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Phylogenetic analyses and nitrate reducing activity of fungal cultures isolated from the permanent, oceanic oxygen minimum zone of the Arabian Sea.

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Abstract

Reports on the active role of fungi as denitrifiers in terrestrial ecosystems have stimulated an interest to study the role of fungi in marine oxygen deficient systems. In this study, the culturable diversity of fungi was investigated from four stations within the permanent, oceanic, oxygen minimum zone (OMZ) of the Arabian Sea. The isolated cultures grouped within the two major fungal phyla *Ascomycota* and *Basidiomycota*; diversity estimates in the stations sampled indicated that the diversity from the oxygen depleted environments (ODEs) are less compared to mangrove regions and deep sea habitats. Phylogenetic analyses of 18S rRNA sequences revealed a few divergent isolates that clustered with environmental sequences previously obtained by others. This is significant as these isolates represent phylotypes which were known so far only from metagenomic studies and are of phylogenetic importance. Nitrate reduction activity, the first step in the denitrification process was recorded for isolates under simulated anoxic, deep-sea conditions showing ecological significance of fungi in the oxygen depleted habitats. This report increases our understanding of fungal diversity in unique, poorly studied habitats and underlines the importance of fungi in the ODEs.

Keywords: Arabian Sea, oxygen minimum zone, culturable fungi, molecular diversity, nitrate reducing activity

Introduction

Oxygen minimum zones (OMZs) in the world's oceans are reported, in the eastern Pacific Ocean, along the west coast of Africa in the Atlantic Ocean and the Indian Ocean region (Stramma et al. 2010). The microorganisms inhabiting these regions are adapted to an alternate respiratory pathway and utilize nitrates, in the absence of oxygen, through the denitrification process (Lam and Kuypers 2011). Due to this, large amounts of fixed nitrogen in the oceanic system are lost to the atmosphere as dissolved nitrogen gas. The global marine nitrogen budget predicts that about 150 Tg N yr⁻¹ of fixed nitrogen is lost due to denitrification in the marine system, 40% (60 Tg N yr⁻¹) of this loss occurs from the Arabian Sea (Bange et al. 2005). Apart from the imbalance in the oceanic nitrogen (N) budget (Codispoti 1995), denitrification decreases the amount of nitrogen available for phytoplankton uptake (Liu et al. 2003), and the intermediary products such as NO and N₂O produced during the denitrification process are harmful green house gases. Hence, the major concern that drives the intensive research on this system is the direct effect of this pathway on the global climate.

Fungi have an incomplete denitrification pathway and can reduce nitrate or nitrite only to N₂O. The pathway mainly stops there, continuously generating harmful green house gas (Shoun et al. 1992). Studies from terrestrial habitats such as the grassland ecosystem (Laughlin and Stevens 2002) and semiarid soil (McLain and Martens 2006) have shown that fungi account for nearly 80% of the N₂O production. Hence, our interest is to study the ecological role of fungi as they are known to predominantly produce only nitrous oxide which directly affects the ecosystem from the Arabian Sea oxygen-depleted habitats.

Knowledge on fungal diversity associated with the OMZs of the world was mostly obtained from cultivation-independent studies of the total micro-eukaryotic communities and there are very few reports on fungal specific molecular surveys or cultivation based studies (Stoeck et al. 2014; Manohar and Raghukumar 2013; Orsi and Edgcomb 2013; Jebaraj et al. 2012; Orsi et al. 2012). In spite of meagre records on the culturable fungal diversity from extreme marine habitats, obligate marine fungi such as *Eurotium herbariorum*, were isolated from hydrothermal vent regions (Burgaud et al. 2009). There are also reports of fungal isolates, from marine sediments collected from the west coast of South Africa with denitrification potentials and extra-cellular enzyme activity (Mouton et al. 2012). Isolation of fungi from the coastal, anoxic regions of the Arabian Sea retrieved about 54 different marine-derived fungi (Jebaraj and Raghukumar 2009). Molecular analyses of these isolates revealed a number of novel taxa (Jebaraj et al. 2010). One of the isolates was recently described as a new species, *Tritirachium candoliense* sp. nov., based on its morphological and phylogenetic features. Electron microscopy revealed that although sporulation and the internal organelles of the isolate were affected by low

oxygen levels, still it could thrive under low oxygen conditions (Manohar et al. 2014). A recent study reported dissimilatory nitrate reduction activity resulting in ammonia fermentation and nitrous oxide production by *Aspergillus terreus*, obtained by enrichment culture techniques from the seasonal OMZ of the Arabian Sea (Stief et al. 2014). These reports clearly point out the importance of fungi in the marine oxygen depleted habitats.

The OMZ of the Arabian Sea extends between 45°W, 73°E, 0°S and 30°N, in the water column between 200 m to 1200 m, and in the Bay of Bengal it spans from 73°W, 100°E, 0°S and 30°N at a water column depth between 104 m to 389 m. The oxygen concentration in these regions drops to almost 0.1 mL L⁻¹ (4 µM) or lower, compared to the average ocean oxygen concentration that ranges between 6 - 8 mL L⁻¹ (260 – 350 µM). These regions are of microbiological importance since major biogeochemical processes are catalysed by the microbial communities which inhabit it (Lam and Kuypers 2011; Goregues et al. 2005). The Arabian Sea also harbors an oxygen deficient system in the coastal regions along the south-west coast of India. This is a seasonal phenomenon which develops during the month of June and extends to December, with maximum intensity in September and October every year (Naqvi et al. 2000). Studies on the fungal abundance and diversity, from the seasonally occurring, coastal oxygen deficient system of the Arabian Sea, show that fungal diversity is affected by seasonal changes in dissolved oxygen levels. Direct detection of fungal filaments, observed in the sediments collected from these locations; reveal that fungi are an inherent biotic component of anoxic sediments (Jebaraj and Raghukumar 2009). However, there are no reports of the diversity and isolation of fungi from a permanent OMZ. In our present study, we report on the fungal diversity from a permanent, open-ocean OMZ of the Arabian Sea where the oxygen levels are close to zero, and have remained anoxic since the time of its discovery during the International Indian Ocean Expedition (Wyrski 1971; Naqvi et al. 2006).

