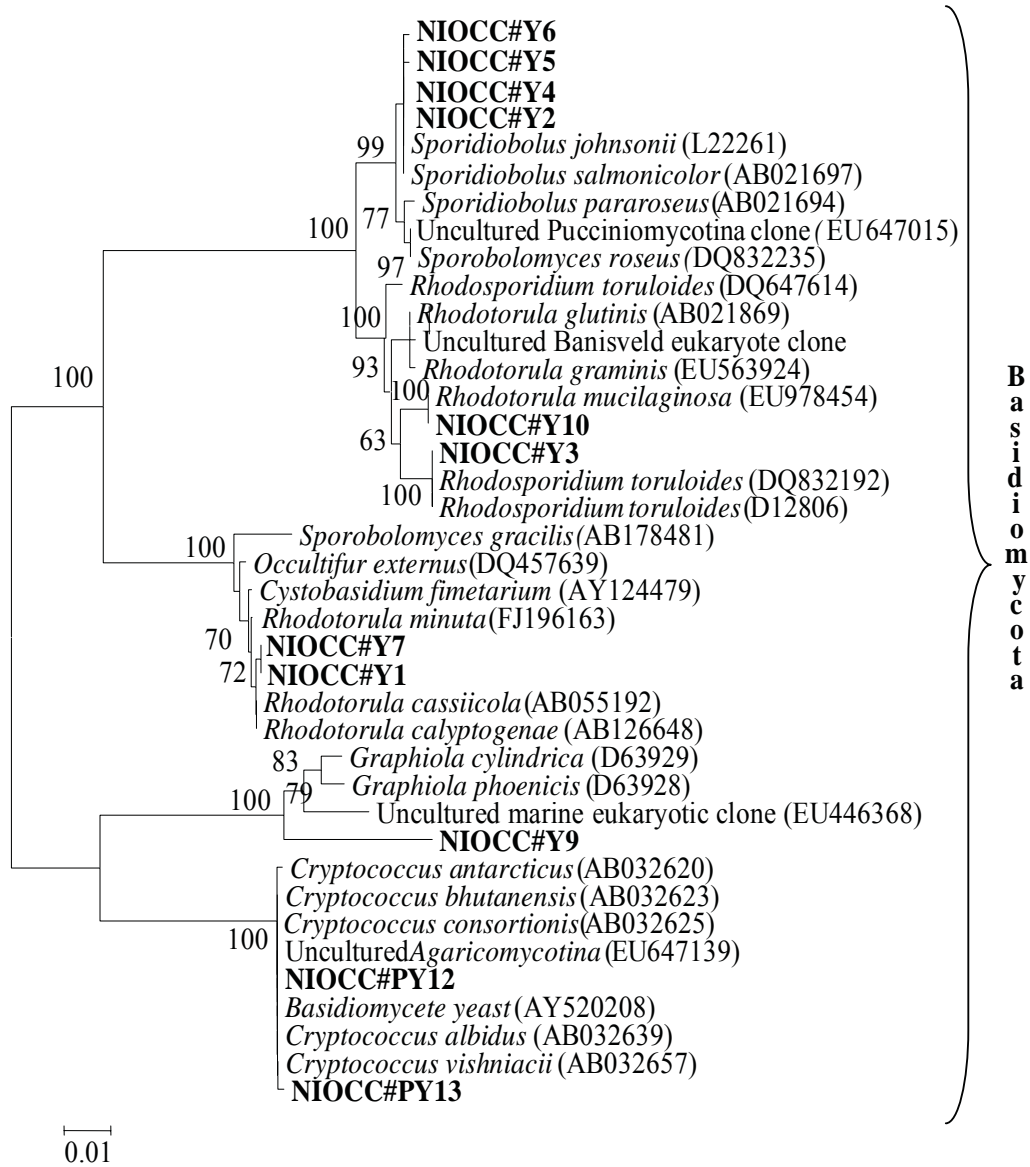


Fig 2.8 Neighbor-joining phylogenetic tree for yeasts isolated during cruise #ABP26 based on rDNA-18S sequences (~1100 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).

