RESEARCH ARTICLE

Fungal diversity in oxygen-depleted regions of the Arabian Sea revealed by targeted environmental sequencing combined with cultivation

Cathrine S. Jebaraj[1,2], Chandralata Raghukumar[1], Anke Behnke[2] & Thorsten Stoeck[2]

[1]National Institute of Oceanography (Council of Scientific and Industrial Research), Dona Paula, Goa, India; and [2]School of Biology, University of Kaiserslautern, Kaiserslautern, Germany

Correspondence: Thorsten Stoeck, School of Biology, University of Kaiserslautern, Erwin Schrödinger Str. 14, D-67663 Kaiserslautern, Germany. Tel.: +49 631 205 2502; fax: +46-631-2052496; e-mail: stoeck@rhrk.uni-kl.de

Received 18 May 2009; revised 17 September 2009; accepted 14 October 2009.
Final version published online 25 November 2009.

DOI:10.1111/j.1574-6941.2009.00804.x

Editor: Riks Laanbroek

Keywords
fungal diversity; Arabian Sea; cultivation; SSU rDNA; oxygen minimum zone; PCR primers.

Abstract

In order to study fungal diversity in oxygen minimum zones of the Arabian Sea, we analyzed 1440 cloned small subunit rRNA gene (18S rRNA gene) sequences obtained from environmental samples using three different PCR primer sets. Restriction fragment length polymorphism (RFLP) analyses yielded 549 distinct RFLP patterns, 268 of which could be assigned to fungi (Dikarya and zygomycetes) after sequence analyses. The remaining 281 RFLP patterns represented a variety of nonfungal taxa, even when using putatively fungal-specific primers. A substantial number of fungal sequences were closely related to environmental sequences from a range of other anoxic marine habitats, but distantly related to known sequences of described fungi. Community similarity analyses suggested distinctively different structures of fungal communities from normoxic sites, seasonally anoxic sites and permanently anoxic sites, suggesting different adaptation strategies of fungal communities to prevailing oxygen conditions. Additionally, we obtained 26 fungal cultures from the study sites, most of which were closely related (> 97% sequence similarity) to well-described Dikarya. This indicates that standard cultivation mainly produces more of what is already known. However, two of these cultures were highly divergent to known sequences and seem to represent novel fungal groups on high taxonomic levels. Interestingly, none of the cultured isolates is identical to any of the environmental sequences obtained. Our study demonstrates the importance of a multiple-primer approach combined with cultivation to obtain deeper insights into the true fungal diversity in environmental samples and to enable adequate intersample comparisons of fungal communities.

Introduction

Fungi are primarily aerobic heterotrophs that play an essential role as decomposers of organic matter in a variety of environments. In marine ecosystems, fungi are the major decomposers of woody and herbaceous substrates and their importance lies in their ability to aggressively degrade lignocellulose (Newell, 1996). Marine fungi also contribute to the degradation of dead animals and animal parts (Kohlmeyer & Kohlmeyer, 1979), and are important pathogens of animals and plants or partners in mutualistic symbioses (Raghukumar, 1986; Alsumard *et al.*, 1995; Pivkin, 2000).

Fungi were long thought to play only a minor role in the ecosystem processes of anoxic environments (Dighton, 2003). However, many fungal taxa were recently shown to possess metabolic adaptations to utilize nitrate and (or) nitrite as an alternative for oxygen (Shoun *et al.*, 1992). This testifies to their potential to participate in anaerobic denitrification processes in biogeochemically highly active ecosystems such as the oxygen minimum zones (OMZs) of the Arabian Sea, which are characterized by high microbially mediated denitrification rates (Naqvi *et al.*, 2006). Indeed, in a recent study, Jebaraj & Raghukumar (2009) showed that fungi isolated from anoxic marine waters of the Arabian Sea are capable of growth under

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

oxygen-deficient conditions while performing anaerobic denitrification.

Such findings suggest that the abundance and ecological role of fungi in anoxic marine systems is probably underestimated. Support for this assumption comes from analyses of the small subunit rRNA locus (18S rRNA gene) amplified from genomic DNA isolated from environmental samples. While usually rare in open ocean surface waters, the majority of environmental 18S rRNA gene diversity surveys conducted in oxygen-depleted aquatic environments report large proportions of fungal 18S rRNA gene sequences (reviewed in Epstein & López-García, 2007). Many of these environmental sequences appear as unique phylogenetic branches that are highly divergent from previously described 18S rRNA gene fungal sequences (e.g. Takishita *et al.*, 2005, 2007a, b; López-García *et al.*, 2007; Stoeck *et al.*, 2007; Laurin *et al.*, 2008). As a rule, such molecular diversity surveys targeting microbial eukaryotes have used domain-specific PCR primers to amplify 18S rRNA gene fragments from environmental samples. As such primers target most eukaryotic organisms across all major clades and only sample a small fraction of amplicon diversity present in a sample (Epstein & López-García, 2007), it is reasonable to assume that many fungi may have escaped these surveys. Thus, the full extent of fungal diversity may be orders of magnitude higher than these domain-targeted environmental diversity studies have shown. Indeed, PCR primers with specificity for fungal DNA, while reducing coamplification of DNA from nonfungal sources, were applied successfully for estimating the extent of fungal diversity in soil (e.g. Borneman & Hartin, 2000; Anderson *et al.*, 2003; Malosso *et al.*, 2006). To date, this targeted strategy that applies fungal-specific primers to analyze fungal diversity has gone untested for anoxic aquatic habitats.

Using previously published primers designed for the specific amplification of fungal 18S rRNA gene from mixed-origin genomic DNA (Maiwald *et al.*, 1994; Kappe *et al.*, 1996; Vainio & Hantula, 2000; Gomes *et al.*, 2003), we analyzed fungal diversity in samples from the OMZ of the Arabian Sea. Therefore, we constructed clone libraries with two fungal-specific PCR primer sets and one domain-specific PCR primer set, routinely used in environmental diversity surveys. This strategy not only allowed an insight into fungal diversity in OMZs of the Arabian Sea but also revealed the substantial proportion of fungal diversity that is missed in a domain-specific PCR primer approach. Additionally, we used standard cultivation techniques to complement the molecular diversity surveys, unearthing a different subset of the fungal communities under study than the molecular approach. Finally, community structure analyses suggest that fungal assemblages thriving under different oxygen regimes are significantly distinct from each other, probably reflecting different adaptations to geochemically distinct environments.

Materials and methods

Sampling sites

Samples were collected from three sites in the Arabian Sea (Table 1). (1) Sampling of sediments at the coastal station (15°29.951′N, 73°40.827′E) off Goa at 25 m depth was carried out in October 2005 during the anoxic season (25_ANS), with no dissolved oxygen (DO) detectable, and in January 2006 during the oxic season (25_OXS), when oxygen saturation was restored. (2) Sampling in the perennial OMZ off Goa (15°20.10′N, 72°54.11′E) at a depth of 200 m was carried out during May 2007, when we collected anoxic sediment (200_ANS) and anoxic near-bottom water (200_ANW). (3) As a comparison, we sampled the permanently oxygenated sediment from a shallow 3-m-depth site (03_CRS) in the coral reef region off Kavaratti (10°34.588′N, 72°38.019′E) in February 2007. Coral sand samples were collected by divers in sterile containers. All other sediment samples were collected using an ~60-cm-long gravity corer while water was collected in 5-L Niskin bottles. All samples were divided into aliquots for the isolation of fungi for cultivation and for total genomic DNA extraction. Samples for isolation were stored at 5 °C and processed within 24 h. Water samples for molecular analyses (5 L of water) were drawn on Durapore filters [0.45 μm; Millipore (India) Pvt Ltd, Bangalore] under the

Table 1. Sampling sites and characteristics

Sampling site	Designation (color coding in Figs 1–3)	Sampling date	DO (μM)	Temp (°C)	Salinity (PSU)	Sampling material	Sample volume
Near a shore off Goa (25 m)	25_ANS (brown)	October 2005	ND	22.5	35.4	Sediment	~500 mg
Near a shore off Goa (25 m)	25_OXS (orange)	January 2006	118. 4	26.9	34.7	Sediment	~500 mg
OMZ off Goa (200 m)	200_ANS (blue)	May 2007	ND	14.8	35.6	Sediment	~500 mg
OMZ off Goa (200 m)	200_ANW (turquoise)	May 2007	ND	14.8	35.6	Water	~5 L
Coral reef off Kavaratti (3 m)	03_CRS (green)	February 2007	93.74	28.7	34.0	Sediment	~500 mg

ND, below detection limit; Temp, temperature.