Materials and Methods

Sampling site and collection of samples

Sampling was carried out from four stations SSt-500: 500 m depth, 17° 33.50' N, 71° 11.35' E, SSt-700: 700 m depth, 17° 32.28' N, 71° 11.09' E, SSt-800: 800 m depth, 17° 31.50' N, 71° 10.31' E and SSt-1100: 1100 m depth, 17° 31.44' N, 71° 04.88' E, situated within the OMZ of the Arabian Sea (Fig. 1). Samples were collected during the cruise YK08-11 on board R/V Yokosuka and its deep-sea submersible Shinkai 6500 (Japan Agency for Marine-Earth Science and Technology, Japan) between September and November 2008. From each of the stations, sediment, overlying and near-bottom water samples were collected for analyses. The overlying water (OLW) which gets collected in the core liner above the sediment was siphoned out and stored. Sediment samples were collected using a ~ 8.5 cm x

20 cm push corer, where after samples were cut at 2 cm intervals down to 8 cm, and extruded into alcohol sterilized clean plastic containers. Near bottom water, about 1 – 2 m above the sediment surface was collected in 2 L Niskin bottles and the dissolved oxygen (DO) level in the near bottom was determined simultaneously using an optical oxygen sensor (Oxygen Optode 3830; Aanderaa Data Instruments AS, Norway) mounted upon the submersible. Samples were processed for fungal isolation on the same day that they were collected on.

Isolation of fungi

Fungi were isolated from all the sediment sections by modified particle plating technique (Bills and Polishook 1994). Approximately 1 g of sediment slurry was sieved successively through a mesh size of 200 μm and 100 μm screens. Those particles that passed through the 200 μm mesh, but retained on the 100 μm mesh were plated in triplicates on 1/5 times diluted media plates. Diluted media were used to discourage the growth of fast growing fungi. The different media used were Malt extract agar, Corn meal agar, Czapek Dox agar and Brewer's anaerobic agar (HiMedia Pvt. Ltd. India). They were prepared with seawater and supplemented with streptomycin (0.75 g L^{-1}) and penicillin (0.9 g L^{-1}). Aliquots (100 ml) of the overlaying water and near bottom water were filtered using sterile Durapore filters (0.45 μm , Millipore India Pvt. Ltd. India) and placed on the culture media plates for growth of fungal colonies. The colonies appearing after incubation at room temperature ($\sim 28^\circ\text{C}$) for 15 – 20 days were picked using sterile needles and sub-cultured on 1/5 times diluted malt extract agar plates. Precaution was taken during the entire isolation protocol to avoid contamination and media plates were exposed to identify any common aerial contaminants from the sampling and working area. The fungal isolates were subsequently grouped based on colony morphology and morphological characters using identification keys provided in literature (Domsch *et al.* 1980). A single representative for each group was designated with a unique isolate number and catalogued. This resulted in twenty-eight "morphotypes" that were subsequently analysed.

Identification of fungi

The isolates from different sampling stations were identified based on the small subunit region of the ribosomal RNA (18S rRNA) gene sequence. For sequence analysis, each fungal culture was grown for 5 days at room temperature in 50 ml of malt extract broth (HiMedia Pvt. Ltd. India) contained in a 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 and Epstein 2003).

The 18S rRNA gene sequence was amplified using fungal specific primers NS1 and FR1 (Vainio and Hantula 2000; Gomes et al. 2003). The 50 µl PCR reaction included 1x Hot Start Taq buffer (Sigma, USA), in a reaction buffer provided by the company containing Tris-HCl, KCl, (NH₄)₂SO₄, 15 mM MgCl₂ (pH 8.7), 200 µM concentrations of each deoxynucleoside triphosphate, and 0.5 µM concentrations of each oligonucleotide primer and the final volume was adjusted to 50 µl 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 (Sigma, USA). The PCR products were cloned separately for each fungal culture using the TA cloning kit (Invitrogen, USA) according to the manufacturer's instructions. Plasmids were isolated from positive overnight cultures using the Fast Plasmid Mini Prep kit (Eppendorf AG, Germany). One representative clone for each culture was sequenced bidirectionally in 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 the sequences deposited are JN546116-JN546143.

Phylogenetic analyses

The 18S rRNA sequences of the isolates from this study, the closest taxa of each culture identified by using BLASTn analysis (Altschul et al. 1997) and the environmental representatives of novel clusters identified within Ascomycota and Basidiomycota (Suppl. Table 1) were used for phylogenetic analyses. Multiple alignments of all these sequences were carried out along with their closest match in ClustalW (Thompson et al. 1994) and refined with the G-Blocks program (<http://www.phylogeny.fr>). The resulting alignments were manually checked and subjected to Maximum Likelihood (ML) analyses in MEGA 5 (Tamura et al. 2011). ML algorithm employed the GTRGAMMAI model and bootstrapping was performed with 1000 replicates. Additionally, Neighbour Joining (NJ) trees were constructed in SeaView version 4 (Gouy et al. 2010). Robustness of NJ trees in the analyses was assessed with 1000 NJ bootstrap replicates.

Distribution of fungi

The percentage frequency of fungal distribution in the water, sediment and the overlying water sampled from within the OMZ stations was calculated from the CFUs.

$$\text{Percentage frequency} = \frac{\text{Total number of cfu belonging to each morphotype}}{\text{Total number of cfu}} * 100$$

The occurrence of fungal CFU from each sampling site and across the changing oxygen concentrations were studied based on Pielou's evenness (J'), Shannon-Wiener index, (H' log_e) and Simpson's Index of

Diversity (1- λ). Pielou's evenness is a measure of biodiversity which quantifies how equal the community is numerically and is constrained between 0 and 1; higher value signifies that the variation in communities between the samples is less. The Shannon-Wiener index values ($H' \log_e$) is generally in the range of 0 to ~4.6 using natural log (\log_e). A value near 0 would indicate that every species in the sample is the same and near 4.6 would indicate that the numbers of individuals are evenly distributed between all the species. We also calculated the Simpson's Index of Diversity (1- λ) which generates values between 0 and 1 with the greater value signifying greater sample diversity. The statistical analysis was carried out using the software PRIMER 5, v 5.2.4 (Primer-E Ltd. ©).

