

## Chapter 3

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*Culturable and unculturable diversity of fungi from the  
denitrification zones of the Arabian Sea.*

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### 3.1 Introduction

Fungi live in very diverse habitats and display specialized adaptations in structure and biochemistry to thrive in each of these habitats (Maheshwari, 2005). Some of the unique habitats they are known to occupy include the outer stratosphere (Wainwright *et al.*, 2003; ISRO News Letter, 2009), the psychrophilic fungi inhabiting the cold arctic and antarctic regions (Robinson, 2001), deep sea sediments (Raghukumar *et al.*, 2004), those living in extreme high temperatures ranging upto 62 °C (Maheshwari *et al.*, 2000) and the xerotolerant fungi living on the bark of trees and on dead, dry wood stumps (Petrovič *et al.*, 2006).

Presence of fungi in the marine environment was first studied in 1930 by Hönkh and Sparrow, followed by Barghoorn and Linder (1944). Detailed study on the diversity and physiology of fungi living in marine environment was carried out by Kohlmeyer and Kohlmeyer (1979). Following this, research on the fungal diversity in the marine habitats was carried out to understand their role in the marine ecosystems such as the detritus of salt marsh grass (Gessner, 1982), in mangrove detritus (Sathe-Pathak *et al.*, 1993) and from plant detritus in the coastal regions (Newell, 1996). Studies of fungi was also carried from deep sea environments, during which fungi was isolated from 4450 m depth (Roth *et al.*, 1964) and the barotolerance in fungi isolated from deep sea habitat was studied (Raghukumar *et al.*, 1992). These studies are based on the classical, cultivation-dependent, isolation and enumeration technique. Traditional work has been carried out to study the fungal communities colonizing the floating and submerged wood in the marine system and those associated with algae and marsh plants (Jones, 1985; Jones *et al.*, 1998). These studies of the fungal diversity are very incomplete because of the inability of many fungi to grow and sporulate in the culture media (Jones & Hyde, 2002). In spite of the short comings in the cultivation-based diversity

assessment, about 800 species of obligate marine fungi could be isolated so far (Hyde *et al.*, 2000).

Advancements in the field of molecular ecology have revolutionized the study of fungal diversity. Molecular biological techniques, particularly the analysis of nucleotide sequences and phylogenetic approaches has greatly influenced the fungal systematics (Guarro *et al.*, 1999). Molecular tools such as RAPD and T-RFLP analysis of the amplified ITS and 18S rDNA region were used to identify the fungal cultures isolated from the marine ecosystem (Roberts *et al.*, 1996, Buchan *et al.*, 2002). These studies showed that the diversity of the marine fungi was more extensive than documented so far (Pang & Mitchell, 2005). Identification of the isolated fungal cultures using molecular markers was not without drawbacks, as all the fungal cultures could not be retrieved and maintained in laboratory conditions.

The introduction *in-situ* community profiling without the need to isolate cultures brought to light numerous hitherto undescribed organisms from various environments. Some of the groups described only based on environmental DNA analysis are presumably of high relevance for a variety of research disciplines like ecology, evolution, physiology and biogeochemistry (Epstein & Lopez-Garcia, 2007; Lopez-Garcia & Moreira, 2008). The well established phylogenetic analysis of prokaryotic SSU rRNA genes isolated from various environments has expanded our understanding of the diversity and ecological role of bacteria in the ecosystems (Giovannoni & Rappé, 2000; Freitag & Prosser, 2003; Molina *et al.*, 2007; Lehours *et al.*, 2007). In contrast to prokaryotes, molecular diversity surveys targeting microbial eukaryotes started only in recent times (Moon-van der Stay *et al.*, 2001; Lopez-Garcia *et al.*, 2001; Edgcomb *et al.*, 2002; Dawson & Pace, 2002).

A strong focus of eukaryote diversity research has been on a variety of environments such as shallow water anoxic sediments (Dawson & Pace, 2002; Stoeck *et al.*, 2003), hydrothermal vent systems (Edgcomb *et al.* 2002, Lopez-Garcia *et al.*, 2003, 2007), oxygen-depleted deep basins (Stoeck *et al.*, 2003b, 2006), anoxic fjords (Behnke *et al.*, 2006, Stoeck *et al.*, 2007, Zuendorf *et al.*, 2006), saline meromictic lakes (Takishita *et al.*, 2007a), unique deep sea habitats (Takishita *et al.*, 2005, Takishita *et al.*, 2007b), hypersaline systems (Alexander *et al.*, 2009), a variety of oxygen-depleted freshwater environments (Dawson and Pace 2002, Luo *et al.* 2005, Slapeta *et al.* 2005) and terrestrial habitats (Schadt, *et al.* 2003). These studies using eukaryote specific primers has revealed a much larger diversity comprising of Green algae, Ciliates, Cercomonads, Stramenopiles, Radiolarians and Ophisthokonts (Dawson & Pace, 2002, Stoeck & Epstein, 2003; Stoeck *et al.*, 2003, Takishita *et al.*, 2005; Lou *et al.*, 2005; Bhenke *et al.*, 2006; Alexander *et al.*, 2009). Most, if not all these studies have shown that fungal representatives constitute a large proportion in most of the environmental libraries.

The fungal sequences obtained in many instances, especially during the initial studies were restricted to known and well described taxa (Dawson and Pace 2002, Stoeck and Epstein 2003, Brad *et al.*, 2007). Community patterns from the supersulphidic anoxic Fijords of Norway (Bhenke *et al.*, 2006), anoxic Mariager Fjord in Denmark (Zunderof *et al.*, 2006), hyper saline anoxic basin in the Mediterranean etc. (Alexander *et al.*, 2009) show that the fungal diversity is very low and a multiple PCR approach too does not improve the fungal diversity from the previously studied Cariaco Basin (Stoeck *et al.*, 2006). However there is a possibility that this could be due to the other dominant groups present in the sampling site that might have masked the presence of fungal sequences.

Recent reports of novel fungal lineages have been identified from anoxic, deep sea environments (Takishita *et al.*, 2005, 2007a, 2007b, Bass *et al.*, 2007). Survey in the Lost City, hydrothermal vent system in the Mid-Atlantic ridge has also reported a unique clade “hydrothermal and/or anaerobic fungal group” (Lopez-Garcia *et al.*, 2007). With increasing group specific studies based on 18S rRNA and internal transcribed spacer (ITS) rRNA gene sequences, novel clusters of fungal sequences from diversified locations such as the tundra regions and temperate forest soil (Schadt, *et al.* 2003, O’Brien *et al.*, 2005) are reported.

Several of these environmental fungal sequences are highly divergent from previously deposited sequences of known fungi, indicating, that oxygen-depleted aquatic habitats may hold great promise for the discovery of novel fungi. Culture-independent techniques offer an alternative and a more detailed insight into the diversity, but it may not be an exclusive method to monitor the communities (Pang & Mitchell, 2005). Incorporation of molecular information into diversity studies and defining the fungal communities can provide a near complete picture of the ecological functioning. Hence in this study fungal specific PCR primers have been used to study the extent of fungal diversity in combination with cultivation based approaches.

## **3.2 Materials and methods**

### **3.2.1 Diversity of culturable fungi**

Fungal colonies were isolated from the sediments collected from the three coastal stations and five open ocean slope stations by particle plating technique. Sampling procedure and the oceanographic instruments

used for the collection are described in Chapter 2 (Fig. 2.4). Fungal colonies were also isolated from the oxygen depleted water and the overlying water (OLW) of the sediment core collected from four open ocean stations (SSt-500, SSt-700, SSt-800 and SSt-1100). Water was collected approximately 1 m above the sediment bed using the Niskin sampler. About 100ml of water was filtered on sterile Durapore filters (0.45  $\mu\text{m}$ , Millipore (India) Pvt Ltd, Bangalore). The filter papers were plated on malt extract agar (MEA), Corn meal agar (CMA), Czapek Dox agar (CDA) and Brewer's agar (BA). All the media (HiMedia Pvt. Ltd., India) were used at 1/5 strength to discourage the growth of fast growing fungi and the plates were fortified with antibiotics as described in section 2.2.3. Fungal colonies appearing after incubation at 30°C for 15 – 20 days were picked using sterile needles and each of them was designated with a unique isolate number. OLW was collected from top of the sediment core in clean sterile 15 mm teflon tubes. Fungal colonies were isolated from OLW following the similar method which was used for isolating fungal colonies from water as described above. Fungal cultures were also isolated from the coral reef sand collected from Lakshwadeep sampling station (LSt\_03) by particle plating technique.

Individual fungal colonies identified as unique colonies based on the colony morphology were catalogued as separate isolates. Fungal colonies isolated from the three coastal stations, SSt-200 station and LSt\_03 are given unique isolate numbers (# 1, # 2, etc.). Fungal colonies isolated from the SSt stations (SSt-500, SSt-700, SSt-800 and SSt-1100) were designated with a suffix YK-1, YK-2, YK-3 etc., representing the research vessel RV-Yokosuka used for sampling. The distribution of the fungal cultures obtained is represented as percentage frequency.