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

exclusion of oxygen (Stoeck *et al.*, 2003), and ~5–10 g of sediment was frozen immediately in liquid nitrogen before storage at −80 °C until further processing. The DO of the near-bottom water was determined spectrophotometrically (Pai *et al.*, 1993).

Isolation and identification of culturable fungi

Culturable fungi were isolated using the particle-plating technique of Bills & Polishook (1994), with slight modifications. In brief, approximately 1 g of sediment slurry was serially sieved through a 200-μm and a 100-μm mesh. The particles that passed through the 200-μm mesh, but were retained on the 100-μm mesh were spread-plated on different fungal media such as malt extract agar, corn meal agar and Czapek Dox agar (HiMedia Pvt Ltd, India) prepared in seawater and fortified with streptomycin (0.1 g in 100 mL medium) and penicillin (40 000 U in 100 mL medium) to inhibit bacterial growth. Incubation was carried out under oxic conditions. The cultures isolated from the different sampling stations were identified based on their morphology and partial (~1600 bp) 18S rRNA gene sequence. Genomic DNA was extracted from the freeze-dried mycelial mats of each culture grown aerobically for 5 days at room temperature in 50 mL of malt extract broth (HiMedia Pvt Ltd) in 250-mL Erlenmeyer flasks. Total genomic DNA was extracted from the freeze-dried mycelial mats of each culture using a high salt concentration extraction buffer (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). One milliliter of buffer was added to approximately 500 mg of mycelia and the total genomic DNA was extracted using chloroform–phenol extraction and isopropanol precipitation as described previously (Stoeck & Epstein, 2003). The 18S region of the rDNA was amplified using fungal-specific primers NS1 and FR1 (Table 2). The 50-μL PCR reactions included 0.5 U HotStart Taq DNA polymerase (Qiagen, Hildesheim, Germany) in 1 × HotStart Taq buffer, 200 μM of each dNTP and 0.5 μM of each oligonucleotide primer. PCR was performed using initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 2 min, with a final extension at 72 °C for 7 min. 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, Hamburg, Germany). One representative clone of 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. In order to evaluate the intrastrain variability of the 18S rRNA gene, for five strains (FCAS35, FCAS36, FCAS41, FCAS89 and FCAS125), we have sequenced four amplicons. Sequences were included in phylogenetic analyses as described below. GenBank accession numbers of sequences from cultured isolates are GQ120154–GQ120179 and GU072534–GU072548 for duplicate amplicons.

PCR primer selection, environmental 18S rRNA gene clone libraries construction and operational taxonomic unit (OTU) calling

Total DNA from the five environmental samples (25_ANS, 25_OXS, 200_ANS, 200_ANW and 03_CRS) was extracted using a high salt concentration extraction buffer, followed by chloroform–phenol extraction and precipitation with isopropanol as described previously (Stoeck & Epstein, 2003). Amplification of 18S rRNA gene was carried out using three different primer sets (Table 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 coamplifies a range of non-fungal eukaryote genes such as Metazoa, *Cercozoa*, *Viridiplantae*, Alveolata, Centroheliozoa 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 a relatively high fungal specificity and previously amplified only a few nontarget taxa (green algae and *Pseudomonas*) from Antarctic soil samples (Malosso *et al.*, 2006). We also applied the universal

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

Table 2. Primer sets used in this study to amplify 18S rRNA gene sequences from genomic environmental DNA

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

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

eukaryote primer pair EukA and EukB, routinely used in environmental eukaryote diversity surveys (Massana *et al.*, 2004; Stoeck *et al.*, 2006; Euringer & Lueders, 2008), which amplifies nearly the full length of the 18S rRNA gene (Table 2) of a wide range of higher eukaryote taxon groups (Medlin *et al.*, 1988).

Amplicons were ligated into pGEM-T vector and transformed into *Escherichia 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 Qiagen's 96-well Directprep Kit. The presence of 18S rRNA gene inserts was confirmed by a standard M13-PCR amplification of extracted plasmids. In sum, 1440 positively screened plasmids were subjected to restriction fragment length polymorphism (RFLP) analyses. Therefore, between 200 and 400 ng of amplification products with expected sizes were digested with 7.5 U of the restriction endonuclease HaeIII (New England Biolabs, Beverly, MA) 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 2–3 h. At least one representative clone of each RFLP pattern ($n = 549$) was partially sequenced (*c.* 600 nucleotides) at MWG-Biotech as described above.

Because partial fragments could only be adequately aligned within each primer set (the sequence overlap was too small for partial fragments to be aligned between sequences obtained by different primer sets), sequences of each individual primer set were grouped separately into OTUs based on 99.0% sequence similarity using three independent (one for each primer pair-derived sequence set) DOTUR analyses (Schloss & Handelsman, 2005). One representative of each OTU that was identified as a fungal sequence using a BLASTN search in the GenBank nr-database (Altschul *et al.*, 1997) was chosen for full-fragment sequencing ($n = 100$). The obtained 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)]. Nine potentially chimeric sequences were identified and removed before subsequent sequence analyses. This left us with 91 full-fragment sequences that were analyzed together in a final DOTUR analysis. Similarities between two sequences were calculated using a custom program (PAIRALIGN) provided by M. Nebel (University of Kaiserslautern), which uses IUB matrix-based pairwise alignments. In total, 549 partial and 91 full-fragment environmental sequences have been deposited in the GenBank database under accession numbers GU071985–GU072533, GU072549–GU072590 and GQ120105–GQ120153.

Phylogenetic analyses

Environmental 18S rRNA gene sequences were compared initially with 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. Maximum-likelihood analyses were conducted using RAXML (Stamatakis *et al.*, 2008) on the CIPRES Portal v. 1.15 (http://www.phylo.org). The relative stability of tree topologies was assessed using 1000 bootstrap replicates. Heuristic searches for bootstrap analyses used stepwise addition, starting trees with simple addition of sequences and tree bisection and reconnection branch swapping. Maximum-likelihood bootstrapping analyses were carried out using RAXML with all free model parameters estimated by RAXML as described in Stamatakis *et al.* (2008). Details on model parameters for the individual alignments are given in the legends of Figs 1–3.

Community comparisons

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 the abundance of environmental fungal OTUs ($J_{abundance}$). An unweighted pair group mean average (UPGMA) cluster analysis 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 of species richness for all clone libraries were calculated in ESTIMATES (Colwell, 2005).

Results and discussion

We subjected 1440 environmental clones obtained from five individual sampling events in the Arabian Sea (Table 1) to RFLP analyses. The clones grouped into 549 distinct RFLP patterns, which were subjected to 18S rRNA gene sequence analyses. Surprisingly, GenBank BLASTN analyses of one representative sequence from each RFLP pattern identified only 268 unique RFLP patterns as members of the kingdom Fungi. Considering the replicates of these unique patterns, in total, we could putatively identify 455 out of the initial 1440 clones as taxonomically affiliated to the kingdom Fungi. The remaining RFLP patterns ($n = 281$) represented sequences falling into a number of different taxonomic