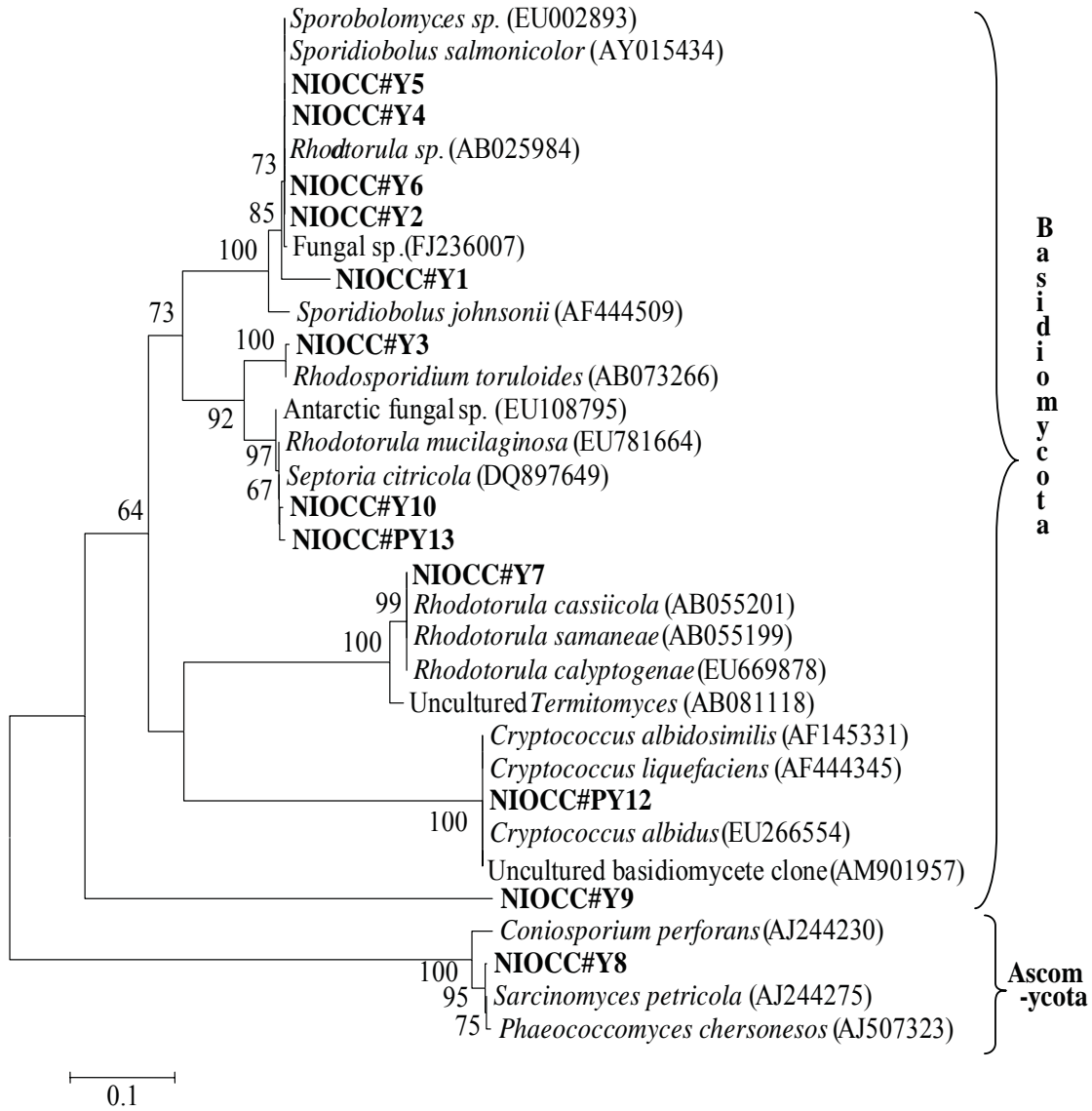


Fig 2.9 Neighbor-joining phylogenetic tree for yeasts isolated during cruise #ABP26 based on rDNA-ITS sequences (~600 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).