The occurrence of the total fungal species from each sampling site and across the changing oxygen concentrations were studied based on

the species richness and Pielou's evenness values. The diversity of fungi from the coastal stations (CSt) during the field trips with the changing oxygen levels and in the oxygen depleted slope stations (SSt) was assessed based on the Shannon's and Simpson's diversity index. The statistical analysis was carried out using the software PRIMER 5 (Clarke & Gorley, 2001).

### **3.2.2 Isolation by enrichment culturing**

Isolation of cultures by enrichment method was carried out from the sediment collected during the anoxic season (Ft-1) at the coastal station (CSt-26). Approximately, 5 g of sediment samples were incubated in airtight glass bottles in 80 ml of enrichment media that consisted only of 10 mM sodium nitrate solution prepared in artificial seawater and were supplemented with antibiotics to inhibit the growth of bacteria. The medium was then flushed with nitrogen gas and incubated for 30 days. At the end of the incubation period, fungal colonies were isolated from these sediments by particle plating as described in section 2.2.3.

### **3.2.3 Identification of culturable fungi**

The sporulating cultures isolated from the different sampling stations were identified based on the taxonomic keys described in Domsch et al., (1980) or based on the partial (~ 1600 bp) 18S rDNA gene sequence. For the 18S rDNA sequence analysis each fungal culture was grown for 5 days at room temperature in 50 ml of malt extract broth (MEB, HiMedia Pvt. Ltd., India) in 250-ml Erlenmeyer flask. The total genomic DNA was extracted from the freeze-dried mycelial mats of each culture

using a high salt concentration extraction buffer. The components of the buffer were 100 mM Tris HCl (pH 8), 100 mM sodium phosphate buffer (pH 8), 1.5 M NaCl, 100 mM EDTA (pH 8.) and 1 % CTAB. Total genomic DNA was extracted using the buffer followed by chloroform-phenol extraction and precipitation with isopropanol as described previously (Stoeck & Epstein, 2003). The 18S region of the rDNA was amplified using fungal specific primers NS1 and FR1 (Table 3.2). The 50  $\mu$ l PCR reaction included 1x HotStart Taq buffer (Qiagen, Hildesheim, Germany), in a reaction buffer provided by the company containing Tris-Cl, KCl,  $(\text{NH}_4)_2\text{SO}_4$ ], 15 mM  $\text{MgCl}_2$  (pH 8.7), 200  $\mu$ M concentrations of each deoxynucleoside triphosphate (dNTPs), and 0.5  $\mu$ M concentrations of each oligonucleotide primer and the final volume was adjusted to 50  $\mu$ l with sterile water. PCR was performed using initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 2 min, with a final extension at 72°C for 7 min with Hot Start Taq DNA polymerase (Qiagen, Valencia, CA). The PCR products were cloned separately for each fungal culture using the TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids were isolated from positive overnight cultures using the Fast Plasmid Mini Prep kit (Eppendorf AG, Hamburg, Germany). One representative clone for each culture was sequenced bidirectionally (M13 sequencing primers) by MWG-Biotech on an Applied Biosystems (ABI) 3730 DNA Stretch Sequencer, with the XL Upgrade and the ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit. Sequences were included in phylogenetic analyses as described below. GenBank accession numbers of sequences deposited so far from cultured isolates are GQ120154- GQ120179.

### 3.2.4 Diversity of unculturable fungi

Environmental clone libraries were constructed from samples collected from five sampling sites (Table 3.1). Total genomic DNA from the sediment or water filters were extracted using a high salt concentration extraction buffer containing 100 mM Tris HCl (pH 8), 100 mM sodium phosphate buffer (pH 8), 1.5 M NaCl, 100 mM EDTA (pH 8.) and 1 % CTAB, followed by chloroform-phenol extraction and precipitation with isopropanol as described previously (Stoeck & Epstein, 2003).

**Table 3.1** Sampling sites for construction of five environmental clone libraries

Sampling site	Material	Quantity	DO( $\mu$ M)
CSt-26 (Ft-1)	sediment	~ 500 mg	~ 0
CSt-26 (Ft-3)	sediment	~ 500 mg	118.4
SSt-200	sediment	~ 500 mg	4.28
SSt-200	water	~ 5 l	4.28
LSt-03	sediment	~ 500 mg	93.74

DO = dissolved oxygen

Amplification of 18S rDNA was carried out using three different primer sets (Table 3.2). The primer sets were chosen based on their specificity and adequate length of amplified fragments in order to carry out robust phylogenetic analyses. The first primer set (Fung1) consisted of the fungal specific NS1 and FR1 primers resulting in ~ 1650 bp-fragments. According to homology searches this primer set has only moderate specificity and co-amplifies a range of non-fungal eukaryote genes like Metazoa, Cercozoa, Viridiplantae, Alveolata, Centrohelioczoa and Bangiophyceae

(Pang & Mitchell, 2005). The second primer set (Fung2) included the fungal specific UF1 and S3 primers amplifying ~ 900 bp-fragments. This primer set has relatively high fungal specificity and previously amplified only few non-target taxa (green algae and *Pseudomonas*) from maritime Antarctic soil samples (Malosso *et al.*, 2006). We also used the universal eukaryote primer pair EukA and EukB, routinely used in environmental eukaryote diversity surveys (Euringer & Lueders, 2008; Marshall *et al.*, 2008; Stoeck *et al.*, 2006; Massana *et al.*, 2004) that amplifies nearly the full-length of the 18S rDNA (Table 3.2) of a wide range of higher eukaryote taxon groups (Medlin *et al.*, 1988).

**Table 3.2** Primer sets used in this study to amplify 18S rDNA sequences from genomic environmental DNA

Primer set	Primers	Primer Sequence (5' -3')	Reference
<b>Fung1</b>	NS1	GTA GTC ATA TGC TTG TCT C	Vainio & Hantula,2000
	FR1	AIC CAT TCA ATC GGT AIT	Gomes <i>et al.</i> , 2003
<b>Fung2</b>	UF1	CGA ATC GCA TGG CCT TG	Kappe <i>et al.</i> , 1996
	S3	AGT CAA ATT AAG CCG CAG	Maiwald <i>et al.</i> , 1994
<b>EukAB</b>	EukA	AAC CTG GTT GAT CCT GCC AGT	Medlin <i>et al.</i> , 1988
	EukB	TGA TCC TTC TGC AGG TTC ACC TAC	Medlin <i>et al.</i> , 1988

Amplicons were ligated into pGEM-T vector and transformed into *E. coli* cells (TOP 10 strain) using Invitrogen's TA-cloning kit as described above. For each library and primer set we selected nearly 100 positively screened colonies (blue-white screening) for overnight growth and plasmid extraction using the 96 well Directprep Kit (Qiagen, Valencia, CA). The presence of 18S rDNA inserts was confirmed by the standard M13-PCR

amplification of extracted plasmids. Between 200 and 400 ng of amplification products with expected sizes were digested with restriction enzyme *Hae III* (New England Biolabs, USA) because its recognition site GGCC is reported to have a relatively high frequency of occurrence in the genomic DNA compared to many other enzymes. Digestion was carried out with 7.5 U enzymes for 60 min at 37°C, followed by an inactivation step for 20 min at 80°C. The resulting bands were separated by electrophoresis in a 2.5% low-melting point agarose gel at 80 V for 2 to 3 h. Clones with identical amplified ribosomal DNA restriction fragment analysis (ARDRA) patterns were considered members of the same operational taxonomic unit (OTU). At least one clone of each OTU was sequenced bidirectionally using M13 forward and reverse primers at MWG-Biotech as described above.

Sequences of each individual primer set were grouped separately into OTUs based on a 99.0% sequence similarity cutoff value using DOTUR (Schloss & Handelsman, 2005). ARDRA patterns were then assigned to their respective sequences. The sequences were checked for chimeras using the Bellerophon Chimera Check program and the Check\_Chimera utility (Ribosomal Database Project (Cole *et al.*, 2003), as well as partial treeing analysis (Robison-Cox *et al.*, 1995). Putative chimeras and low quality reads were removed prior to subsequent sequence analyses. Similarities between two sequences were calculated using a custom program (PairAlign) provided by M. Nebel (University of Kaiserslautern), which employs IUB matrix-based pairwise alignments. These environmental sequences have been deposited in Genbank under the accession numbers GQ120105- GQ120153.

### 3.2.5 Phylogenetic analyses

Environmental 18S rDNA gene sequences initially were compared to those in GenBank using BLAST analysis to determine their approximate phylogenetic affiliation. Sequences of environmental clones together with their closest GenBank cultured and uncultured matches were aligned using the ARB FastAligner utility. Alignments were manually refined using phylogenetically conserved secondary structures. The conserved and unambiguously aligned positions were used in subsequent phylogenetic analyses. Evolutionary distance analyses under maximum likelihood criteria were carried out in PAUP\* v4.0b8 (Swofford, 2002), with all characters equally weighted and unordered. The TBR heuristic option was used to search tree space, running ten random additions with the MulTree option on. The evolutionary model that best fit each of our aligned data sets was chosen among 56 possible models using Modeltest (Posada & Crandall, 2001). Support for the evolutionary distance analyses under Maximum Likelihood came from 1000 bootstrap replicates using heuristic searches. Bayesian analyses were carried out using MrBayes v3.2.1 (Huelsenbeck & Ronquist, 2001) with posterior probability support values calculated using four chains/two runs and running 10 million generations for each alignment. Trees were sampled every 1000th generation. The first 25% of sampled trees were considered 'burn-in' trees and were discarded. A 50% majority rule consensus of the remaining trees was used to calculate posterior probability values. The hLTR in MrModeltest v2 was used to estimate the best fitting evolutionary model and based on this GTR+I+G was used for analysis.