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

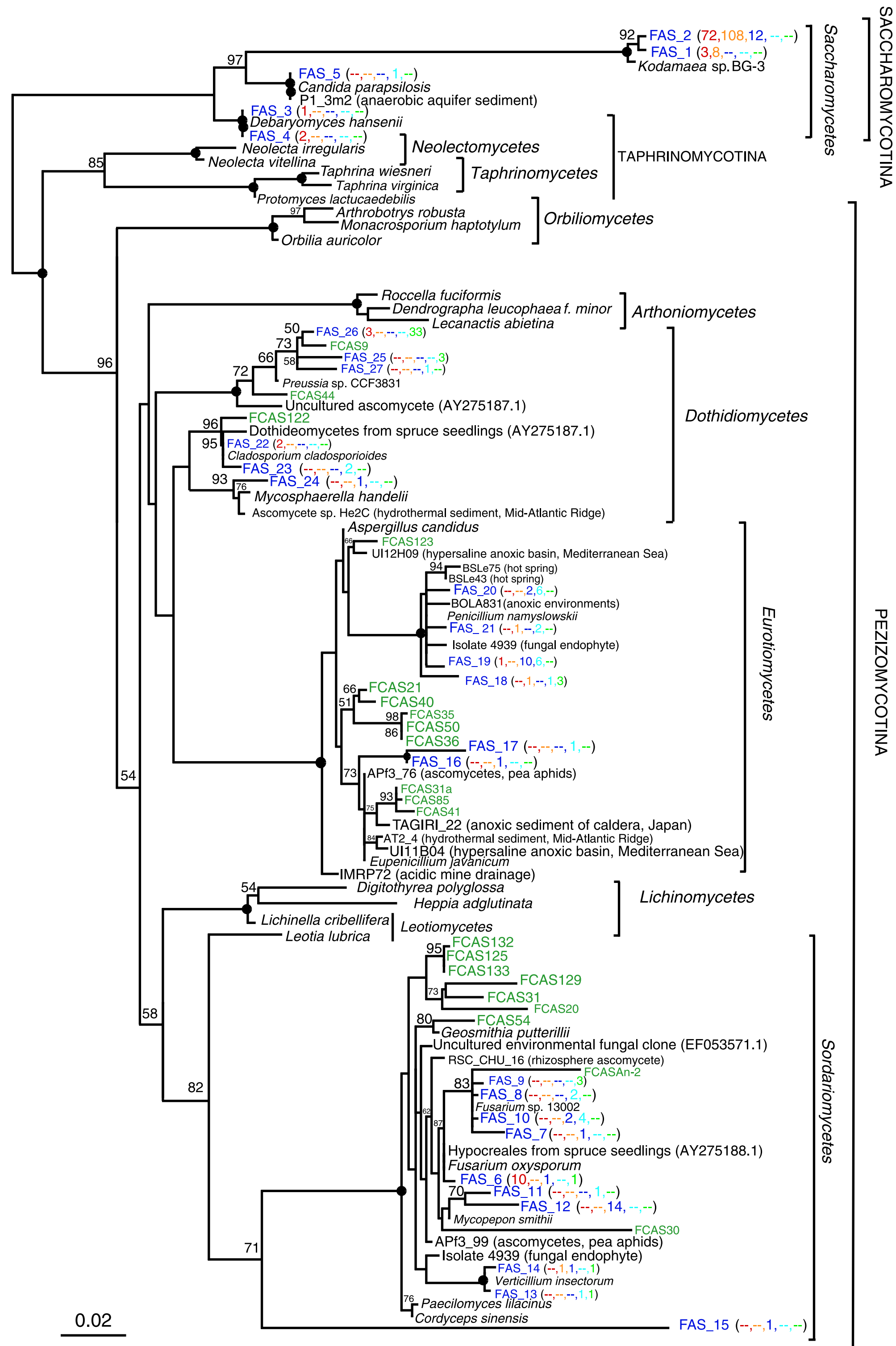

Fig. 1. Maximum-likelihood phylogenetic tree of the 18S rRNA gene sequences of phylum *Ascomycota* showing the positions of environmental OTUs and from cultured isolates obtained from the Arabian Sea. The tree was constructed using a GTR+I+G evolutionary model with the variable-site γ distribution shape parameter at 0.645 and the proportion of invariable sites at 0.312, based on 925 unambiguously aligned positions. Full support from 1000 bootstrap replicates is indicated by a black-filled dot at the respective node. Other support values are only displayed when > 50. Numbers in parentheses following environmental OTU names indicate the number of sequences of this OTU that were found in the individual libraries. Sequences from cultured isolates are in green and designated as FCAS followed by the culture identification number. Color coding for environmental libraries is given in Table 1.

groups (Table 3, Supporting Information, Table S1). The partial sequences ($n = 268$) obtained from representatives of each unique RFLP pattern grouped into 91 distinct OTUs called at 99% sequence similarity: 25 OTUs for primer set Fung1, 54 OTUs for primer set Fung2 and 12 OTUs for primer set EukAB. After we sequenced one complete fragment from at least one representative of each individual OTU, we were able to analyze all sequences in one individual

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

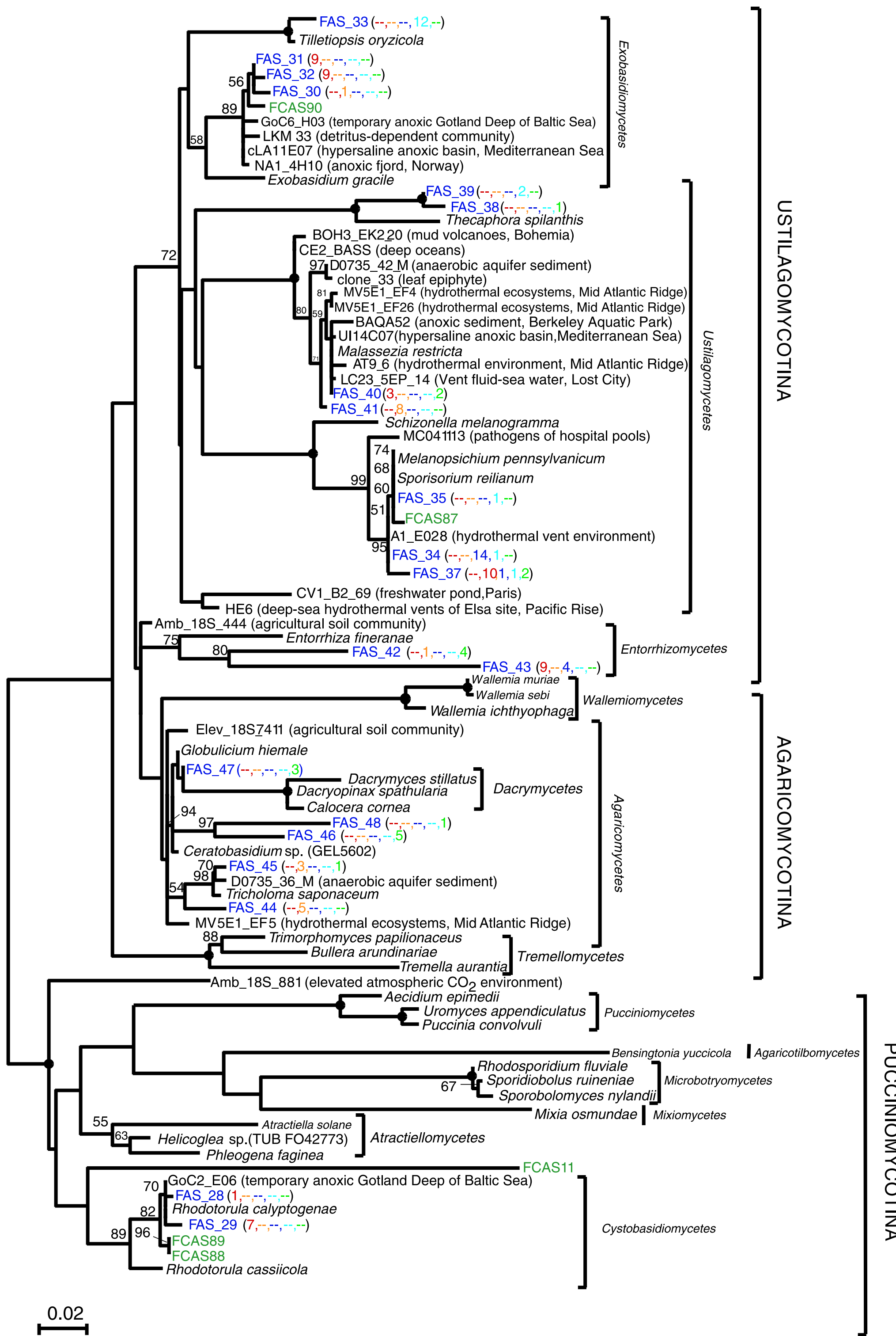

Fig. 2. Maximum-likelihood phylogenetic tree of the 18S rRNA gene sequences of phylum *Basidiomycota* showing the positions of environmental OTUs and from cultured isolates obtained from the Arabian Sea. The tree was constructed using a GTR+I+G evolutionary model with the variable-site γ distribution shape parameter at 0.737 and the proportion of invariable sites at 0.336, based on 785 unambiguously aligned positions. Further legend as in Fig. 1.