Nitrate reduction ability of isolates under simulated deep-sea, anoxic conditions

Selected fungal cultures were examined for their nitrate reducing capacity at two different growth conditions ie., 1) Oxidic /1 bar / 28°C representing the oxidic, coastal habitat and 2) Anoxic /100 bar / 10°C which simulates the anoxic, deep-sea conditions with high pressure, low oxygen and temperature. Starter cultures were grown separately for each isolate, in 20 ml of malt extract broth in 100 ml conical flasks for seven days. Approximately 5 mg (dry weight) of the mycelial suspension or yeast cells was used as inoculum. For the experimental study, the cultures were grown in 20 ml of nitrate broth containing 10 mM of potassium nitrate, in sterile, polypropylene pouches under the respective growth condition for 15 days. The pouches for anoxic growth were flushed with nitrogen gas for 3 min as described earlier (Jebaraj and Raghukumar 2009) to simulate the anoxic conditions, and were suspended in a deep-sea culture vessel filled with sterile distilled water, and pressurized to 100 bar and incubated at 10 °C, as described by Singh et al. (2010) to simulate deep-sea (at 1000 m depth) conditions. In the case of oxidic growth, the pouches were sealed without flushing and incubated at 1 bar pressure in a laboratory incubator at 28°C. The nitrate reducing capacity of the isolates was determined by using the Griess test which is based on the activity of the enzyme, nitrate reductase (Smibert and Krieg 1994). The Nitrate test was performed by addition of the Griess reagent to the culture supernatant, at the end of the incubation time, to detect the presence of nitrite as a result of nitrate reduction, indicated by a pink colour formation. This was followed by addition of powdered zinc to check for the presence of residual nitrate. The isolates which showed no nitrate reduction and tested positive for residual nitrate, were categorized as non-reducers (--). The isolates which were positive for nitrite production but still had some residual nitrate were marked as incomplete nitrate reducers (+-), while the isolates which were positive for nitrite accumulation and (or) negative for residual nitrate are grouped as nitrate reducers (++) .

Results

Dissolved oxygen levels

The open ocean OMZ of the Arabian Sea extends from 200 m - 1200 m in the mid-oceanic water column, and in regions where the depth is less than 1200 m, the oxygen-depleted waters directly impinges its effect on the sediment. The four sampling stations selected for the study were situated within the OMZ and less than 1200 m in depth, where the oxygen-depleted water column had its effect on the sediments (Fig. 1). The DO of the near bottom water in SSt-500 and SSt-700 was less than 1 μM , i.e. 0.4 μM and 0.52 μM , respectively; and at SSt-800 and SSt-1100 it was 2.75 μM and 20 μM respectively. We have studied the diversity of fungi from the water, OLW and the sediments collected from the four stations.

Distribution of fungi

A total of 1954 fungal colonies were obtained from the sampling; of these a large majority of nearly 43.9 % of mycelial colonies and 12.5 % of yeast colonies could not be identified as they did not survive the initial sub-culture under laboratory conditions. The colonies which grew in the laboratory were grouped based on the colony morphology and morphological characters using the identification keys (Domsch *et al.* 1980). This resulted in twenty-eight “morphotypes” which were used for the study and denoted by isolate numbers with a suffix “YK”. BLASTn analysis showed that 22 isolates belonged to *Ascomycota* and 6 belonged to *Basidiomycota* and it included both mycelial and yeast forms (Table 1).

The distribution of the fungal “morphotypes” in the water, sediment and OLW samples collected from all the four stations were estimated by measuring their percentage frequency (Table 2). Yeasts were the most dominant group which contributed to 32.3 % of the total distribution. Morphotypes represented by isolates YK-6 and YK-19 were basidiomycete yeasts; isolates YK-4 and YK-18 belonged to *Ascomycota*, however 12.5 % of yeasts could not be identified. Yeasts were isolated from all the four stations from the water or OLW samples, except yeasts represented by YK-18, which was isolated from the sediment sample of SSt-700. Among the mycelial cultures, maximum representation was by *Cladosporium* sp. and taxa synonymous to it such as *Zasmidium* sp. and *Davidiella* sp. represented by isolates YK-7, YK-10, YK-16, YK-24 and YK-30. These isolates all together accounted for 22.7 % of the total distribution. They were isolated from stations SSt-500, SSt-700 and SSt-1100 and could be isolated from water, OLW and sediment samples.

The isolates that grouped within *Ascomycota* predominantly belonged to subphyla *Pezizomycotina*. YK-4 clustering with *Pichia* sp. was the only representative within subphyla

Saccharomycotina and there was no representation of any isolates within *Taphrinomycotina*. Basidiomycetous Isolates belonged to all the three subphyla *Pucciniomycotina*, *Ustilagomycotina* and *Agaricomycotina* (Table 2). Diversity estimated based on Pielou's evenness ranged between 0.1 – 1.0 within the different samples analysed. The Shannon's and Simpson's diversity index ranged between 0.1 - 1.4 and 0.02 – 0.6 respectively (Table 2).

Diversity of fungi

The 18S rRNA sequence of 28 cultures isolated from the Arabian Sea OMZ, their closest identified taxa from BLASTn analysis, representative sequences from all fungal phyla according to the classification by Hibbett et al. (2007) and environmental sequences retrieved from various marine OMZs was subjected to phylogenetic analyses. The environmental phylotypes belonging to major environmental clusters such as the *Pezizomycotina* clone group (Manohar and Raghukumar 2013), Deep-Sea fungal group (Nagano et al. 2010) and Soil clone group-I (Nagahama et al. 2011) identified within *Ascomycota*, and the environmental phylotypes identified within *Agaricomycotina*, as well as sequences belonging to An-Hydrothermal fungal group (López-García et al. 2007) and Env clade A (Jebaraj et al. 2012), within *Basidiomycota*, were used for analyses (Supp. Table 1). Of the 22 isolates which grouped within *Ascomycota* 21 of them were belonged to *Pezizomycotina* (Fig. 2). YK-4 was the only representative within *Saccharomycotina* and there was no representation of *Taphrinomycotina* (Fig. 2). Isolates YK-30, YK-10, YK-16, YK-24 clustered close to *Cladosporium* sp., *Zasmidium* sp. and *Davidiella* sp. which were their closest known taxa from the BLASTn analysis (Table 1). However, YK-7, which has only 97 % similarity with *Davidiella* sp. grouped within this cluster but branched out separately (Fig. 2). Isolate YK-17 clustered with *Aureobasidium* sp. as well as with environmental sequences retrieved from the Gotland Basin, Lost city vent region and Greenland anoxic habitat. YK-31 clustered with environmental sequence obtained from the anoxic sediments of Greenland and Arctic Ocean. Isolate YK-18 clustered with *Hortea* sp., ascomycetous yeasts and the isolates YK-33, YK-5, YK-9, YK-34, YK-28, YK-1, YK-3, YK-2, YK-20 and YK-4 clustered together with the taxa identified as their closest match through BLASTn analysis (Fig. 2). Isolate YK-27 branched close to *Nectria* sp., but with low support from phylogenetic analyses and only a 97 % similarity in BLASTn analysis revealed that this isolate is highly divergent and could be a new species. YK-22, YK-35 and YK-29 clustered with strong bootstrap close to *Aspergillus* spp., *Eupenicillium* and *Penicillium* sp., and the environmental sequences identified as *Pezizomycotina* clone group. But isolates YK-35 and YK-29 branch out separately within this cluster and are possible novel species isolated from the Arabian Sea OMZ.