### 3.2.6 Statistical analyses

The program package SPADE (Chao & Shen, 2003-2005) was used to calculate the Jaccard index as a measure of similarity between two communities based on abundance of environmental fungal OTUs ( $J_{abundance}$ ). An unweighted pair group mean average (UPGMA) cluster analyses based on  $J_{abundance}$  data was performed using the Cluster analysis module of Statistica v. 7 (StatSoft, Tulsa OK). A Venn diagram was constructed to display the overlap in OTU composition between the different primer sets using VENNY (Oliveros, 2007).

Species accumulation curves and Abundance-based Coverage Estimator (ACE) of species richness for all the clone libraries were calculated in EstimateS (Colwell, 2005).

## 3.3 Results

### 3.3.1 Diversity of culturable fungi

Fungal cultures could be isolated from the coastal stations during all the season by particle plating technique. Maximum fungal diversity was seen in the pre-monsoon season in April 06 during the field trip 5 (Table 3. 3). During Ft-7, in Dec 06 no culturable fungal colonies could be isolated, this may be due to faulty incubator when the temperature set at 30 °C increased upto 37- 40 °C and absence of recoverable fungi may not necessarily mean absence of fungi in the natural ecosystem during this season.

About 57 different fungal isolates were recovered from the coastal stations during the eight field trips to the three coastal stations. Among the mycelial fungi that formed CFUs, *Aspergillus* species showed the highest

**Table 3.3** List of cultures isolated from the sediments collected from the coastal stations (CSt) during the different field trips and their percentage frequency

Isolate number	Identification	Ft-1 (Oct 05)	Ft-2 (Nov 05)	Ft-3 (Jan 06)	Ft-4 (Mar 06)	Ft-5 (Apr 06)	Ft-6 (Oct 06)	Ft-7 (Dec 06)	Ft-8 (Feb 07)
# 1	<i>Aspergillus</i> sp.	28	3	—	37	22	—	—	—
# 2	<i>Aspergillus</i> sp.	6	13	—	9	3	—	—	—
# 4	<i>Isaria</i> sp.	0.5	—	—	—	—	—	—	—
# 6	<i>Aspergillus</i> sp.	2.5	—	—	—	—	—	—	—
# 8	<i>Aspergillus</i> sp.	5	—	—	—	—	—	—	—
# 9	<i>Ulospora bilgramii</i>	0.5	—	—	—	—	—	—	—
# 10	<i>Scolicobasidium</i> sp.	0.5	—	—	—	—	—	—	—
# 11	<i>Tritirachium</i> sp.	5	—	—	—	10	—	—	—
# 12	<i>Humicola</i> sp.	17	—	—	16	—	—	—	—
# 20	<i>Beauveria</i> sp.	0.5	—	—	—	—	—	—	—
# 21	<i>Aspergillus wentii</i>	0.5	—	—	1	2	—	—	—
# 24	<i>Cladosporium</i> sp.	22	—	—	—	—	—	—	—
# Th	Thraustochytrid colony	—	50	25	—	19	—	—	—
# 28	Yeast colony	—	3	—	—	—	—	—	—
# 30	<i>Microascus cirrosus</i>	—	3	—	—	—	—	—	—
# 31	<i>Myrothecium verrucaria</i>	—	3	—	—	—	—	—	—
# 31a	<i>Penicillium namyslowskii</i>	—	1	—	—	—	—	—	—
# 32	Cleistothecial form	—	1	—	—	—	—	—	—
# 33	<i>Aspergillus</i> sp.	—	1	—	—	—	—	—	—
# 35	<i>Aspergillus penicillioides</i>	—	—	9	—	—	—	—	—
# 36	<i>Aspergillus penicillioides</i>	—	—	9	—	—	—	—	—

Table 3.3 contd.

Isolate number	Identification	Ft-1 (Oct 05)	Ft-2 (Nov 05)	Ft-3 (Jan 06)	Ft-4 (Mar 06)	Ft-5 (Apr 06)	Ft-6 (Oct 06)	Ft-7 (Dec 06)	Ft-8 (Feb 07)
# 41	<i>Penicillium</i> sp.	—	—	5	—	—	—	—	—
# 46	Unidentified mycelial fungi	—	—	6	—	2	—	—	—
# 50	<i>Aspergillus</i> sp.	—	—	6	—	—	—	—	—
# 42-y	Yeast colony	—	—	1	—	—	—	—	—
# 43-y	Yeast colony	—	—	3	—	—	—	—	—
# 40	<i>Aspergillus candidus</i>	—	—	3	—	—	—	—	—
# 44	<i>Ermodothis angulata</i>	—	—	1	—	—	—	—	—
# 45	Unidentified mycelial form	—	—	14	—	—	—	—	—
# 51	Yeast colony	—	—	—	17	—	—	—	—
# 57 a	Yeast colony (orange)	—	—	—	1	—	—	—	—
# 57 b	White filamentous yeast	—	—	—	1	—	—	—	—
# 52 a	non-sporulating fungal colony	—	—	—	3	5	—	—	—
# 53	Ascomycetes with ascocarps	—	—	—	1	—	—	—	—
# 54	<i>Geosmithia putterillii</i>	—	—	—	2	—	—	—	—
# 55	Unidentified yeast colony	—	—	—	2	—	—	—	—
# 56	<i>Aspergillus</i> sp.	—	—	—	1	—	—	—	—
# 65a	<i>Cladosporium</i> sp.	—	—	—	—	2	—	—	—
# 65b	<i>Aspergillus</i> sp.,	—	—	—	—	2	—	—	—
# 66	Unidentified mycelial form	—	—	—	—	2	—	—	—
# 68	<i>Aspergillus</i> sp.	—	—	—	—	2	—	—	—
# 67	Unidentified mycelial fungi	—	—	—	—	5	—	—	—
# 60	Unidentified yeast	—	—	—	—	2	—	—	—
# 61	<i>Aspergillus</i> sp.	—	—	—	—	2	—	—	—

Table 3.3 contd.

Isolate number	Identification	Ft-1 (Oct 05)	Ft-2 (Nov 05)	Ft-3 (Jan 06)	Ft-4 (Mar 06)	Ft-5 (Apr 06)	Ft-6 (Oct 06)	Ft-7 (Dec 06)	Ft-8 (Feb 07)
# 62	<i>Aspergillus</i> sp.	—	—	—	—	3	—	—	—
# 71-Y	Filamentous yeast colony	—	—	—	—	—	1	—	—
# 72	<i>Aspergillus</i> sp.	—	—	—	—	—	1	—	—
# 74	Unidentified mycelial colony	—	—	—	—	—	1	—	—
# 75	mycelial fungi (pink)	—	—	—	—	—	1	—	—
# 76	Unidentified mycelial colony	—	—	—	—	—	1	—	—
# 73	Unidentified mycelial colony	—	—	—	—	—	5	—	—
# 77	non-sporulating fungal colony	—	—	—	—	—	5	—	—
# 100	Filamentous yeast colony	—	—	—	—	—	—	—	4
# 104	<i>Aspergillus</i> sp.	—	—	—	—	—	—	—	4
# 103	Orange yeast	—	—	—	—	—	—	—	4
# 101	Yeast colonies (white)	—	—	—	—	—	—	—	15
# 102	Filamentous yeast	—	—	—	—	—	—	—	18
	Unidentified mycelial fungi	12	22	17	8	19	84	0	55
	Total Species (S)	13	10	12	13	16	8	0	6
	Species richness (d)	2.6	2.0	2.4	2.6	3.2	1.5	0	1.1
	Pielou's evenness (J')	0.8	0.7	0.9	0.7	0.8	0.3	0	0.7
	Shannon's diversity index (H')	1.9	1.5	2.2	1.9	2.3	0.7	0	1.3
	Simpson's diversity index (1-λ)	0.8	0.7	0.9	0.8	0.9	0.3	0	0.6