DOTUR run, which resulted in 48 distinct OTUs overall, indicating that a number of OTUs obtained by the individual primer-set analyses were shared among two or three primer sets. Table 4 presents an overview of the numbers of clones, RFLP patterns, sequences and OTUs analyzed in this study.

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

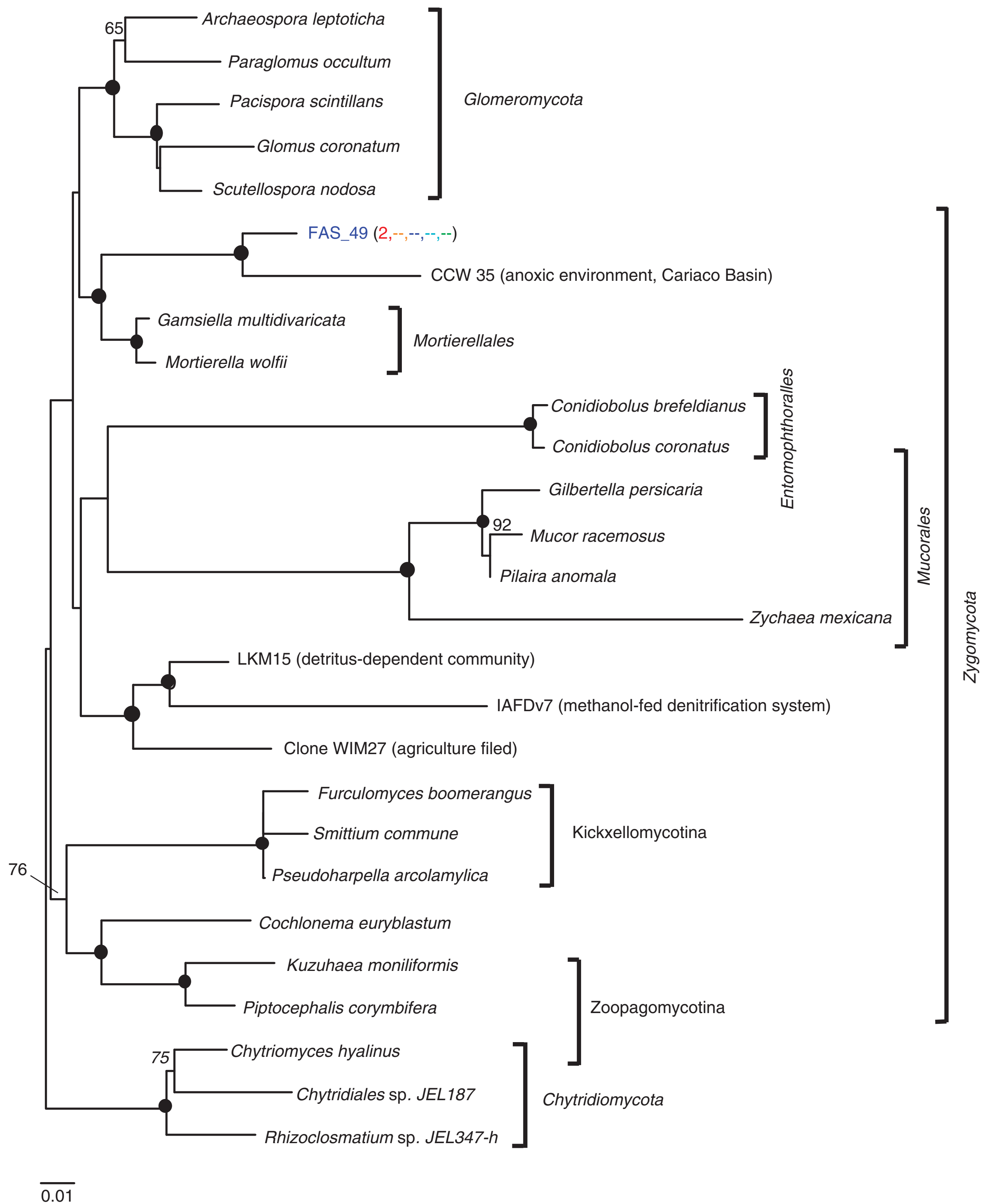

Fig. 3. Maximum-likelihood phylogenetic tree of the 18S rRNA gene sequences of phyla *Zygomycota*, *Glomeromycota* and *Chytridiomycota* showing the position of the environmental OTU obtained from the Arabian Sea branching as sister to *Mortierellales*. The tree was constructed using a GTR+I+G evolutionary model with the variable-site γ distribution shape parameter at 0.571 and the proportion of invariable sites at 0.39 based on 1436 unambiguously aligned positions. Further legend as in Fig. 1.

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Selectivity of fungal-specific primers

More than half of the unique RFLP patterns obtained and analyzed by sequencing and BLASTN ($n = 549$) turned out to be of nonfungal origin ($n = 281$) (Table 4). As expected, the largest proportion (74.07%) of such nonfungal sequences was retrieved within the RFLPs obtained with the universal eukaryote primer set EukAB, but also putatively fungal-specific primer sets retrieved 48.18% (Fung1) and 35.05% (Fung2) unique nonfungal taxa (Table 3). These findings met our expectations, considering that primer set Fung1 has a lower specificity to fungi compared with primer set Fung2 (see Materials and methods).

Specific PCR primers for environmental studies aim to amplify all members of the fungal community without bias, while excluding the coamplificaton of other eukaryote sequences. As noted earlier (Anderson *et al.*, 2003), this is particularly difficult using the highly conserved 18S rRNA gene region (Page & Holmes, 1998). Several authors claim that they have designed or applied truly specific fungal 18S rRNA gene primer pairs (Smit *et al.*, 1999; Borneman & Hartin, 2000). However, there is conflicting evidence for this specificity as other authors reported amplification of non-fungal templates using the same primer pairs (Borneman & Hartin, 2000; Anderson *et al.*, 2003). It seems that most, if not all, fungal 18S rRNA gene primers are prone to observable cross-kingdom amplifications. Further evidence for this comes from BLAST homology analyses for fungal 18S rRNA gene primers indicating a relatively low success rate for most primer pairs in recovering fungal sequences, but coamplification of nonfungal targets (Pang & Mitchell, 2005). The degree to which cross-kingdom amplification occurs largely depends on the choice of specific primers, PCR conditions but also the abundance of specific fungal taxon groups in relation to the co-occurrence of eukaryote nontarget organisms in the environmental samples under study, i.e. when nontarget DNA is much more abundant in an environmental sample than target DNA, it is reasonable to assume that there is a greater than usual chance of nontarget DNA being amplified. Such systematic biases can distort diversity assessments. Furthermore, different fungal-specific primer sets may amplify different subsets of the fungal community (Fig. 4; Anderson *et al.*, 2003). This severely affects the comparability of different fungal 18S rRNA gene data sets obtained from PCR amplification with different primers. A solution may arise from the application of a multiple-primer approach as suggested for general microbial eukaryote diversity surveys (Stoeck *et al.*, 2006).

Table 3. Taxonomic assignment of unique nonfungal RFLPs ($n = 281$) after sequencing and BLASTN analysis of one representative sequence from each RFLP pattern ($n = 549$), obtained after restriction digest of 1440 clones

Primer set	Taxonomic assignment of nonfungal RFLPs	Proportion of unique nonfungal RFLPs obtained with each primer set (%)
Fung1	*Corallochytrium*; Choanoflagellates; Metazoa (*Gastrotricha*)	48.18
Fung2	Bacteria (*Proteobacteria*); *Viridiplantae*; Rhizaria; *Corallochytrium*; Metazoa (*Gastrotricha*)	35.05
EukAB	Bacteria (*Proteobacteria*, *Bacteroidetes*, *Firmicutes*); Choanoflagellates; Rhizaria, *Eugleonozoa*; Alveolata; Metazoa (Arthropods, Nematodes, Annelids)	74.07

The three primer sets used to generate the individual clone libraries are described in Table 2. Values given in the last column depict the proportion of unique nonfungal RFLP patterns (identified after BLASTN analyses of obtained sequences from each unique RFLP) relative to the number of total distinct RFLP patterns obtained with the respective primer set (193 for Fung1; 194 for Fung2; and 162 for EukAB). A table detailing the taxonomic affiliation of each individual nontarget sequence (RFLP pattern) is given as Supporting Information.