Six basidiomycetes with two representative isolates for each of the three subphyla *Pucciniomycotina* (YK-6, YK-19), *Ustilagomycotina* (YK-8, YK-23) and *Agaricomycotina* (YK-25, YK-32)

were obtained during this study (Fig. 3). YK-6 has a strong affiliation with environmental sequences and phylogenetic analyses showed that they are divergent from *Rhodotorula* spp. Isolates YK-19 and YK-23 grouped with known taxa and environmental representatives, possibly of the same taxa. Two of the isolates YK-25 and YK-23 clustered close to the known taxa *Cernia unicolor* and *Tilletiopsis* sp. respectively, and YK-8 branches out separately from *Melanopsichium pennsylvanicum* and *Sporisorium formosanum* (Fig. 3).

Nitrate reduction ability of isolates under simulated deep-sea, anoxic condition

Isolates YK-2, YK-5, YK-17, YK-31, YK-35 belonging to the phylum Ascomycota and YK-6, YK-8, YK-19 belonging to the phylum Basidiomycota were screened for their ability to reduce nitrate. This includes representative isolates identified as well described taxa (YK-2, YK-5), putatively new species (YK-8), isolates which cluster with known taxa and environmental phylotypes (YK-17, YK-19, YK-35) and isolates YK-31 and YK-6 which cluster with environmental sequences away from any of the described fungal taxa. *Fusarium oxysporum* (MT-811), a known denitrifier from a terrestrial habitat was used as a positive control (Table 3). Isolates YK-2, YK-17, YK-19 and YK-29 along with the positive control were able to reduce nitrate partially under oxic conditions at 1 bar pressure and 28 °C. All the isolates tested for nitrate reduction activity were capable of reducing nitrate under anoxic conditions at 100 bar pressure and 10 °C. This shows that the nitrate reduction capacity is highly prevalent among the fungal isolates and they can utilize nitrate in the absence of oxygen and participate in the denitrification process, even under conditions of elevated pressure and low temperature, which is the characteristic feature of the deep-sea environments. However, the positive control was able to reduce the nitrate completely and tested negative for the presence of residual nitrate. This clearly shows that terrestrial isolates are also capable of nitrate reduction under simulated deep-sea conditions. Refined isolation procedures using mineral media and enrichment incubation could be possible techniques to cultivate complete denitrifiers from marine habitats.

Discussion

Fungi are classically categorized as obligate aerobes and it is considered as one of the major metabolic difference between bacteria and fungi (Foster 1949). Pioneering studies have shown that colonization of fungi on wood in marine environment is slower or almost negligible in oxygen minimum zones (Kohlmeyer and Kohlmeyer 1979). However, recent molecular studies have shown the presence of fungi through cultivation techniques and molecular methods from various marine, OMZs of the world (Stoeck et al. 2014; Orsi and Edgcomb 2013; Jebaraj et al. 2012; Nagahama et al. 2011; Jones et al. 2011; Nagano et al. 2010; Gao et al. 2008, 2010; López-García et al. 2007).

In this study, nearly 2000 fungal colony forming units were cultured on media plates from the sediment and water samples collected from the permanent OMZs of the Arabian Sea. Diversity index based on Pielou's evenness ranged between 0.1 – 1.0, but most of the values were more than 0.4 which shows that there is not much difference between the samples. Diversity estimates based on Shannon's index ranged between 0.1 – 1.4 (Table 2) which is lower than the diversity indices of the deep sea environment (1.2 and 2.0) when the isolations were carried out at lower temperature (Damare et al. 2006). The diversity index values reported from mangrove ecosystems was between 3.2 to 5, based on isolating fungi from mangrove twigs and drift wood by moist chamber technique at room temperature (Prasannarai and Sridhar 2001; Maria and Sridhar 2002). The isolation techniques are specific for each habitat and substrata and they are designed to maximize the possibility of isolation of cultures native to the area. A direct comparison of the Shannon's index is possible, only if the studies have used the same base value for log in the calculations and this limits the conclusions based on this index. However, the Simpson's index of Diversity ($1-\lambda$) which ranges between 0 and 1 provides a better picture of the diversity in each region, as the higher values signifies higher diversity. The values of $1-\lambda$ from this region is between 0.02 – 0.6 and these values are much less compared to the mangrove regions which is reported to be in the range of 0.84 – 0.97 (Prasannarai and Sridhar 2001; Maria and Sridhar 2002). Though the diversity of fungi is less compared to the mangrove regions, the nitrate reduction ability recorded in the isolates from these regions clearly shows that they are adapted to survive in the oxygen depleted habitats (Table 3).