**Table 3.4** List of cultures and their percentage frequency from the slope stations within the oxygen depleted region.

Isolate number	Closest named species / colony morphology	SSt-200			SSt-500			SSt-700			SSt-800			SSt-1100		
		wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed
# 90	clone LKM33	—	—	8	—	—	—	—	—	—	—	—	—	—	—	—
# 122	<i>Zasmidium cellare</i>	—	—	8	—	—	—	—	—	—	—	—	—	—	—	—
# 123	<i>Aspergillus versicolor</i>	—	—	8	—	—	—	—	—	—	—	—	—	—	—	—
# 132	<i>Tritirachium</i> sp.	—	—	8	—	—	—	—	—	—	—	—	—	—	—	—
# 129	<i>Cordyceps sinensis</i>	—	—	8	—	—	—	—	—	—	—	—	—	—	—	—
# 133	<i>Tritirachium</i> sp.	—	—	8	—	—	—	—	—	—	—	—	—	—	—	—
# 125	<i>Tritirachium</i> sp.	—	—	8	—	—	—	—	—	—	—	—	1	—	—	—
YK-1	Mycelial fungi (pink)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-2	Mycelial fungi (yellow)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-3	<i>Acremonium</i> sp. KR21-2	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
YK-4	White mycelial form	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—
YK-5	<i>Alternaria alternata</i>	—	—	—	—	—	—	—	—	—	—	1	—	—	12	—
YK-6A	Orange yeasts	—	—	—	—	—	—	—	—	—	50	—	—	—	—	—
YK-6B	Orange yeasts	—	—	—	—	—	—	—	—	—	50	—	—	—	—	—
YK-6C	<i>Rhodotorula graminis</i>	—	—	—	28	5	—	10	78	—	—	2	—	78	—	—
YK-6D	Yeast colonies (orange)	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
YK-6E	Yeast colonies (orange)	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
YK-6F	Yeast colonies (orange)	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
YK-6G	Yeast colonies (orange)	—	—	—	—	—	—	—	6	—	—	—	—	—	—	—
YK-6H	Yeast colonies (orange)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-7	Yeast colonies (brown)	—	—	—	—	—	—	5	—	—	—	—	—	—	—	—
YK-8B	<i>Pichia guilliermondii</i>	—	—	—	—	—	—	—	11	—	—	1	—	—	—	—
YK-8C	<i>Melanopsichium pennsylvanium</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—
YK-8D	Yeast colonies (white)	—	—	—	—	—	—	—	6	—	—	—	—	—	—	—

wat : water, OLW: overlaying water, sed: sediment

Table 3.4 contd.

Isolate number	Closest named species / colony morphology	SSt-200			SSt-500			SSt-700			SSt-800			SSt-1100		
		wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed
YK- 9	<i>Cochliobolus</i> sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-10	Pale brown mycelia	—	—	—	—	—	—	—	—	—	—	—	—	—	26	—
YK-11	<i>Phialophorophoma litoralis</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	12	—
YK-12	Cream coloured mycelia	—	—	—	—	—	—	—	—	—	—	—	—	—	6	—
YK-13	<i>Aspergillus unguis</i>	—	—	42	—	—	64	—	—	—	—	1	—	3	—	—
YK-14	Unidentified mycelial colony	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11
YK-15	Unidentified mycelial colony	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
YK-16	<i>Zasmidium cellare</i>	—	—	—	68	83	—	18	—	—	—	—	—	3	9	—
YK-17	Unidentified mycelial colony	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-18C	<i>Hortaea werneckii</i>	—	—	—	—	—	—	—	—	56	—	—	—	—	—	—
YK-19C	Yeast colony (pale orange)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-19D	<i>Rhodotorula nymphaeae</i>	—	—	—	1	5	—	9	—	—	—	—	—	—	—	—
YK-20	<i>Fusarium oxysporum</i>	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
YK-21	<i>Aspergillus</i> sp.,	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—
YK-22	<i>Aspergillus</i> sp.,	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—
YK-23	<i>Tilletiopsis albescens</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-24	<i>Cladosporium cladosporioides</i>	—	—	—	—	—	14	—	—	—	—	—	—	—	—	—
YK-25	<i>Cerrena unicolor</i>	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-26	Thraustochytrid colony (??)	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-27	<i>Fusarium</i> sp. MBS1	—	—	—	—	—	—	—	—	33	—	—	—	—	—	—
YK-28	<i>Chaetomium globosum</i>	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-29	Unidentified mycelial colony	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-30	<i>Zasmidium cellare</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11
YK-31	<i>Lepidosphaeria nicotiae</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11
YK-32	<i>Corioloopsis byrsina</i>	—	—	—	—	—	—	—	—	11	—	—	—	—	—	—

wat : water, OLW: overlaying water, sed: sediment

Table 3.4 contd.

Isolate number	Closest named species / colony morphology	SSt-200			SSt-500			SSt-700			SSt-800			SSt-1100		
		wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed
YK-33	Non-sporulating fungi	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-34	<i>Arthrimum marii</i>	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
YK-35	Unidentified mycelial colony	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-36	Unidentified mycelial colony	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-37	Unidentified mycelial colony	—	—	—	—	—	—	—	—	—	—	—	—	—	—	44
YK-38	<i>Aspergillus</i> sp. (Brown)	—	—	—	2	2	—	—	—	—	—	—	—	—	—	22
	<i>Penicillium</i> sp.	—	—	—	—	—	—	—	—	—	—	—	1	2	32	—
	Unidentified mycelia	—	—	—	—	4	—	55	—	—	—	51	92	13	—	—
	Unidentified yeast colony	—	—	—	—	—	—	—	—	—	—	43	—	—	—	—
	Total Species (S)	—	—	8	4	6	8	7	4	3	2	6	6	9	7	5
	Species richness (d)	—	—	1.5	0.7	1.1	1.5	1.3	0.7	0.4	0.2	1.1	1.1	1.7	1.3	0.9
	Pielou's evenness (J')	—	—	0.9	0.5	0	0.6	0.7	0.6	0.8	1.0	0.5	0.2	0.4	0.9	0.9
	Shannon's diversity index	—	—	1.8	0.7	0.7	1.3	1.3	0.8	0.9	0.7	0.9	0.4	0.9	1.7	1.4
	Simpson's diversity index	—	—	0.8	0.5	0.3	0.6	0.6	0.4	0.6	0.5	0.6	0.2	0.4	0.8	0.7

wat : water, OLW: overlaying water, sed: sediment

frequency of occurrence during most of the sampling period. *Cladosporium* sp. was also frequent during the anoxic period during Ft-1 in Oct 05. The straminipilan fungi, thraustochytrids were the next most abundant fungi (Table 3.3). The total species recovered from the different seasons varied from 6 to 16, the species richness ( $d$ ) and Pielou's evenness ( $J'$ ) calculated also reflects that there was difference between the sampling seasons. The Shannon's diversity index varied between 2.3 and 1.3, and the Simpson's diversity index was between 0.9 and 0.3 in the coastal stations during the eight field trips. During all the stations a large percentage of fungal CFUs were unidentified (Table 3.3).

Fungal cultures were isolated from five stations situated within the open ocean oxygen depleted environment along the continental slope region. Fungal cultures were isolated from water, overlaying water and the sediment collected from these stations. Yeast colonies and filamentous yeasts were the most dominant culturable fungi isolated from this region (Table 3.4). Among the culturable fungi *Zasmidium cellare* followed by *Aspergillus* sp. were the most dominant forms. A large number of mycelial colonies and yeasts colonies were unidentified and could not be retrieved during subsequent sub-culturing. The fungal diversity in the five sampling stations was uniform and the diversity was similar in the water, overlaying water and in the sediment. The species richness was very low in the slope stations when compared to the coastal stations (Table 3.4). Shannon's diversity index and Simpson's diversity index also reflected a poor diversity in the slope stations. The Values ranged between 0.4 and 1.8 for the former and between 0.2 and 0.8 for the latter (Table 3.4).

### **3.3.2 Isolation by enrichment culturing**

Fungal cultures were isolated from the sediment incubated in anaerobic condition. Particle plating of sediment after incubation yielded four fungal

CFUs. They were identified to be one *Fusarium* sp. and three *Aspergillus* spp. (Table 3.5).

**Table 3.5** List of cultures obtained by enrichment culturing

Isolate No.	Identification
# An-1	<i>Aspergillus</i> sp.
# An-2	<i>Fusarium</i> sp.
# An-4	<i>Aspergillus</i> sp.
# An-3	<i>Aspergillus</i> sp.

### 3.3.3 Identification of culturable fungi

**Table 3.6:** Selected list of cultures identified based on 18S rDNA sequence

Isolate No.	Isolated from	closest BLAST hit	Acc No	% similarity
<b>Phylum Ascomycota</b>				
# 9	CSt-26	<i>Ulospora bilgramii</i>	DQ384071.1	97.8
# 44	CSt-26	<i>Eremodothis angulata</i>	DQ384067.1	99.4
# 36	CSt-26	<i>Aspergillus penicillioides</i>	AB003077.1	99.7
# 35	CSt-26	<i>Aspergillus penicillioides</i>	AB003077.1	99.8
# 41	CSt-26	<i>Penicillium namyslowskii</i>	AB028190.1	99.4
# 31a	CSt-26	<i>Penicillium namyslowskii</i>	AB028190.1	99.5
# 40	CSt-26	<i>Aspergillus candidus</i>	AB008396.1	100.0
# 21	CSt-26	<i>Aspergillus wentii</i>	AB002063.1	99.6
# 50	CSt-26	<i>Aspergillus penicillioides</i>	AB003077.1	99.7
# 20	CSt-26	<i>Beauveria felina</i>	AY261369.1	99.9
# 30	CSt-26	<i>Microascus cirrosus</i>	M89994.1	97.5
# An-2	CSt-26	<i>Fusarium oxysporum</i>	AB110910.1	99.9
# 31	CSt-26	<i>Myrothecium verrucaria</i>	AJ301999.1	98.7
# 54	CSt-26	<i>Geosmithia putterillii</i>	AB031390.1	99.1
# 122	SSt-200	<i>Zasmidium cellare</i>	EF137362.1	99.9
# 123	SSt-200	<i>Aspergillus versicolor</i>	AB008411.1	99.6
# 132	SSt-200	<i>Tritirachium</i> sp.	AB003951.1	99.5
# 133	SSt-200	<i>Tritirachium</i> sp.	AB003951.1	99.5
# 129	SSt-200	<i>Cordyceps sinensis</i>	AJ301999.1	98.5

Table 3.6 contd.