Diversity patterns of fungal communities in the Arabian Sea

A UPGMA cluster analysis of the $J_{abundance}$ index (Fig. 5) reveals that the fungal communities from the different sampling sites (Table 1) are distinctly different from each other. Of all the comparisons, the two coastal sites sampled under anoxic and oxic conditions (25_ANS, 25_OXS) are most similar in their fungal community membership ($J_{abundance} = 0.48$). The sediment (200_ANS)- and water column (200_ANW)-derived fungal communities from the OMZ offshore site cluster together with a $J_{abundance}$ of 0.35. The fungal community from the coral reef reference site (03_CRS) showed the highest dissimilarity to all other samples ($J_{abundance} = 0.06–0.1$)

The Indian Ocean has a characteristic seasonal anoxic condition that develops along the western margin during October to January (Naqvi *et al.*, 2006). In this process, the coastal sites are subjected to seasonal oxygen fluctuations. Our analysis suggests a clear separation of fungal communities adapted to permanently oxic conditions, temporal anoxia and the ones adapted to permanent anoxia. Recent studies have shown that numerous fungi can adapt to alternate modes of respiration depending on oxygen availability (Daiber *et al.*, 2005; Jebaraj & Raghukumar, 2009). It is then reasonable to assume that the fungal community of the coastal site (25_ANS, 25_OXS), considered as an entity, may be capable of physiological adaptation when the oxygen

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Table 4. Overview of the total number of clones analyzed per library and primer set, total number of distinct RFLP patterns obtained, number of unique RFLP patterns that could be assigned to fungi and nonfungal taxon groups (for each distinct RFLP, we obtained a partial sequence that was analyzed by BLASTN) and number of distinct OTUs (called at 99% sequence similarity) obtained after DOTUR analyses of full-length fragments for each primer set

Clone library	Total number of clones analyzed by RFLP	Total number of distinct RFLP patterns	Number of unique RFLP patterns assigned to fungi after sequencing	Number of unique fungal OTUs	Number of unique RFLP patterns assigned to nonfungal taxon groups after sequencing
Primer set: *Fung1*					
25_ANS	96	38	35	10	3
25_OXS	96	24	23	6	1
200_ANS	96	36	4	3	32
200_ANW	96	62	18	5	44
03_CRS	96	33	20	3	13
Total	480	193	100	25*	93
Primer set: *Fung2*					
25_ANS	96	46	29	10	17
25_OXS	96	30	27	11	3
200_ANS	96	37	26	9	11
200_ANW	96	55	20	17	35
03_CRS	96	26	24	11	2
Total	480	194	126	54*	68
Primer set: *EukAB*					
25_ANS	96	42	17	6	25
25_OXS	96	17	7	2	10
200_ANS	96	36	17	6	19
200_ANW	96	28	0	0	28
03_CRS	96	39	1	1	38
Total	480	162	42	12*	120
Overall	1440	549	268	48†	281

The total number of OTUs does not equal the sum of OTUs of the individual libraries, but reflects the number of unique OTUs for all five individual libraries (obtained with the same primer set) together. For details, see Materials and methods.
†The overall number of OTUs does not equal the sum of OTUs of the three individual primer sets (= 91), but is a result from a separate DOTUR analysis that determined the number of unique OTUs for all three individual primer sets together. For details, see the Materials and methods.

concentration decreases. This adaptation is neither a prerequisite for survival in oxygenated coral reef sediment (03_CRS) nor for life under permanently anoxic conditions (200_ANS, 200_ANW).

Apart from a few exceptions (e.g. some *Chytridiomycetes*), no strictly anaerobic fungi have been described. This, however, contrasts with molecular diversity surveys in anaerobic aquatic environments, where fungal sequences sometimes account for a significant fraction of the clones (Dawson & Pace, 2002; Edgcomb *et al*., 2002; Stoeck & Epstein, 2003; Luo *et al*., 2005; Stoeck *et al*., 2006, 2007). Some recent studies in permanently anoxic habitats have found novel fungal clades to be restricted to these environments (Luo *et al*., 2005; Brad *et al*., 2008; Laurin *et al*., 2008). Support for the adaptation of fungi to anoxia comes from reports of some ascomycetes (Dumitru *et al*., 2004; Sonderegger *et al*., 2004) and some basidiomycetes (Fell *et al*., 2001) that are capable of fermentation and anaerobic growth. Some of these fungi have even been isolated from anaerobic deep-sea environments (Nagahama *et al*., 2003).

Considering these wide ecological and physiological adaptations of different fungi, it is not unexpected that fungal communities thriving under oxygenated conditions (25_OXS) are distinctively different from fungal communities living under anoxic conditions (25_ANS). The same applies to those from anoxic sediments (200_ANS) and anoxic water samples (200_ANW). However, the actual degree of dissimilarity is difficult to assess with our data due to severe undersampling (Fig. 6).

Phylogeny of environmental fungal rDNA sequences

The analyses of the intrastrain variability of the 18S rRNA gene of five strains obtained in this study revealed a maximum divergence of 0.77% (FCAS35, 0.18–0.36%; FCAS36, 0.17–0.5%; FCAS41, 0.25–0.77%; FCAS89, 0–0.14%; and FCAS125, 0%). Thus, we are confident that a 1% divergence between two different OTUs is sufficient to represent distinct genotypes. The environmental OTUs identified in this study are designated as FAS (fungal OTUs

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

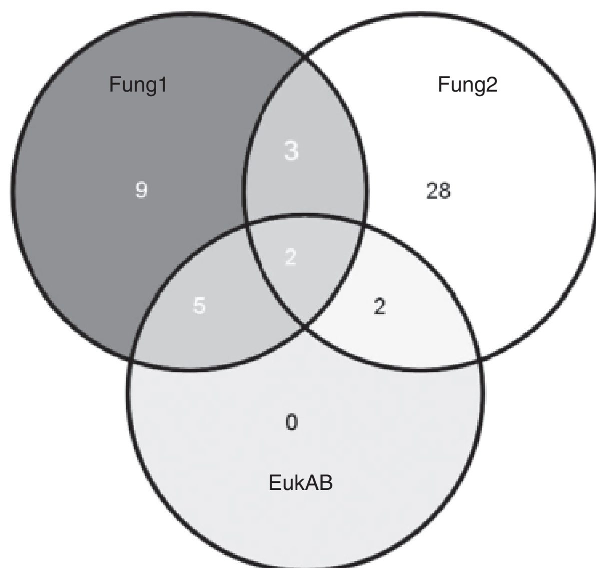

Fig. 4. Venn diagram showing the number of OTUs that were recovered simultaneously by two or three of the primer sets used in this study (Fung1, Fung2 and EukAB; see Table 2) and number of OTUs recovered exclusively with a single primer set. All OTUs, which were retrieved by the universal eukaryote primer set (EukAB), were also detected with the two fungal-specific primer sets (Fung1 and Fung2).

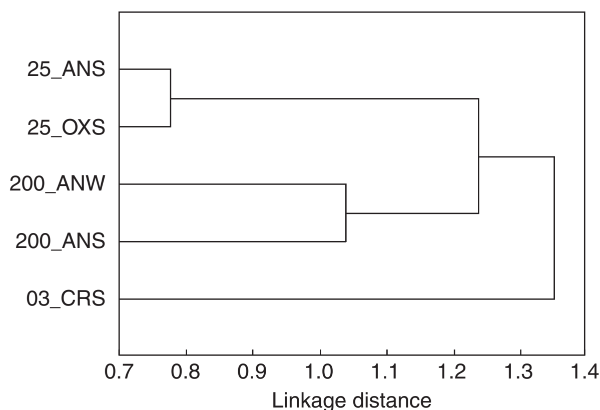

Fig. 5. Dendrogram resulting from calculated Jaccard index based on incidence ($J_{incidence}$) of unique OTUs, as a measure of community similarity between the samples under study. For information on sampling sites, see Table 1. Similarity values were transformed into a distance matrix and subsequent cluster analysis was performed using the UPGMA algorithm.

from the Arabian Sea). The OTUs from the five sample sites were assigned to the Dikarya (*Ascomycota*, 27 OTUs; *Basidiomycota*, 20 OTUs), and one OTU branched within the subphylum Mucoromycotina (phylum *Zygomycota*).