The limited studies on the diversity of fungi from marine sediments and water from vent habitats and deep sea regions show that the fungal cultures isolated from these regions are very diverse and have physiological adaptations to thrive in these regions (Burgaud et al. 2009; Singh et al. 2010). The inability of more than 50 % of the isolates obtained during our to thrive under laboratory conditions, suggests that the diversity could be very complex and to cultivate the novel groups will be a challenge. However, having access to living isolates are crucial for an in-depth analysis of their morphology, physiology and ecology. This is a decisive advantage of the culturable over un-culturable diversity analysis; in spite of the limitations with the former technique. Of the 28 isolates that we obtained from the OMZ, we could clearly identify that at least five are diverse and novel species. Cultivation-independent studies of fungi from marine habitats has aided in the identification of a large number of diverse novel fungi and environmental clusters from various marine habitats (López-García et al. 2007; Gao et al. 2008; Nagano et al. 2010; Nagahama et al. 2011; Jebaraj et al. 2012). However, some phylotypes which belong to these environmental clusters could not be assigned to their rightful place within these clades because representatives of these clusters were not included during analysis (Lai et al. 2007; Jebaraj et al. 2010). Increasing the availability of molecular data for each taxonomic group

and diligent implementation of phylogenetic analysis are promising approaches to unravel the yet to be described marine fungal diversity (Manohar and Raghukmar 2013). Hence in this study, we included representative environmental sequences from all the major environmental studies that have been reported so far from the marine environment within the fungal phyla Ascomycota and Basidiomycota (Supplemental material 1).

Phylogenetic analyses of the 18S rRNA sequences of the isolates obtained from our study along with the representative culture and environmental sequences showed four different types of clustering. The majority of the isolates, 13 belonging to *Ascomycota* and 2 belonging to *Basidiomycota* branched close to well described taxa with strong support (Fig. 2 and 3). Isolates retrieved, grouped within all the three subphyla, *Pucciniomycotina*, *Ustilagomycotina* and *Agaricomycotina* of the *Basidiomycota*; *Pezizomycotina* and *Saccharomycotina* of *Ascomycota*. Isolates belonging to *Saccharomycotina* of *Ascomycota* and *Agaricomycotina* of *Basidiomycota* were isolated for the first time from the OMZ and were not retrieved during earlier studies on the coastal OMZ of the Arabian Sea (Jebaraj et al. 2010). Some of the isolates YK-27, YK-7, YK-16 and YK-24 belonging to *Ascomycota* and YK-8 belonging to *Basidiomycota* branched out separately from well described culture sequences and these isolates could be new species. Further investigation of these isolates can help in identifying the novelty of these isolates retrieved from the OMZs. A few isolates formed a cluster with well known taxa and environmental phylotypes, eg. isolate YK-19 clustered with *Rhodotorula minuta* and an environmental sequence from the anoxic sediment of Gotland. Such clustering was exhibited by three other isolates YK-17, YK-35 and YK-32. These clusters represent fungal taxa which were retrieved using both cultivation dependent and independent techniques. Interestingly, isolates YK-31 and YK-6 clustered with good bootstrap support with environmental sequences D2P04H12 and UI11BO6, obtained from the oxygen-depleted Arctic and Mediterranean region respectively. These isolates branched out separately from other known taxa and represent the environmental phylotypes which were known so far only from environmental studies (Figs. 2, 3).

Environmental fungal phylotypes are reported from a large number of marine OMZs (Stoeck and Epstein, 2003; Nagano et al., 2010; Takishita et al., 2007, Jebaraj et al. 2010; Stoeck et al., 2010, Thaler et al., 2012). Meta-analysis of the phylotypes shows that some environmental sequences are very similar to well defined taxa and grouped within the fungal phyla *Basidiomycota*, *Ascomycota*, *Chytridiomycota* or as basal fungal lineages. Some of the sequences clustered into major environmental clades such as the deep-sea fungal group-I (Nagahama et al. 2011; Takishita et al. 2007), soil clone group-I identified within *Ascomycota*, environmental clades within *Basidiomycota*, *Chytridiomycota* and at the base of the tree (Jebaraj et al. 2012). The inclusion of representative

sequences from the reported environmental studies has also helped to understand that some of the environmental sequences are very divergent and form a completely novel environmental cluster (Fig. 3).

Very little is known about fungi and its importance in the marine habitat and far less is known about its role in special niches such as the marine OMZs. This investigation represents a minor fraction of the fungal diversity from the permanent OMZ of the Arabian Sea. Although metagenomic analyses have retrieved environmental sequences representing novel clusters from various oxygen depleted habitats of the world, there are no previous reports of attempts to isolate and cultivate such novel cultures which are, so far, known only as environmental phylotypes. In this study, we were able to isolate a few novel fungal cultures which are similar to highly divergent phylotypes and could represent the first cultivable forms of these environmental sequences. These isolates are maintained in the culture collection at CSIR-National Institute of Oceanography, Goa, India in the Biological Oceanography Division. Further investigations will be carried out to describe the new species and to study their physiological adaptations to live in oxygen-depleted marine habitats. This can eventually aid in determining the ecological role of fungi in marine habitats.

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Table 1

List of “morphotypes” represented as isolates and its closest identified taxon based on the BLASTn analysis of the 18S rRNA sequence.

Isolate No.	GenBank accession no.	Closest identified taxon and its GenBank accession no.	Similarity (% identity)
Phylum Ascomycota			
YK-1	JN546116	<i>Paecilomyces</i> sp. (DQ401104.1)	99
YK-2	JN546124	<i>Engyodontium album</i> (AB106650.1)	99
YK-3	JN546131	<i>Acremonium</i> sp. (AB108787.1)	99
YK-4	JN546137	<i>Pichia guilliermondii</i> (EU784644.1)	99
YK-5	JN546135	<i>Alternaria alternata</i> (U05194.1)	99
YK-7	JN546121	<i>Davidiella tassiana</i> (EU343087.1)	97
YK-9	JN546136	<i>Cochliobolus lunatus</i> (DQ337381.1)	99
YK-10	JN546117	<i>Cladosporium</i> sp.(FJ716252.1)	99
YK-16	JN546120	<i>Cladosporium</i> sp. (FJ716252.1)	99
YK-17	JN546123	<i>Aureobasidium pullulans</i> (EU707932.1)	99
YK-18	JN546122	<i>Hortaea werneckii</i> (Y18700.1)	99
YK-20	JN546125	<i>Fusarium oxysporum</i> (DQ916150.1)	99
YK-22	JN546126	<i>Eupenicillium javanicum</i> (EF413620.1)	99
YK-24	JN546118	<i>Cladosporium cladosporioides</i> (DQ678004.1)	99
YK-27	JN546129	<i>Nectria cinnabarina</i> (AB003949.1)	97
YK-28	JN546130	<i>Chaetomium globosum</i> (AY545725.1)	99
YK-29	JN546127	<i>Aspergillus cervinus</i> (AB008397.1)	99
YK-30	JN546119	<i>Zasmidium cellare</i> (EF137362.1)	99
YK-31	JN546133	<i>Phaeosphaeria</i> sp. S93-48 (EU189216.1)	93
YK-33	JN546132	<i>Didymocreasa dasivanii</i> (DQ384066.1)	99
YK-34	JN546134	<i>Apiospora montagnei</i> (AB220229.1)	99
YK-35	JN546128	<i>Aspergillus versicolor</i> (AB008411.1)	99
Phylum Basidiomycota			
YK-6	JN546143	<i>Rhodosporidium diobovatum</i> (AB073271.1)	99
YK-8	JN546142	<i>Melanopsichium pennsylvanicum</i> (DQ363314.1)	99
YK-19	JN546138	<i>Rhodotorula minuta</i> (FJ196163.1)	99
YK-23	JN546139	<i>Tilletiopsis albescens</i> (D83188.1)	98
YK-25	JN546140	<i>Cerrena unicolor</i> (AY850007.1)	98
YK-32	JN546141	<i>Corioloropsis byrsina</i> (AY336773.1)	99