Isolate No.	Isolated from	closest BLAST hit	Acc No	% similarity
<b>Phylum Ascomycota</b>				
# 125	SSt-200	<i>Tritirachium sp.</i> IAM 14522	AB109761.1	99.9
YK-24	SSt-500	<i>Cladosporium cladosporioides</i>	DQ678004.1	98.0
YK-28	SSt-500	<i>Chaetomium globosum</i>	AY545725.1	98.0
YK-16	SSt-500	<i>Zasmidium cellare</i>	EF137362.1	96.0
YK-18C	SSt-700	<i>Hortaea werneckii</i>	Y18700.1	99.0
YK-34	SSt-500	<i>Apiospora montagnei</i>	AB220229.1	95.8
YK-13	SSt-700	<i>Aspergillus unguis</i>	EF067336.1	99.0
YK-5b	SSt-800	<i>Alternaria alternata</i>	U05194.1	97.0
YK-8B	SSt-1100	<i>Pichia guilliermondii</i>	DQ534403.1	98.0
YK-9	SSt-1100	<i>Cochliobolus sp.</i>	FJ235087.1	97.0
YK-11	SSt-1100	<i>Phialophorophoma litoralis</i>	EU754077.1	95.0
YK-30	SSt-1100	<i>Zasmidium cellare</i> strain	EF137362.1	98.0
YK-31	SSt-1100	<i>Lepidosphaeria nicotiae</i>	DQ384068.1	96.0
# 85	LSt-03	<i>Penicillium namyslowskii</i>	AB028190.1	99.5
<b>Phylum Basidiomycota</b>				
# 11	CSt-26	<i>Rhodotorula aurantiaca</i>	AB030354.1	92.1
# 90	SSt-200	<i>Graphiola cylindrica</i>	D63929.1	97.1
YK-6C	SSt-500	<i>Rhodotorula graminis</i>	EU563924.1	95.0
YK-19C	SSt-500	<i>Aureobasidium pullulans</i>	EU707928.1	98.0
YK-19D	SSt-500	<i>Rhodotorula minuta</i>	FJ196163.1	98.0
YK-25	SSt-500	<i>Cerrena unicolor</i>	AY850007.1	98.0
YK-8C	SSt-700	<i>Melanopsichium pennsylvanicum</i>	DQ363314.1	99.0
YK-32	SSt-700	<i>Corioloopsis byrsina</i>	AY336773.1	99.0
# 87	LSt-03	<i>Sporisorium reilianum</i>	DQ832229.1	62.5
# 88	LSt-03	<i>Rhodotorula calyptogenae</i>	AB126648.1	99.8
# 89	LSt-03	<i>Rhodotorula calyptogenae</i>	AB126648.1	99.8

Culturable forms of fungi could be retrieved from all the sampling locations, from these 49 different cultures were sequenced for 18S rDNA region (Table 3.6).

Thirty eight of them belonged to Phylum Ascomycetes and most of them predominantly belonged to Eurotiomycetes. This is one of the biggest division and most of the commonly occurring and well studied forms belong

to this group. Eleven cultures grouped with the phylum Basidiomycetes. A majority of the sequences had more than 95 % similarity with the sequences available in the NCBI database with the different environmental sequences obtained from this study (Table 3.6). Except for two cultures # 87 and # 11 which had a greater percentage of dissimilarity compared to other cultures. # 87 has only 62.54 % similarity with *Sporosorium reilianum* and #11 belonging to Basidimycota had 92.13 % similarity with *Rhodotorula aurantica*.

### 3.3.4 Unculturable diversity

Five clone libraries were constructed using the total genomic DNA extracted from the environmental samples collected from a CSt and SSt station and a reference sample from the coral reef station (Table 3.1). Each of the libraries were constructed using two fungal specific primer sets (Fung1 and Fung2) for selectively amplifying fungal sequences and a universal eukaryote specific primer set EukAB that is routinely used in environmental eukaryote SSU rRNA diversity surveys (Table 3.2).

About 1440 clones obtained from five clone libraries were analyzed for their phylogenetic affiliation. Surprisingly, GenBank BLASTn analyses of these clones resulted in only 456 fungal targets, 19 of which were of low quality sequences (insufficient lengths, including Ns), and were not considered for further analyses. Of the 456 fungal targets obtained, 152 fungal sequences (grouping into 19 OTUs) were obtained with the Fung1 primer set, 172 sequences (35 OTUs) with the Fung2 primer set, and the universal eukaryotic primer set retrieved 113 fungal sequences (9 OTUs). Nearly two-third of the clones analyzed (984 clones) using BLASTn emerged as non-fungal sequences. As expected, numerous (n = 346) such non-fungal sequences were retrieved with the universal eukaryote

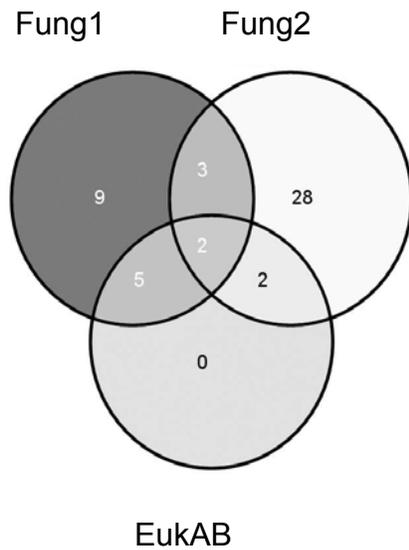
primer set EukAB. But also putatively fungal specific primer sets retrieved 313 (Fung1) and 281 (Fung2) non-fungal amplicons (Table 3.7).

**Table 3.7** Taxonomic distribution of non-fungal operational taxonomic units (OTUs)

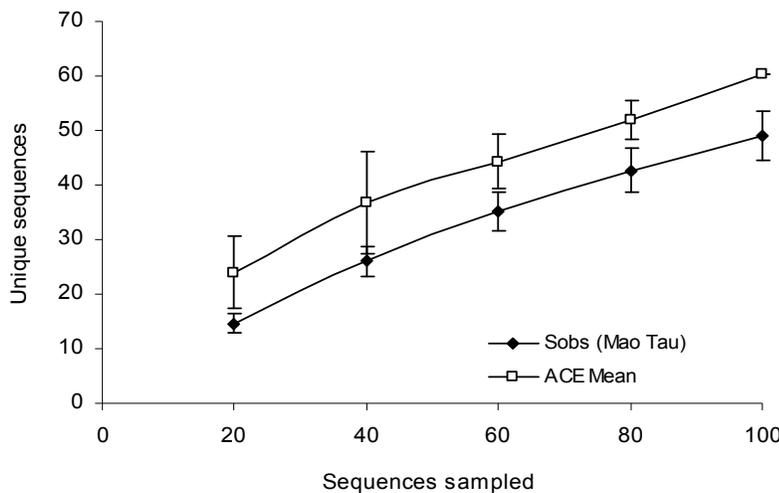
Primer set	Non-fungal GenBank BLAST matches
Fung1	<i>Corallochytrium limacisporum</i> ; Choanoflagellates; Metazoa (Gastrotricha)
Fung2	Bacteria: (Proteobacteria); Viridiplantae; Rhizaria; <i>Corallochytrium</i> ; Metazoa (Gastrotricha)
EukAB	Bacteria (Proteobacteria, Bacteroidetes, Firmicutes); Choanoflagellates, Rhizaria, Euglenozoa; Alveolata; Metazoa (Arthropods, Nematodes, Annelids)

The Venn diagram (Figure 3.1) shows that the number of OTUs that were retrieved simultaneously with both fungal-specific primer sets is only small (n=5) indicating that each of the two primer sets amplified a different subset of taxa from the fungal communities under study. Fung1 and Fung2 primer set retrieved about 9 and 28 unique OTUs, but EukAB derived OTUs were only an overlap of the OTUs obtained from the fungal specific primer sets. Though, we were able to retrieve about 37 unique OTUs by using a combination of two fungal specific primer sets, the sequences belonged primarily to either phylum ascomycota or basidiomycota and only one OTU belonging to phylum zygomycota was obtained. There were no sequences belonging to phylum chytridiomycota.