OTUs belonging to the *Ascomycota* (Fig. 1) grouped within the subphyla Saccharomycotina and Pezizomycotina. FAS_2 is the most frequently represented OTU with a close similarity to the yeast *Kodamaea* sp. (99.67%) belonging to *Saccharomycetes*. *Saccharomycete* yeasts are able to cope with anoxia while producing ethanol (Prior *et al*., 1989). A large number of OTUs (FAS_6, FAS_7, FAS_8, FAS_9, FAS_10, FAS_11 and FAS_12) belong to *Sordariomycetes*. Their closest known relatives belong to the genus *Fusarium*, which serves as a model to study fungal denitrification under low-oxygen conditions (Takaya *et al*., 1999; Daiber *et al*., 2005). Six OTUs from the Arabian Sea (Fig. 1) branch within the *Eurotiomycetes*. These sequences are affiliated with a number of environmental sequences obtained from a hypersaline anoxic Mediterranean deep-sea basin (Alexander *et al*., 2009), anoxic meromictic lake sediments (Takishita *et al*., 2007a), anoxic coastal sediments (Dawson & Pace, 2002), acidic mine drainages (Baker *et al*., 2004), hydrothermal vent habitats (López-García *et al*., 2007) and boiling springs' lake sediment (Wilson *et al*., 2008). This strongly indicates that the respective phyloclade includes extremophile fungi that are capable of thriving in the most extreme aquatic environments known to date. Described representatives in this clade are *Penicillium* and *Aspergillus*, versatile ubiquitously distributed species that are capable of anaerobic denitrification (Takasaki *et al*., 2004). This agrees with our observation that under laboratory conditions, isolate FCA-S31a (99.45% similarity to *Penicillium namyslowskii*) grows anaerobically while reducing nitrate (Jebaraj & Raghukumar, 2009). The widespread distribution of ascomycete fungi in oxygen-depleted and extreme habitats is also indicated by the close affiliation of a number of further OTUs from the Arabian Sea to environmental clones obtained from other anoxic habitats and extreme environments. For example, FAS_5 is 99.88% similar to P1_3m2 obtained from anoxic aquifer sediment (Brad *et al*., 2008), and the dothideomycete OTU FAS_24 is 98.92% similar to an environmental clone from the Mid-Atlantic hydrothermal vent sediment (López-García *et al*., 2007).

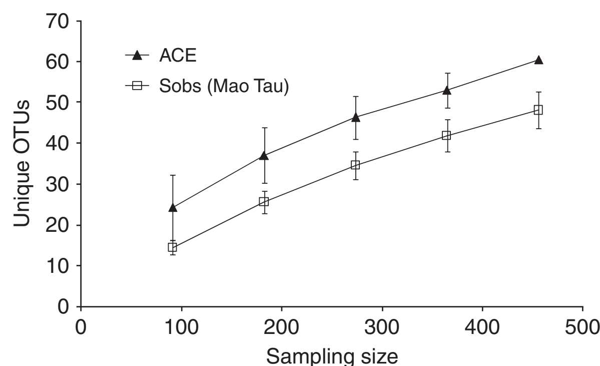

Fig. 6. Sampling saturation analyses of our fungal molecular diversity survey in the Arabian Sea. Species accumulation curve and cumulative abundance-based coverage estimator (ACE) total diversity estimate for all libraries (see Table 1) are plotted.

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

Within the phylum *Basidiomycota* (Fig. 2), we identified sequences branching in the subphyla Pucciniomycotina, Agaricomycotina and Ustilagomycotina. Only a few are closely (> 97% sequence similarity) related to previously deposited sequences of described basidiomycetes. For example FAS_47 is 99.46% similar to the wood-decaying saprophyte *Globulicium hiemale*, and FAS_28 and FAS_29 are similar to the nitrogenous compound scavenger *Rhodotorula cassiicola* (99.83% and 98.60% sequence similarity, respectively). Most OTUs are more divergent from sequences of described taxa such as the *Ustilagomycetes* FAS_38 and FAS_39, exhibiting only 93% sequence similarity to the plant pathogen *Thecaphora spilanthis*. Many basidiomycete OTUs from the Arabian Sea branch in clades that include other environmental fungal sequences from a variety of oxygen-depleted habitats. Examples are three exobasidiomycete OTUs (FAS_30, FAS_31 and FAS_32), which are related to sequences from an anoxic Norwegian Fjord (A. Behnke, K. Barger, J. Bunge & T. Stoeck, unpublished data), an anoxic Mediterranean deep-sea basin (Alexander *et al*., 2009) and an anoxic basin in the central Baltic Sea (Stock *et al*., 2009). The closest described relative of this sequence clade is the plant pathogen *Exobasidium gracile*, with at least 37.3% sequence divergence. The OTUs FAS_40 and FAS_41 branch together with sequences from hydrothermal vent ecosystems (López-García *et al*., 2007; T. Le Calvez, S. Mahe & P. Vandenkoomhuyse, unpublished data), anoxic intertidal sediment (Dawson & Pace, 2002), an anoxic Mediterranean deep-sea basin (Alexander *et al*., 2009) and other deep-sea sites (Bass *et al*., 2007). This cluster, belonging to Ustilagomycotina, is within a clade that has been recognized as a 'hydrothermal and/or anaerobic fungal group' (López-García *et al*., 2007). 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 OTUs FAS_40 and FAS_41. Finding a sequence related to a human skin pathogen in anoxic marine environments seems unusual at first glance. However, sequences closely related to *M. restricta* were also reported from hydrate-rich deep-sea sediments of South China Sea (Lai *et al*., 2007), indicating a wide ecological diversity of taxa falling in this sequence clade. Interestingly, OTUs obtained exclusively from oxic regions of our study grouped together within the agaricomycetes, along with culture sequences and environmental clones obtained from anaerobic aquifers, hydrothermal sediment and agricultural soil (Euringer & Lueders, 2008; Le Calvez *et al*., submitted; Lesaulnier *et al*., 2008).

One OTU (FAS_49) obtained from our study (Fig. 3) originates from a fungus other than Dikarya. This clone, together with clone CCW35 from anoxic salt marsh water (Stoeck & Epstein, 2003, sequence similarity 90.45%), branches among zygomycetes. The closest named species was *Mortierella wolfii*, subphylum Mucoromycotina, with only 90.02% sequence similarity. There are contrasting reports on the capability of zygomycetes to grow anaerobically (Kurakov *et al*., 2008; Schmidt *et al*., 2008). Even though we have a poor representation of zygomycetes in our study, they are reported to be a major component of fungal communities in oxygen-depleted environments (Slapeta *et al*., 2005; Takishita *et al*., 2007b; Brad *et al*., 2008). Similarly, we did not discover *Chytridiomycetes* in our clone libraries. This was unexpected, because the latter taxon group includes fermentative anaerobes (Orpin, 1977) and was found in previous 18S rRNA gene diversity surveys in anoxic habitats (Stoeck *et al*., 2007; Takishita *et al*., 2007a, b). This poor representation of fungal groups other than ascomycetes and basidiomycetes may be due to undersampling (Fig. 6), combined with a higher efficiency of the primers to amplify genes from *Ascomycota* and *Basidiomycota* (Anderson *et al*., 2003; Malosso *et al*., 2006). Indeed, previous studies also failed to obtain chytridiomycete sequences using Fung1 (Gomes *et al*., 2003) and Fung2 primers (Malosso *et al*., 2006).

Even though we are not able to distinguish native from transitory fungi (both spores and mycelium contain rDNA; Osherov *et al*., 2002), it is reasonable to assume that the majority of fungi detected in this study are indigenous. The reasoning for this assumption is their close relation to a number of fungal sequences detected (some of them exclusively) in oxygen-deficient environments. Furthermore, we conducted a laboratory experiment to largely exclude the possibility of PCR amplification of DNA from nonindigenous fungi. Wind or water currents are vectors to disperse fungal spores. Such transitory material could theoretically be sources of genomic DNA, although the corresponding organisms are not active members of the fungal communities under study. Therefore, we harvested spores from two cultured isolates originating from the sampling sites (the basidiomycete isolate FCAS11 and the ascomycete isolate FCAS21). The protocol that we used to extract nucleic acids from the environmental samples under study failed to extract DNA from these spores. This largely (even though certainly not entirely) excludes the possibility that the clone libraries constructed from the Arabian Sea samples include significant proportions of nonindigenous transitory fungi (spores) (results not shown, but available from the authors upon request).