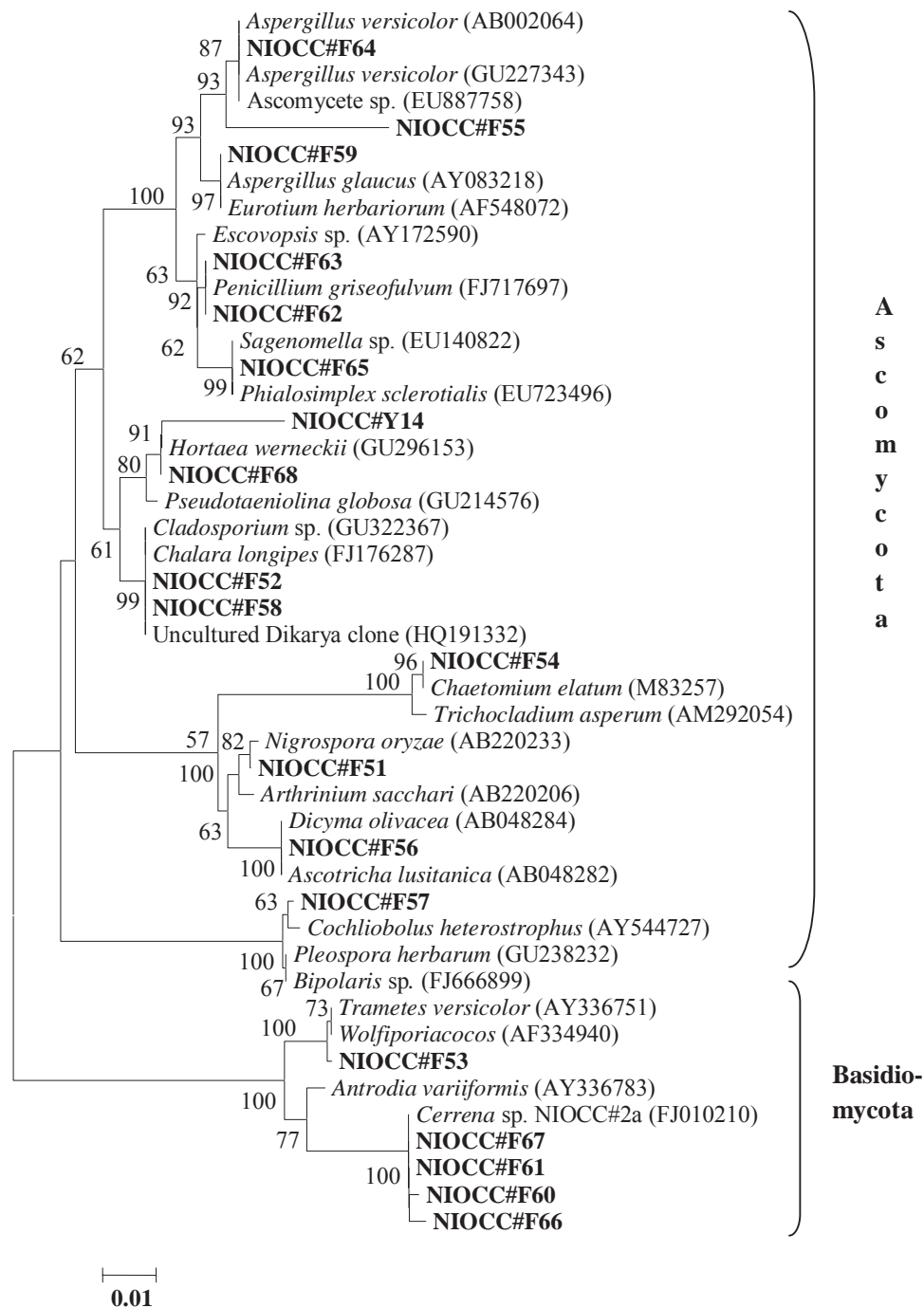


Fig 2.10 Neighbor-joining phylogenetic tree for mycelial fungi isolated during cruise #ABP38 based on rDNA-18S sequences (~1100 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).