Sampling saturation analysis (Figure 3.2) of the fungal OTUs showed that sampling was not done up to saturation. This may be due to the inefficiency of the primer sets used to amplify sequences belonging to

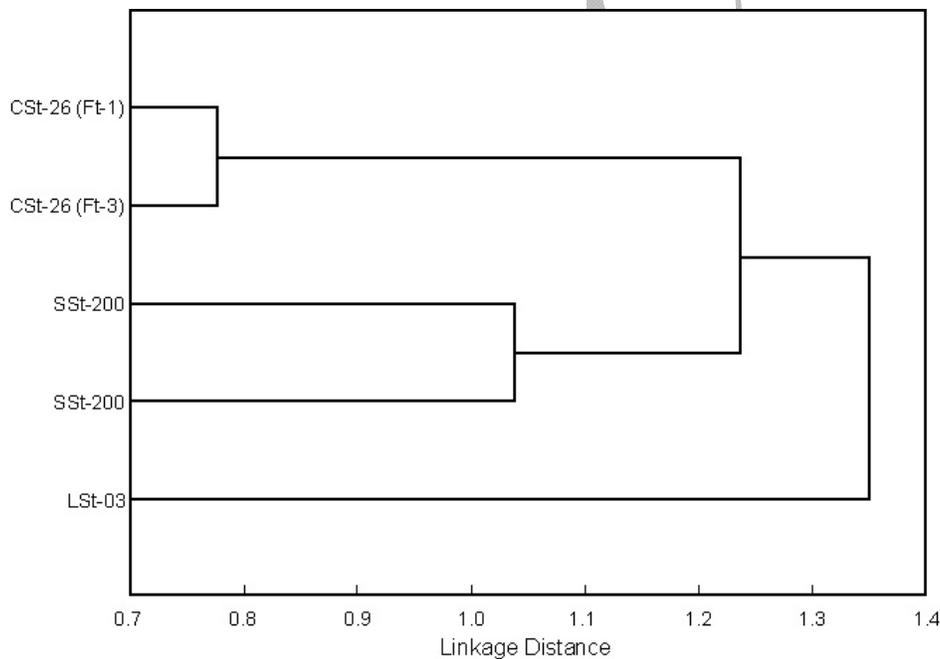


**Fig.3.1** Venn diagram showing the number of OTUs that were recovered simultaneously by two or three of the primer sets used in this study (Fung1, Fung2, EukAB, see Table 3. 2) and number of OTUs recovered exclusively with a single primer set.



**Fig. 3.2** Collectors curves of fungal OTUs. Species accumulation curve (Sobs) gives the number of species expected in the pooled samples, for the given empirical data and Abundance-based Coverage Estimator (ACE) is an estimator of species richness (mean among runs) for the clone libraries are plotted.

zygomycota and chyrtidiomycota. The primer sets also retrieved a large number of non-target OTUs, nearly two-third of the clones analysed (940 clones) on BLAST analysis matched with non-fungal data base entries and 44 sequences did not show similarity to any known sequences. Numerous ( $n = 346$ ) non-fungal sequences were retrieved with the universal eukaryote primer set. Interestingly, also the putative fungal specific primer sets retrieved 313 (Fung1) and 281 (Fung2) non-fungal clones. Each primer set retrieved sequences belonging to a wide range of metazoa and unicellular eukaryotes (Table 3.7). The primer sets EukA-EukB and Fung2 even amplified SSU rRNA fragments that could be assigned to the domain Bacteria.



**Fig. 3.3** Dendrogram resulting from calculated Jaccard index based on incidence ( $J_{incidence}$ ) of unique operational taxonomic units (OTUs), as a measure of community similarity between the samples under study. For information on sampling sites, see Table 3. 1. Similarity values were transformed into a distance matrix and subsequent cluster analysis was performed using the unweighted pair group mean average (UPGMA) algorithm.

A UPGMA cluster analysis of  $J_{abund}$  index (Fig. 3.3) reveals that the fungal communities from the different sampling sites (Table 1) are distinctly different from each other. Of all comparisons, the two coastal sites sampled during anoxic and oxic conditions (CSt-26 during Ft-1 & Ft-3) are most similar in their fungal community membership ( $J_{abund} = 0.48$ ). The sediment and water column-derived fungal communities from the open ocean (SSt-200) OMZ site cluster together with a  $J_{abund}$  of 0.35. The fungal community from the coral reef reference site (03\_CRS) showed highest dissimilarity to all other samples ( $J_{abund} = 0.06-0.1$ ). Our analysis suggests a clear separation of fungal communities adapted to temporal anoxic condition and those adapted to permanent anoxic zone. There is no difference in the community structure of fungi between the anoxic and oxic field trip samplings in the CSt-26 station. This may be due to the physiological adaptation of the fungal community to the changing oxygen concentrations.

### 3.3.5 Phylogenetic analysis:

Environmental OTUs identified in this study are designated as “Fungal OTUs from the Arabian Sea”: FAS\_1 to FAS\_49. The OTUs from the five sample sites were assigned to the Dikarya (Ascomycota: 27 OTUs; Basidiomycota: 21 OTUs), and one OTU branched within the subphylum Mucoromycotina.

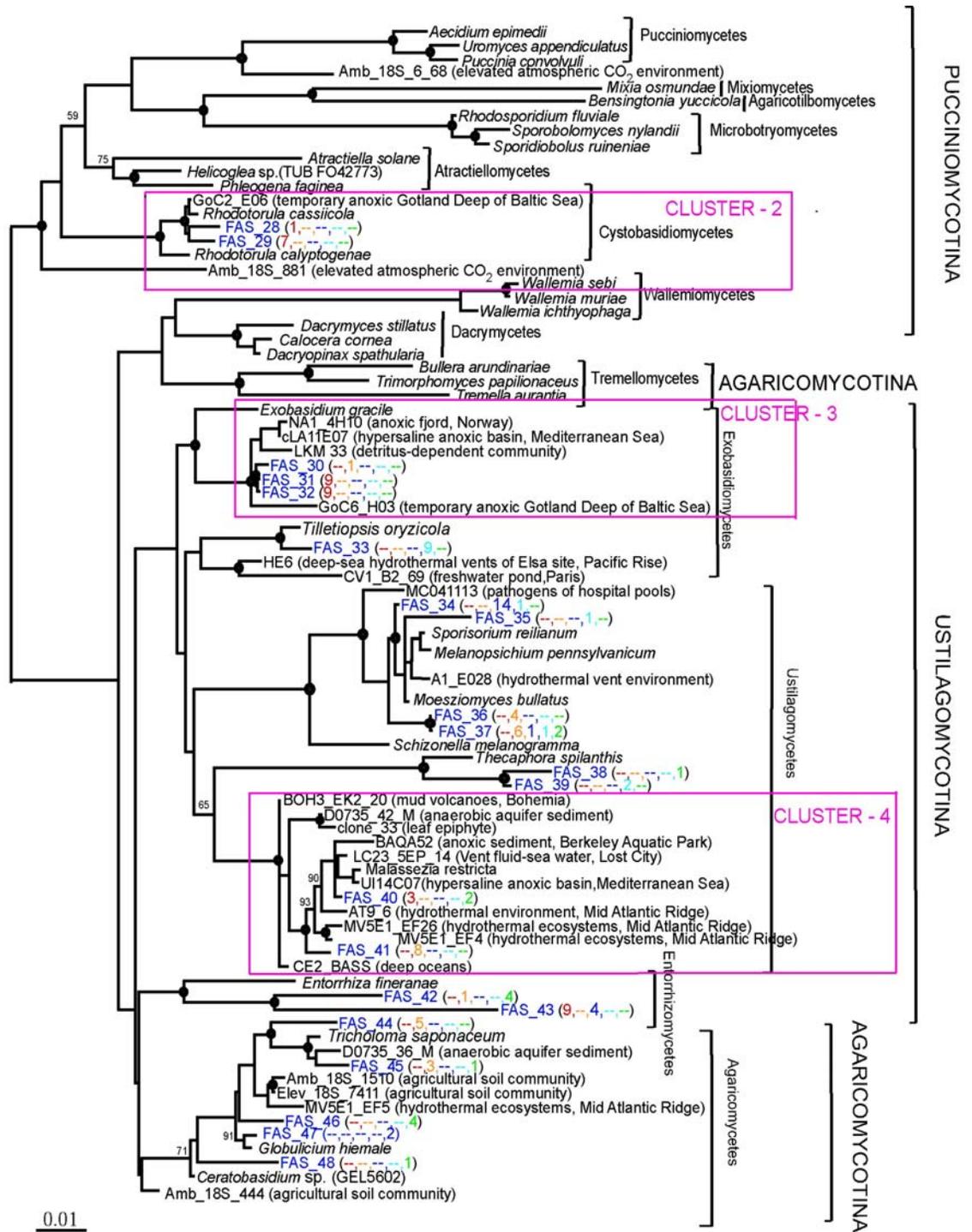
Ascomycota OTUs (Fig.3.4) belonged to the subphylum Saccharomycotina and Pezizomycotina. FAS\_2 was the most frequently represented phylotype and is closely related to the yeast *Kodamaea* sp. with a similarity of 99.67%. Maximum number of environmental clones FAS\_6, FAS\_7, FAS\_8, FAS\_9, FAS\_10, FAS\_11 and FAS\_12 belonged to Sordariomycetes and their closest known named species being *Fusarium* sp. *Fusarium oxysporum* is the most widely studied fungus of



**Fig. 3.4** Minimum evolution phylogenetic tree of the 18S rDNA sequences of phylum Ascomycota showing the position of environmental OTUs obtained from the Arabian Sea.