Cultured fungi

Traditionally, fungal diversity studies in environmental samples are based on cultivation approaches. Limitations of this strategy – such as the inability to separate biomass from particulate material and lack of growth media and cultivation conditions suitable for all members of the community – are

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

held responsible for our 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). However, this strategy is no panacea, as it has biases such as PCR-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.

We obtained 26 cultures from all the sampling locations. The taxonomic breadth of the isolated strains is restricted: five isolates (FCAS11, FCAS87, FCAS88, FCAS89 and FCAS90) could be assigned to *Basidiomycota*, predominantly Pucciniomycotina (Fig. 2), and 21 of them belonged to Pezizomycotina of *Ascomycota* (Fig. 1). Both divisions of fungi have a high representation of cultured taxa, indicating the accessibility of these groups to cultivation. With the exception of two ascomycetes (FCAS31 and FCAS129) and three basidiomycetes (FCAS87, FCAS90 and FCAS11), all cultures have $> 99\%$ sequence similarities to previously cultured and well-described fungi. This indicates that cultivation using standard techniques generally produces more isolates of already known taxa. We note that cultivation under anoxic conditions may have produced a different set of fungi being more divergent to known taxa. Current efforts are in order to stimulate growth under anoxic conditions using a variety of different media. Thus far, we were able to grow one of the isolates (isolate FCAS11) discussed in this study under anoxic cultivation conditions. This supports the hypotheses that at least some fungi from the OMZ region, subjected to changing oxygen conditions, can readily adapt to anoxia.

We also obtained a few isolates that show only a low similarity to previously described and sequenced fungi. These are, for example the basidiomycete isolates FCAS90 exhibiting 97.10% sequence similarity to *Graphiola cylindrical*, FCAS87 with only 62.5% similarity to *Sporisorium reilianum* and FCAS11 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 a living condition paves the way for an in-depth analysis of their phylogeny, morphology and ecology. A current multigene analysis of isolate FCAS11, along with ultrastructural analyses, points to the discovery of a novel fungal taxon on class level with the capability of anaerobic denitrification (C.S. Jebaraj, T. Boekhout, W. Muller, F. Kauff & T. Stoeck, unpublished data). 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.

Acknowledgements

We are grateful to the Captain, crew and colleagues onboard *CRV Sagar Sukti* for their help during the sampling. This study was funded by grants STO4141/2-3 and STO414/3-1 by the Deutsche Forschungsgemeinschaft to T.S. C.S.J. would like to thank the University of Kaiserslautern for an international fellowship and Dr S.R. Shetye, Director, NIO, for the travel support and encouragement provided for carrying out the work. We are grateful to Dr Seshagiri Raghukumar for his critical review of the manuscript and for helping us with the identification of the fungi. Furthermore, we acknowledge the comments of two anonymous reviewers, who helped to improve our manuscript. This is NIO's contribution number 4639.

References

Alexander E, Stock A, Breiner HW, Behnke A, Bunge J, Yakimov MM & Stoeck T (2009) Microbial eukaryotes in the hypersaline anoxic L'Atalante deep-sea basin. *Environ Microbiol* **11**: 360–381.

Alsumard LT, Golubic S & Priess K (1995) Fungi in corals: symbiosis or disease? Interactions between and fungi cause pearl-like skeleton biomineralization. *Mar Ecol Prog Ser* **117**: 137–147.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Anderson IC, Campbell CD & Prosser JI (2003) Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environ Microbiol* **5**: 36–47.

Baker BJ, Lutz MA, Dawson SC, Bond PL & Banfield JF (2004) Metabolically active eukaryotic communities in extremely acidic mine drainage. *Appl Environ Microb* **70**: 6264–6271.

Bass D, Howe A, Brown N *et al.* (2007) Yeast forms dominate fungal diversity in the deep oceans. *P R Soc B* **274**: 3069–3077.

Bills GF & Polishook JD (1994) Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. *Mycologia* **86**: 187–198.

Borneman J & Hartin RJ (2000) PCR primers that amplify fungal rRNA genes from environmental samples. *Appl Environ Microb* **66**: 4356–4360.

Brad T, Braster M, van Breukelen BM, van Straalen NM & Roling WFM (2008) Eukaryotic diversity in an anaerobic aquifer polluted with land fill leachate. *Appl Environ Microb* **74**: 3959–3968.

Chao A & Shen TJ (2003-2005) *Program SPADE (Species Prediction and Diversity Estimates)*. Available at http://chao.stat.nthu.edu.tw (accessed July 2009).

Cole JR, Chai B, Marsh TL *et al.* (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**: 442–443.

Colwell RK (2005) EstimateS: Statistical estimation of species richness and shared species from samples. Version 7.5. User's guide and application. Available at http://purl.oclc.org/estimates (accessed July 2009).

Daiber A, Shoun H & Ullrich V (2005) Nitric oxide reductase (P450nor) from *Fusarium oxysporum*. *J Inorg Biochem* **99**: 85–193.

Dawson SC & Pace NR (2002) Novel kingdom-level eukaryotic diversity in anoxic environments. *P Natl Acad Sci USA* **99**: 8324–8329.

Dighton J (2003) *Fungi in Ecosystem Processes*. Marcel Dekker Inc., New York.

Dumitru R, Hornby JM & Nickerson KW (2004) Defined anaerobic growth medium for studying *Candida albicans* basic biology and resistance to eight antifungal drugs. *Antimicrob Agents Ch* **48**: 2350–2354.

Edgcomb VP, Kysela DT, Teske A, de Vera Gomez A & Sogin ML (2002) Benthic eukaryotic diversity in the Guaymas Basin hydrothermal vent environment. *P Natl Acad Sci USA* **99**: 7658–7662.

Epstein S & López-García P (2007) 'Missing' protists: a molecular prospective. *Biodivers Conserv* **17**: 261–276.

Euringer K & Lueders T (2008) An optimised PCR/T-RFLP fingerprinting approach for the investigation of protistan communities in groundwater environments. *J Microbiol Meth* **75**: 262–268.

Fell JW, Boekhout T, Fonseca A & Sampaio JP (2001) The Mycota VII. *Systematics and Evolution. Part B* (McLaughlin DJ, McLaughlin EG & Lemke PA, eds), pp. 1–36. Springer-Verlag, Berlin.

Gomes NCM, Fagbola O, Costa R, Rumjanek NG, Buchner A, Mendona-Hagler L & Smalla K (2003) Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. *Appl Environ Microb* **69**: 3758–3766.

Jebaraj CS & Raghukumar C (2009) Anaerobic denitrification in fungi from the coastal marine sediments off Goa, India. *Mycol Res* **113**: 100–109.

Kappe R, Fauser C, Okeke CN & Maiwald M (1996) Universal fungus-specific primer systems and group specific hybridization oligonucleotides for 18S rDNA. *Mycoses* **39**: 25–30.

Kohlmeyer J & Kohlmeyer E (1979) *Marine Mycology. The Higher Fungi*. Academic Press, New York.

Kurakov AV, Lavrent'ev RB, Nechitailo TY, Golyshin PN & Zvyagintsev DG (2008) Diversity of facultatively anaerobic microscopic mycelial fungi in soils. *Microbiology* **77**: 90–98.

Lai X, Cao L, Tan H, Fang S, Huang Y & Zhou S (2007) Fungal communities from methane hydrate-bearing deep-sea marine sediments in South China Sea. *ISME J* **1**: 756–762.

Laurin V, Labbé N, Parent S, Juteau P & Villemur R (2008) Microeukaryote diversity in a marine methanol-fed fluidized denitrification system. *Microb Ecol* **56**: 637–648.

Lesaulnier C, Papamichail D, McCorkle S, Ollivier B, Skiena S, Taghavi S, Zak D & van der Lelie D (2008) Elevated atmospheric CO_2 affects soil microbial diversity associated with trembling aspen. *Environ Microbiol* **10**: 926–941.