Table 2

Percentage frequency of “morphotypes” represented as isolates from the different stations within the OMZ region.

Isolate No.	SSt-500			SSt-700			SSt-800			SSt-1100			Total
	water	OLW	sed	water	OLW	sed	water	OLW	sed	water	OLW	sed	
YK-1	-	-	-	-	-	-	-	0.1	-	-	-	-	0.1
YK-2	-	-	-	-	-	-	-	0.1	-	-	-	-	0.1
YK-3	-	-	-	-	-	-	-	-	0.1	0.1	-	-	0.1
YK-4	-	-	-	-	0.10	-	-	0.4	-	-	-	-	0.5
YK-5	0.1	-	-	-	-	-	-	0.3	-	-	0.2	-	0.5
YK-6	6.5	0.2	-	1.0	0.8	-	0.1	0.6	-	8.0	-	-	17.1
YK-7	-	-	-	0.5	-	-	-	0.1	-	-	-	-	0.5
YK-8	-	-	-	-	0.1	-	-	-	-	-	-	-	0.1
YK-9	-	-	-	-	-	-	-	0.1	-	-	-	-	0.1
YK-10	-	-	-	-	-	-	-	-	-	-	-	0.5	0.5
YK-16	15.9	3.4	-	1.6	-	-	-	-	-	0.3	0.2	-	21.4
YK-17	0.1	-	-	-	-	-	-	-	-	-	-	-	0.1
YK-18	-	-	-	-	-	0.3	-	-	-	-	-	-	0.3
YK-19	0.3	0.2	-	0.9	-	-	-	-	-	-	-	-	1.4
YK-20	-	-	-	0.1	-	-	-	-	-	-	-	-	0.1
YK-22	-	-	0.9	-	-	-	-	0.3	-	0.3	-	-	1.4
YK-23	-	-	-	-	-	-	-	-	0.1	-	-	-	0.1
YK-24	-	-	0.2	-	-	-	-	-	-	-	-	-	0.2
YK-25	-	-	0.1	-	-	-	-	-	-	-	-	-	0.1
YK-27	-	-	-	-	-	0.2	-	-	-	-	-	-	0.2
YK-28	-	-	0.1	-	-	-	-	-	-	-	-	-	0.1
YK-29	-	-	0.1	-	-	-	-	-	-	-	-	-	0.1
YK-30	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1
YK-31	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1
YK-32	-	-	-	-	-	0.1	-	-	-	-	-	-	0.1
YK-33	0.1	-	-	-	-	-	-	-	-	-	-	-	0.1
YK-34	-	0.1	-	-	-	-	-	-	-	-	-	-	0.1
YK-35	0.1	-	0.1	-	-	-	-	-	-	-	-	-	0.2
Unidentified mycelia	0.6	0.3	0.1	5.1	-	-	-	14.8	18.4	1.5	1.3	0.4	42.4
Unidentified yeast colony	-	-	-	0.1	-	-	0.1	12.3					12.5
Total Species (S)	8	5	7	7	3	3	2	10	3	5	3	4	
Pielou's evenness (J')	0.4	0.5	0.7	0.7	0.6	0.9	1.0	0.4	0.1	0.	0.6	0.8	
Shannon-Wiener Index (H' log _e)	0.9	0.7	1.4	1.3	0.6	1.0	0.7	1.0	0.11	0.7	0.7	1.2	
Simpson's Index of Diversity (1-λ)	0.5	0.3	0.6	0.6	0.3	0.6	0.5	0.6	0.02	0.4	0.4	0.6	

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

Table 3

Nitrate reduction ability of isolates under simulated deep-sea, anoxic conditions

Isolate No.	Nitrate reduction	
	Oxic / 1 bar / 28°C	Anoxic / 100 bar / 10°C
YK-2	--	+-
YK-5	+-	+-
YK-6	+-	+-
YK-8	+-	+-
YK-17	--	+-
YK-19	--	+-
YK-29	--	+-
YK-31	+-	+-
YK-35	+-	+-
MT-811	+-	++

Symbols represent -- : no nitrate reduction, +- : incomplete nitrate reduction, ++ : complete nitrate reduction

Figure legends

Fig. 1 Sampling sites in the Arabian Sea within the oxygen minimum zone. A: The extent of the oxygen minimum zone represented with the 0.5 μM nitrite contour which accumulates due to denitrification (adapted from Naqvi and Shailaja, 1993) with the sampling area in grey shade (B). B: Sampling area with depth contours and the sampling locations represented as black dots.

Fig. 2 Phylogenetic tree showing the position of isolates belonging to phylum *Ascomycota* obtained from the OMZ of the Arabian Sea based on the maximum likelihood analysis of the 18S rRNA sequences. Values at nodes indicate bootstrap support from 1000 replicates from ML/NJ analysis.

Fig. 3 Phylogenetic tree showing the position of isolates belonging to phylum *Basidiomycota* obtained from the OMZ of the Arabian Sea based on the maximum likelihood analysis of the 18S rRNA sequences. Values at nodes indicate bootstrap support from 1000 replicates from ML/NJ analysis.

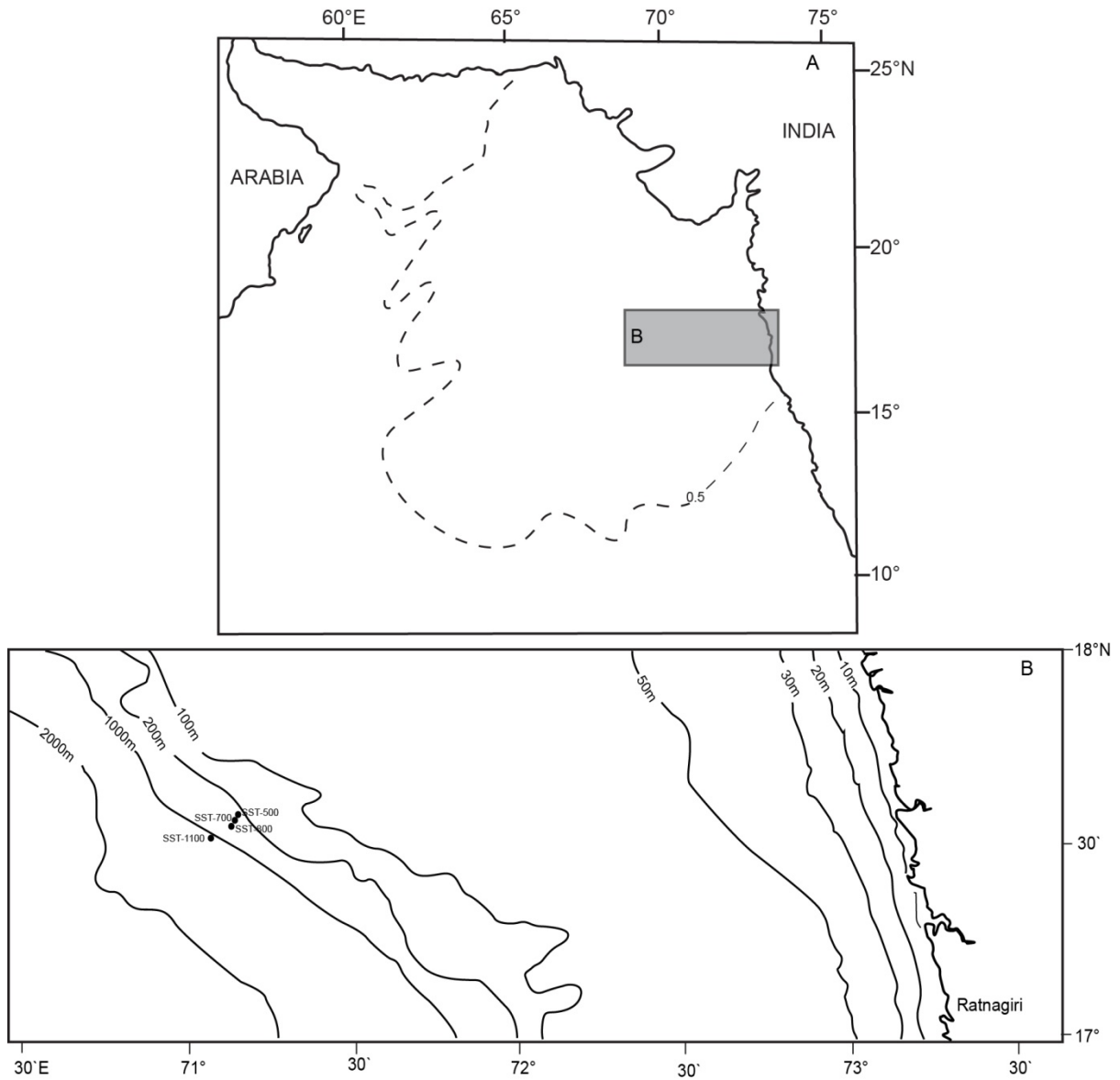


Fig. 1 Sampling sites in the Arabian Sea within the oxygen minimum zone. A: The extent of the oxygen minimum zone represented with the 0.5 μM nitrite contour which accumulates due to denitrification (adapted from Naqvi and Shailaja, 1993) with the sampling area in grey shade (B). B: Sampling area with depth contours and the sampling locations represented as black dots.

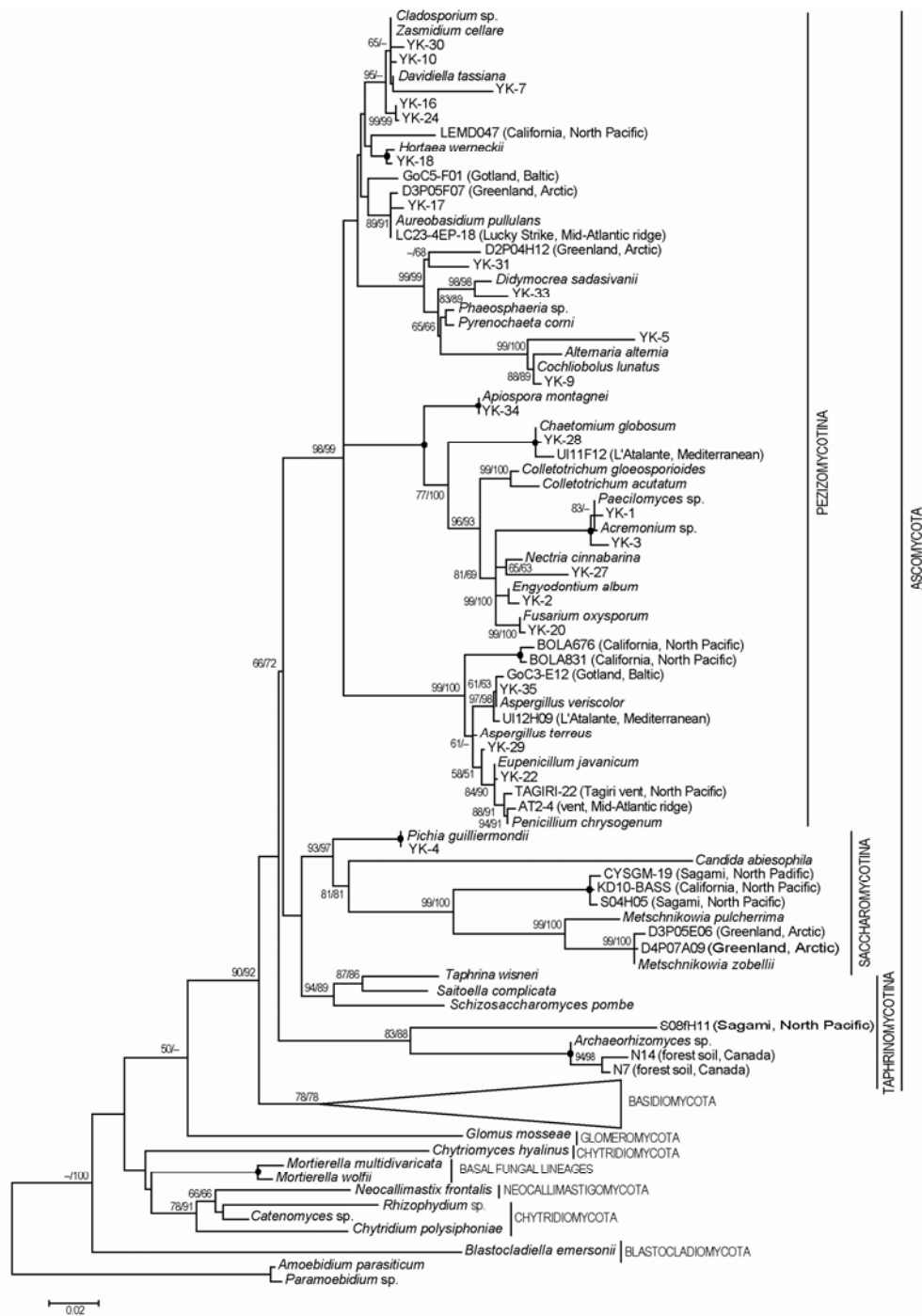


Fig. 2 Phylogenetic tree showing the position of isolates belonging to phylum *Ascomycota* obtained from the OMZ of the Arabian Sea based on the maximum likelihood analysis of the 18S rRNA sequences. Values at nodes indicate bootstrap support from 1000 replicates from ML/NJ analysis.

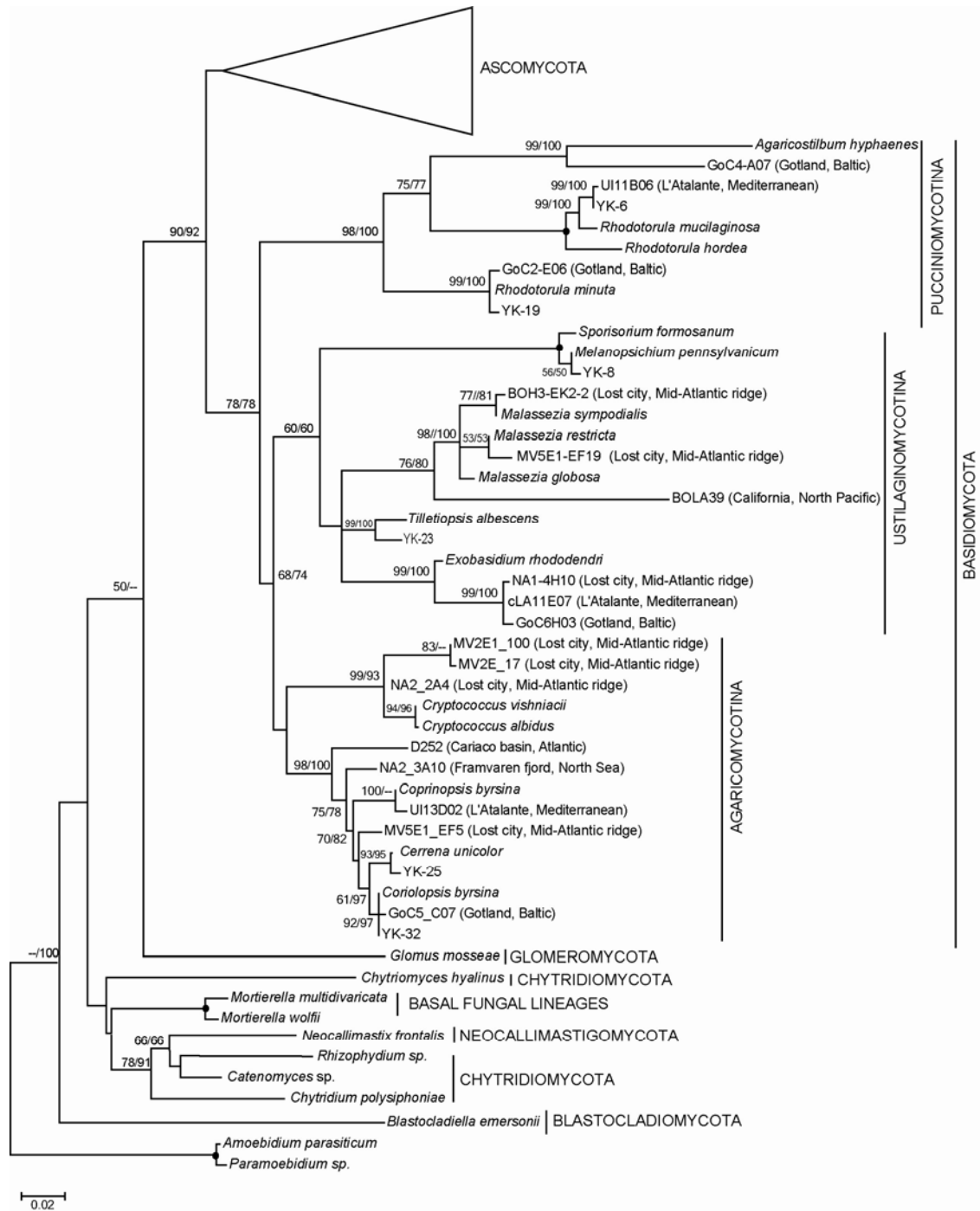


Fig. 3 Phylogenetic tree showing the position of isolates belonging to phylum *Basidiomycota* obtained from the OMZ of the Arabian Sea based on the maximum likelihood analysis of the 18S rRNA sequences. Values at nodes indicate bootstrap support from 1000 replicates from ML/NJ analysis.