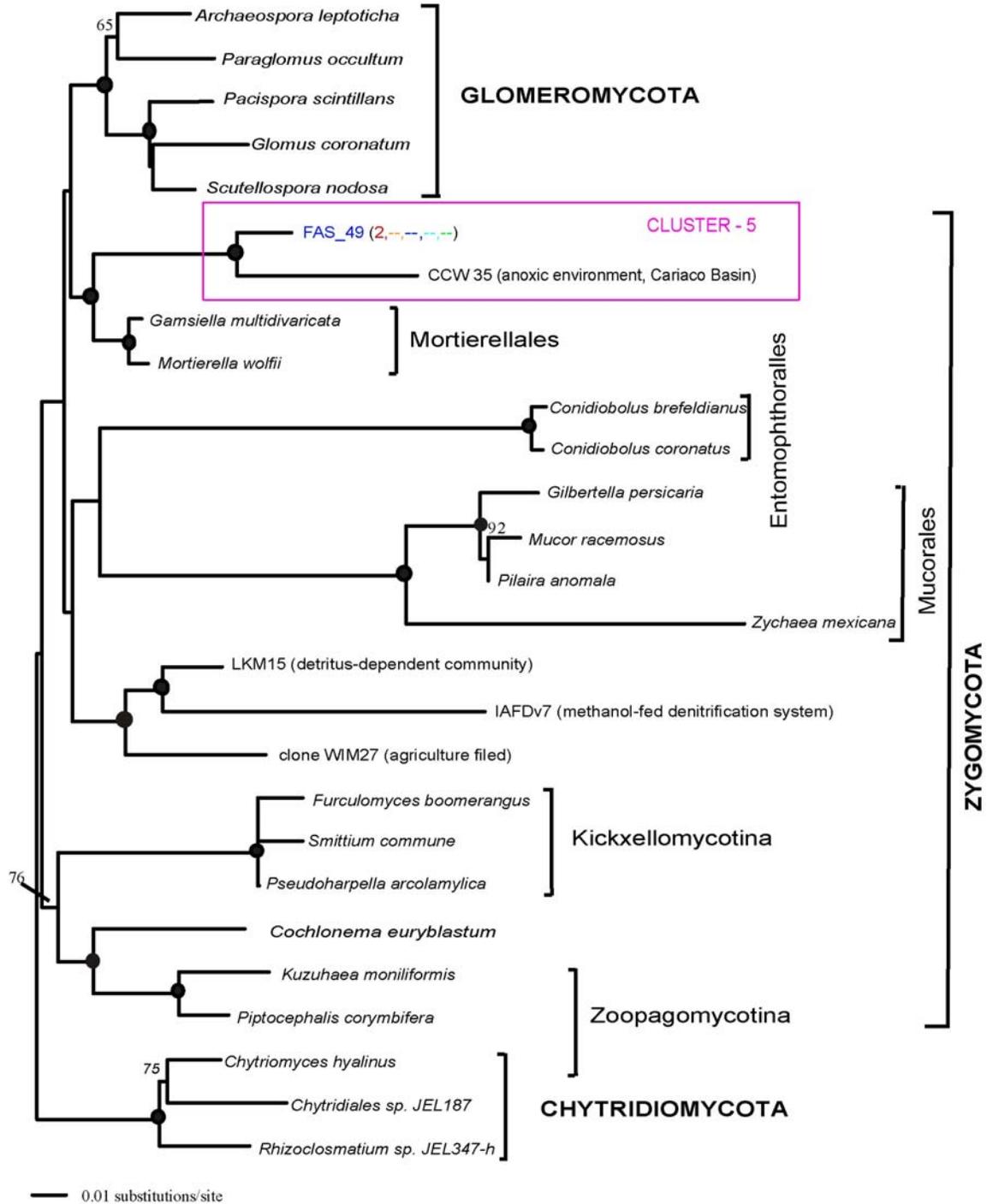
terrestrial origin for its denitrification abilities. The phlotypes obtained from this study grouped to environmental clones obtained from similar anoxic habitats and other extreme environments. FAS\_5 grouped to an environmental clone P1\_3m2 isolated from aquifer sediment (Acc No. EU091828) with 99.88 % similarity and with a named relative *Candida paprapsilopsis*. Environmental clones belonging to Eurotiomycetes, FAS\_16, FAS\_17, FAS\_18, FAS\_19, FAS\_20 and FAS\_21 grouped with a similarity value of 99.11 % to 97.3 % as a single cluster with a number of phlotypes isolated from anoxic environments such as the hypersaline anoxic basin of the Mediterranean Sea (Acc No. EU446318, EU446322), anoxic sediments of a saline meromictic lake in Japan (Acc No. AB191430), anoxic sediments from marine lakes, California (Acc No. AF372705), acidic mine drainages (Acc. No. AY343927), from hydrothermal vent habitats (Acc No. AF530541), boiling springs lake sediment, Volcanic National Park, California (Acc No. EF682467, EF682478) and a few fungal endophytes (Acc Nos. AB074658, DQ979499, AB074664) The environmental sequences have similarity with some of the well described and ubiquitously occurring terrestrial fungal sequences such as *Penicillium namyslowskii*, *Eupenicillium javanicum* and *Aspergillus candidus*. FAS\_24 is a dothidiomycetes clone with a similarity of 99.03 % to *Mycosphaerella handelii* and 98.92% to an environmental clone from the Mid-Atlantic hydrothermal sediment. Environmental sequences closely related to dothideomycetes are reported from the first time from anoxic habitats. These phlotypes group along with common terrestrial forms such as *Cladosporium cladosporioides*, *Preussia* sp. and other uncultured dothideomycetes associated with lichens and seedlings (Acc. No. EF053571.1, AY275188.1).

Basidiomycota OTUs (Fig. 3.5) belonged to the subphyla Pucciniomycotina, Agaricomycotina, and Ustilagomycotina. Only few



**Fig. 3.5** Minimum evolution phylogenetic tree of the 18S rDNA sequences of phylum Basidiomycota showing the position of environmental OTUs obtained from the Arabian Sea.

sequences were closely (> 97% sequence similarity) related to previously deposited sequences of described basidiomycetes such as FAS\_47 exhibiting 99.46% similarity to the wood-decaying saprophyte *Globulicium hiemale* and FAS\_28 and FAS\_29 with the nitrogenous compounds scavenger *Rhodoturula cassicola* as a closest relative with 99.83 % and 98.60 % sequence similarity respectively. Most environmental fungal sequences exhibited more than 3 % sequence divergence from sequences of described taxa of the cystobasidiomycete sequences such as the ustilagomycetes FAS\_38 and FAS\_39 being 93 % divergent to the plant pathogen *Thecaphora spilanthis*. Many fungal sequences from the Arabian Sea branch in clades that include other environmental fungal sequences from a variety of oxygen-depleted habitats. Examples are the three exobasidiomycete sequences FAS\_30, FAS\_31 and FAS\_32 which were related to fungal sequences found in an anoxic Norwegian Fjord (Acc No. EF527154), an anoxic Mediterranean deep-sea basin (Acc No. EU446368) and an anoxic basin in the central Baltic Sea (Acc No. FJ153746). The closest described relative of this sequence clade is the plant pathogen *Exobasidium gracile* with at least 37.3 % sequence divergence. The sequences FAS\_40 and FAS\_41 branch together with sequences from Mid-Atlantic Ridge hydrothermal vents (Acc Nos. AF530542, EF638640, EF638640), the Lost City hydrothermal field (Acc No. DQ504335), mud volcanoes (Acc No. DQ504358), anoxic intertidal sediment (Acc No. AF372708), an anoxic Mediterranean deep-sea basin sequence (Acc No. EU446358) and a few others such as epiphytes and deep ocean fungal sequences (Acc No. FJ153767, EU154971). The closest described species branching in this clade is the rare skin pathogen *Malassezia restricta*, with 0.39% and 2.36% sequence divergence to the OMZ clones FAS\_40 and FAS\_41. Interestingly phlotypes obtained exclusively from oxic regions of our study grouped together within the



**Fig. 3.6** Minimum evolution phylogenetic tree of the 18S rDNA sequences of phylum Zygomycota, Glomeromycota & Chytridiomycota showing the position of environmental OTUs obtained from the Arabian Sea.

agaricomycetes along with culture sequences and environmental clones belonging to anaerobic aquifers, hydrothermal sediment and agricultural soil (Acc Nos. EU647038, EF638641, EF024029, EF025042, EF023155).

Only one phylotype FAS\_49 belonged to zygomycetes (Fig. 3.6), and it grouped with uncultured environmental clone CCW35 (Acc No: AY180022) from the anoxic Cariaco Basin with a similarity of only 90.45 %. The closest named species was *Mortierella wolfii* with 90.02 % similarity belonging to Mortierellales.

### **3.4 Discussion**

#### **3.4.1 Diversity of cultured fungi**

Traditionally, fungal diversity studies in environmental samples are based on cultivation approaches. Limitations of this strategy are the inability to separate biomass from particulate material and lack of growth media and cultivation conditions suitable for all members of the community has led to an underestimation of the total fungal diversity (Anderson *et al.*, 2003). Molecular phylogenetic analyses of clone libraries constructed from environmental samples have become the gold-standard in fungal diversity research (Pang & Mitchell, 2005). But this strategy is no panacea, as biases due to primer (un)specificities as discussed above. Combining cultivation-based and cultivation-independent methods may allow for a more complete picture of fungal diversity as each of the methodological strategies may compensate for the biases of the other.

Fungal cultures could be isolated using cultivation based approach from all the sampling stations (Table 3.3 and 3.4). The diversity among the cultures isolated from the coastal station based on the Shannon's index

shows that the diversity was less compared to the mangrove ecosystem (Maria & Sridhar, 2002). The Shannon's index in the mangrove ecosystem was about 5, whereas our study was only between 1.3 and 2.3. However these values are comparable to the diversity index of fungal cultures obtained from the deep sea environment (Damare *et al.*, 2006) which ranged between 1.2 and 2.0. The fungal diversity studied from the open ocean stations (Table 3.4) was very less even in comparison to the deep sea environment (0.4 to 1.8). Simpson's index also shows that the diversity from the oxygen depleted environment was lower in comparison to the mangrove ecosystem (Maria & Sridhar, 2002). It had an almost 0.9 index whereas our sampling sites showed a variation of 0.2 to 0.9 from the coastal and slope station.

The cultures identified based on the 18S rDNA sequence could be assigned to Basidiomycetes and Ascomycetes (Table 3.5). Eleven isolates belonged to Basidiomycetes, predominantly Pucciniomycotina; twenty-one of them belonged to Ascomycetes, a majority of it belonged to Eurotiomycetes. Both divisions of fungi have a high representation of cultured taxa (Hyde *et al.*, 2000), indicating the accessibility of these groups to cultivation. The 18S rDNA sequence of the cultures studied have >99% sequence similarities to previously studied, cultured and well-described fungi. This indicates that cultivation using standard techniques generally produces more isolates of already known taxa. Possibly, the development of more specific cultivation techniques and media that are adapted to the environmental conditions of the habitat under study might reveal a larger number of unknown taxa.

We obtained few isolates that show only low similarity to previously described and sequenced fungi. These are for example the isolates belonging to Basidiomycota, # 87 with only 62.5% similarity to *Sporisorium reilianum* and isolate # 11 with 92.1% sequence similarity to *Rhodotorula*

*aurantiaca*. These examples reveal another decisive advantage of cultivation over clone library analysis. Having access to (putatively) novel cultures in living condition paves way for an in-depth analysis of their phylogeny, morphology and ecology. A current multi-gene analysis of isolate # 11 along with ultrastructural analyses points to the discovery of a novel fungal taxon on class-level with the capability of anaerobic denitrification (Jebaraj CS, Boekhout T, Muller W, Kauff F, Stoeck T, unpublished). Detailed analysis of the enzymes involved in the nitrate reducing pathways of cultured fungi and their expression profiles *in situ* can help us to understand their role in the OMZ of the Arabian Sea and other anoxic aquatic systems in global biogeochemical cycles.

#### **3.4.2 Diversity of uncultured fungi**

Analysis of fungal communities based on the environmental clone libraries studied across the anoxic habitats of the Indian Ocean shows that they are ubiquitous and the species diversity was not affected by the oxygen concentration (Fig 3.3). Indian Ocean has a characteristic seasonal anoxic condition that develops along the western margin during October to January (Naqvi *et al.*, 2006). Analysis of clone libraries constructed from the CSt-26, which comes under the influence of the seasonal DO changes shows that the community structure was not greatly affected by the seasonal changes of oxygen concentration and there was an overlap between the communities obtained during the anoxic and oxic season (Fig 3.3). Recent studies have shown that fungi can adapt to alternate modes of respiration (Daiber *et al.*, 2005) and the trend showed in our study is possible as the fungal community in large could adapt to the denitrification process during the oxygen depleted condition. But spatial difference was evident in the distribution of fungal communities, as seen in the difference

between the communities of the coastal, open ocean and the coral reef site. This can be seen from the J abundance index which shows that the community structure in the coral reef station was distinctly different from the coastal and slope station communities (Fig 3.3).

A number of recent studies in the anoxic habitats have shown that novel clusters and undescribed clusters are a major component in all the environmental communities studied so far. (Takishita *et al.*, 2005; Takishita *et al.*, 2007; Brad *et al.*, 2008). Exclusive studies on the fungal diversity from the cold Antarctic regions and deep sea environments has also shown the presence of a large number of uncultured sequences and novel clusters which branch out separately (Malosso *et al.*, 2006; Bass *et al.*, 2007). In this study I have attempted for the first time to use fungal specific primers to study the fungal diversity from the anoxic regions of the Arabian Sea. Phylogenetic analysis of the environmental sequences obtained from this study has shown that we can categorize phylotypes specific to each library and at least five clusters of environmental sequences could be identified which groups with sequences obtained from similar anoxic and extreme environments.

### **3.4.3 Phylogenetic analysis**

Of the five unique environmental clusters identified, one cluster with six phylotypes from this study groups within the Eurotiomycetes of Ascomycota (Fig. 3.4). This cluster has sequences obtained from the anoxic Mediterranean basin, anoxic Meromictic Lake sediment and marine anoxic Bolinas Lagoon DNA and a few common culturable forms such as *Penicillium* and *Aspergillus* sp. These commonly occurring species are very versatile and are shown to participate in denitrification activity. Experimental studies with # 31a (99.45% similarity with *Penicillium*

*namyslowskii*) have shown that they can grow under anaerobic conditions by reducing nitrate (Jebaraj & Raghukumar, 2009).

Three clusters were seen within the basidiomycota belonging to Cystobasidiomycetes, Exobasidiomycetes, and Ustilagomycetes (Fig. 3.5). The cluster within the Cystobasidiomycetes groups with environmental sequences of uncultured clones from anoxic Gotland Deep of Baltic Sea, environments with elevated atmospheric CO<sub>2</sub> and with two basidiomycete yeasts *R cassiicola* and *R calyptogenae*. The second environmental cluster within the basidiomycota grouped along with environmental sequences closely related to Exobasidiomycetes. The sequences clustered with phylotypes obtained from anoxic Norwegian fjords, anoxic Mediterranean basin, anoxic Gotland Deep of Baltic Sea and a fungal clone obtained from detritus community. The closest known named species was *Exobasidium gracile* which branched out separately from this environmental cluster which is supported with a high bootstrap support. The third cluster belonging to Ustilagomycotina is within a clade that has been recognized as “hydrothermal and/or anaerobic fungal group” (Lopez-Garcia *et al.*, 2007). This cluster has representatives from mud volcanoes, hydrothermal ecosystems, anoxic Mediterranean basin and anoxic Berkeley Aquatic Park sediment, deep oceans and a leaf epiphyte (Fig. 3.5).

Only one phylotype belonging to zygomycete was obtained from this study and this OTU grouped with CCW35 from the anoxic Cariaco Basin (Fig. 3.6). Though there was a very poor representation of zygomycetes and chytrids in the study, they are reported to be a major component of the fungal environmental community. Zygomycete representatives and a full clade of uncultured fungal sequences were recovered from the anoxic polluted aquifer sediments (Brad *et al.*, 2007). A large number of chytrid sequences have been retrieved from other

anoxic environments (Brad *et al.*, 2007) and universal microeukaryotic libraries (Stoeck *et al.*, 2007). Lack of representatives from these groups may be attributed to the primer sets used for analyses which were efficient to recover the major ascomycetes and basidiomycetes but missed out the other two groups. Similar views have been expressed by researchers studying the fungal diversity from environmental DNA analysis (Anderson *et al.*, 2003; Malosso *et al.*, 2006). It is probable that some fungal sequences present could not be retrieved due to problems associated with primer selection and DNA quality. Increasing studies from across the globe in the anoxic environments has strengthened the database available on the fungal community structure. Hence further studies with this basis should be carried out with more refined primer sets that can amplify fungal taxa belonging to all the phylums. Studies to specifically study individual groups and subgroups may also bring to light many new and interesting dimensions to our understanding of the ecosystem and their functioning.

Aid of molecular tools has definitely broadened the understanding of several ecosystems. In spite of the increasing efficiency of techniques to study the in-situ process at molecular level, an in-depth understanding of the role played by the microorganisms can be achieved only by studying the culturable forms in-vitro conditions. Screening studies of a few cultures isolated from the oxygen depleted environment has been studied for their denitrification ability (Chapter 4).

The major inferences that can be drawn from the study based on the culture dependent and independent approach are: this study represents the first fungal specific SSU rRNA based survey of the oxygen depleted regions of the Arabian Sea and representative environmental sequences from the major fungal groups could be retrieved. Five clusters of environmental sequences that group with sequences from metagenomic libraries constructed from similar anoxic regions could be

identified. Unique fungal phlotypes were obtained from each of the libraries constructed from seasonally and spatially distinct locations. Isolation of cultures from anoxic regions which are divergent from well described taxa gives an opportunity to study them in detail for their phylogenetic and ecological significance. Detailed analysis of the genes that code for the enzymes involved in the nitrate reducing pathways and their expression profiles will help to quantify their role in the OMZ of the Arabian Sea.

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