López-García P, Vereshchaka A & Moreira D (2007) Eukaryotic diversity associated with carbonates and fluid–seawater interface in Lost City hydrothermal field. *Environ Microbiol* **9**: 546–554.

Luo Q, Krumholz LR, Najar FZ, Peacock AD, Roe BA, White DC & Elshahed MS (2005) Diversity of the microeukaryotic community in sulfide-rich Zodletone Spring (Oklahoma). *Appl Environ Microb* **71**: 6175–6184.

Maiwald M, Kappe R & Sonntag HG (1994) Rapid presumptive identification of medically relevant yeasts to the species level by polymerase chain reaction and restriction enzyme analysis. *J Med Vet Mycol* **32**: 115–122.

Malosso E, Waite IS, English L, Hopkins DW & O'Donnell AG (2006) Fungal diversity in maritime Antarctic soils determined using a combination of culture isolation, molecular fingerprinting and cloning techniques. *Polar Biol* **29**: 552–561.

Massana R, Balagué V, Guillou L & Pedrós-Alió C (2004) Picoeukaryotic diversity in an oligotrophic coastal site studied by molecular and culturing approaches. *FEMS Microbiol Ecol* **50**: 231–243.

Medlin L, Elwood HJ, Stickel S & Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S like rRNA-coding regions. *Gene* **71**: 491–499.

Nagahama T, Hamamoto M, Nakase T, Takaki Y & Horikoshi K (2003) *Cryptococcus surugaensis* sp. nov., a novel yeast species from sediment collected on the deep-sea floor of Suruga Bay. *Int J Syst Evol Micr* **53**: 2095–2098.

Naqvi SWA, Naik H, Pratihary A, DeSouza W, Narvekar PV, Jayakumar DA, Devol AH, Yoshinari T & Saino T (2006) Coastal versus open-ocean denitrification in the Arabian Sea. *Biogeosciences* **3**: 621–633.

Newell SY (1996) Established and potential impacts of eukaryotic mycelial decomposers in marine/terrestrial ecotones. *J Exp Mar Biol Ecol* **200**: 187–206.

Oliveros JC (2007) VENNY. An interactive tool for comparing lists with Venn diagrams. Available at http://bioinfogp.cnb.csic.es/tools/venny/index.html (accessed July 2009).

Orpin CG (1977) Invasion of plant tissue in the rumen by the flagellate *Neocallimastix frontalis*. *J Gen Microbiol* **98**: 423–430.

Osherov N, Mathew J, Romans A & May GS (2002) Identification of conidial-enriched transcripts in *Aspergillus nidulans*using suppression subtractive hybridisation. *Fungal Genet Biol* **37**: 197–204.

Page RDM & Holmes EC (1998) *Molecular Evolution: A Phylogenetic Approach*. Blackwell Science, Oxford.

Pai SC, Gong GC & Liu KK (1993) Determination of dissolved oxygen in seawater by direct spectrophotometry of total iodine. *Mar Chem* **41**: 343–351.

Pang KL & Mitchell JI (2005) Molecular approaches for assessing fungal diversity in marine substrata. *Bot Mar* **48**: 332–347.

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Pivkin MV (2000) Filamentous fungi associated with holothurians from the Sea of Japan, off the Primorye Coast of Russia. *Biol Bull* **198**: 101–109.

Prior BA, Kilian SG & du Preez JC (1989) Fermentation of D-xylose by the yeasts *Candida shehatae* and *Pichia stipitis*. *Process Biochem* **24**: 21–32.

Raghukumar C (1986) Fungal parasites of the marine green algae, *Cladophora* and *Rhizoclonium*. *Bot Mar* **29**: 289–297.

Robison-Cox JF, Bateson MM & Ward DM (1995) Evaluation of nearest-neighbour methods for detection of chimeric small-subunit rRNA sequences. *Appl Environ Microb* **61**: 1240–1245.

Schloss PD & Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microb* **71**: 1501–1506.

Schmidt SK, Wilson KL, Meyer AF, Gebauer MM & King AJ (2008) Phylogeny and ecophysiology of opportunistic 'snow molds' from a subalpine forest ecosystem. *Microb Ecol* **56**: 681–687.

Shoun H, Kim DH, Uchiyama H & Sugiyama J (1992) Denitrification by fungi. *FEMS Microbiol Lett* **94**: 277–282.

Slapeta J, Moreira D & López-García P (2005) The extent of protist diversity: insights from molecular ecology of freshwater eukaryotes. *P R Soc B* **272**: 2073–2081.

Smit E, Leeflang P, Glandorf B, van Elsas JD & Wernars K (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Appl Environ Microb* **65**: 2614–2621.

Sonderegger M, Jeppsson M, Larsson C, Gorwa-Grauslund MF, Boles E, Olsson L, Spencer-Martin I, Hahn-Hagerdal B & Sauen U (2004) Fermentation performance of engineered and evolved xylose-fermenting *Saccharomyces cerevisiae* strains. *Biotechnol Bioeng* **87**: 90–98.

Stamatakis A, Hoover P & Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web-servers. *Syst Biol* **75**: 758–771.

Stock A, Bunge J, Jürgens K & Stoeck T (2009) Protistan diversity in the suboxic and anoxic waters of the Gotland Deep (Baltic Sea) as revealed by 18S rRNA clone libraries. *Aquat Microb Ecol* **55**: 267–284.

Stoeck T & Epstein SS (2003) Novel eukaryotic lineages inferred from small-subunit rRNA analyses of oxygen depleted marine environments. *Appl Environ Microb* **69**: 2657–2663.

Stoeck T, Taylor GT & Epstein SS (2003) Novel eukaryotes from the permanently anoxic Cariaco Basin (Caribbean Sea). *Appl Environ Microb* **69**: 5656–5663.

Stoeck T, Hayward B, Taylor GT, Varela R & Epstein SS (2006) A multiple PCR-primer approach to access the microeukaryotic diversity in environmental samples. *Protist* **157**: 31–43.

Stoeck T, Kasper J, Bunge J, Leslin C, Ilyin V & Epstein SS (2007) Protistan diversity in the arctic: a case of paleoclimate shaping modern biodiversity? *PLoS ONE* **2**: e728.

Takasaki K, Shoun H, Nakamura A, Hoshinu T & Takaya N (2004) Unusual transcription regulation of niaD gene under anaerobic conditions supporting fungal ammonia fermentation. *Biosci Biotech Bioch* **68**: 978–980.

Takaya N, Suzuki S, Kuwazaki S, Shoun H, Maruo F, Yamaguchi M & Takoe K (1999) Cytochrome P450nor, a novel class of mitochondrial cytochrome P450 involved in nitrate respiration in the fungus *Fusarium oxysporum*. *Arch Biochem Biophys* **372**: 340–346.

Takishita K, Miyake H, Kawato M & Maruyama T (2005) Genetic diversity of microbial eukaryotes in anoxic sediment around fumaroles on a submarine caldera floor based on the small-subunit rDNA phylogeny. *Extremophiles* **9**: 185–196.

Takishita K, Tsuchiyaa M, Kawatoa M, Ogurib K, Kitazatob H & Maruyamaa T (2007a) Diversity of microbial eukaryotes in anoxic sediment of the saline meromictic lake Namako-ike (Japan): on the detection of anaerobic or anoxic-tolerant lineages of eukaryotes. *Protist* **158**: 51–64.

Takishita K, Yubuki N, Kakizoe N, Inagaki Y & Maruyama T (2007b) Diversity of microbial eukaryotes in sediment at a deep-sea methane cold seep: surveys of ribosomal DNA libraries from raw sediment samples and two enrichment cultures. *Extremophiles* **11**: 563–576.

Vainio EJ & Hantula J (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycol Res* **104**: 927–936.

Wilson MS, Siering PL, White CL, Hauser ME & Bartles AN (2008) Novel archaea and bacteria dominate stable microbial communities in North America's largest hot spring. *Microb Ecol* **56**: 292–305.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Closest BLASTN match of nonfungal sequences.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Downloaded from https://academic.oup.com/femsec/article/71/3/399/489743 by guest on 25 April 2024

© 2009 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved