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Diversity of culturable marine filamentous fungi from deep-sea hydrothermal vents

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Summary

As now very few studies have been carried out on deep-sea marine fungi, this field remains relatively unknown. However, their presence inside benthic microbial eukaryotes at deep-sea vents was recently pointed out from molecular microbial ecology studies. We report here an attempt to describe the culturable part of mycological communities in deep-sea vent ecosystems that is an important step in understanding their diversity, abundance and function. Physiological characterization revealed strains that are more or less adapted to deep-sea conditions. Those results suggest the presence of true marine organisms and other more ubiquitous. Phylogenetical characterization highly correlated to physiological data revealed the presence of fungi that have been previously described and unknown ones until now, belonging to new taxonomic groups. This survey encourages for further work in order to complete descriptions and also to describe the ecological role of these organisms in such extreme environments.

Introduction

Fungi belong to microbial eukaryotes and colonize diverse habitats, terrestrial, aquatic and marine. Marine fungi comprise a small and ecologically defined group of filamentous ascomycetes, their anamorphs and yeasts (Kohlmeyer and Kohlmeyer, 1979), but they have been little studied until now. Described higher marine fungi are listed as 467 species belonging to 244 genera (Kis-Papo, 2005). Fungi from aquatic and marine habitats have received much attention in recent years to assess their diversity and to elucidate their ecological role (Kohlmeyer and Kohlmeyer, 1979; Hyde *et al*., 2000; Shearer *et al*., 2007). Production of bioactive metabolites was also screened and reported (Cuomo *et al*., 1995; Christophersen *et al*., 1998; Cueto *et al*., 2001; Rowley *et al*., 2003; Lin *et al*., 2005; Bhadury *et al*., 2006). Most of the reported studies on marine fungal diversity have focused on tropical mangroves, salt marshes and open oceanic regions (Kohlmeyer and Kohlmeyer, 1979; Jones and Alias, 1997; Buchan *et al*., 2002). In tidal zones, in brackish and estuarine habitats, fungi are involved in the degradation of organic matter; their ecological importance is established as saprobes, pathogens and symbionts in algae, higher plants and animals (Kohlmeyer and Kohlmeyer, 1979; Hyde *et al*., 1998). Concerning yeasts, pink basiodiomycetes seem to be the most common group of yeasts found in the marine environment (Lorenz and Molitoris, 1997), whose presence was correlated with organic matter resulting from polluted waters, water boundaries, decaying plankton blooms and macroalgae. The role of fungi and yeasts in marine habitats seems underestimated (Hyde *et al*., 1998), particularly in extreme environments such as deep-sea. Presence of decaying algae, higher plants and wood in the deep-sea suggest that fungi may be transported to the deep-sea attached to such substratum and/or to particulate organic matters, they could also sediment as spores or mycelial filaments (Lorenz and Molitoris, 1997). Over the past several years, there has been an increasing interest in diversity of microbial eukaryotes in extreme environments. Studies employed culture-independent methods combined with sequence analysis of the small subunit ribosomal RNA genes (SSU rRNA) of microeukaryotes. This approach was applied to survey the microeukaryotic diversity in extreme environments such as acidic and iron rivers (Zettler *et al*., 2001; 2003; Gadanho and Sampaio, 2006), anoxic shallow sediments of marine water and freshwater (Dawson and Pace, 2002; Stoeck *et al*., 2006), suboxic waters and anoxic sediments in salt marsh (Stoeck and Epstein, 2003), anoxic deep-sea waters (Takishita *et al*., 2005; Zuendorf *et al*., 2006) and deep-sea hydrothermal vents (Edgcomb *et al*., 2002; Lopez-Garcia *et al*., 2003; 2007; Gadanho and Sampaio, 2005; Bass *et al*., 2007). These studies revealed a majority of sequences

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belonging to *Alveolata* and *Stramenopiles*. Fungi were scarce or even absent but some fungal sequences harvested at deep-sea vents were novel. Scientists studying microeukaryotic diversity in an acidic iron river revealed new fungal phylotypes with fungal-specific primers that were not detected when universal eukaryotic primers were used (Gadanho and Sampaio, 2006). These results suggested that all the previous studies could have underestimated fungal diversity in extreme environments. Considering the revealed existence of original fungal diversity at deep-sea vents, a new evident following step was isolation and description of corresponding organisms. Yeast diversity was assessed at Mid-Atlantic Ridge (MAR) and revealed 12 phylotypes belonging to *Ascomycota* and 7 to *Basidiomycota* (Gadanho and Sampaio, 2005). Thirty-three per cent of the isolated strains belonged to new phylotypes. In this study, we have decided to assess the presence of culturable fungi with an emphasis on filamentous fungi at deep-sea vent ecosystems. To our best knowledge, no previous report has dealt with culturable filamentous fungi from deep-sea vent ecosystems. Fresh and frozen samples collected at MAR, South-west Pacific-Lau Basin and East Pacific Rise (EPR) during six oceanographic cruises, BIOLAU (1989), DIVANAUT2 (1989), HERO (1991), MARVEL (1997), EXOMAR (2005) and MoMARDREAM-Naut (2007), were used to isolate culturable fungi. We report here a global description of the resulting culture collection of filamentous fungi.

Results

Culturable fungi isolated

A total of 210 samples were processed for fungal growth of which 42 (20%) yielded to isolation of fungi. Consequently, all other samples were free of culturable fungi in our culture conditions (80%). Hydrothermal samples consisted mostly in shrimps (71) and mussels (48) and for lower numbers in smoker rock scrapings (25), alvinellids (20), tubeworms (12), sediments (8), other animals (8), colonization modules (4), seawater (3), corals (3) and gastropods (2). Ocean surface seaweeds and seawater were sampled as control (4 and 2 samples, respectively). The final number of fungi obtained on isolation plates was 97. Morphological characteristics examined under optical microscope permitted to separate filamentous fungi and yeasts. Observations revealed branching hyphae and spores for filamentous fungi (62 isolates) and budding cells for yeasts (35 isolates). Only filamentous fungi have been exploited in this study. Samples used to build our fungal culture collection and their origins are shown in Table 1. Filamentous fungi were mostly isolated from *Bathymodiolus azoricus* mussels (36 isolates) and deep-sea hydrothermal shrimps (14 isolates) when considering absolute frequencies. Those results indicate that fungi were much more associated with animals rather than mineral substrate. Fungal strains were mainly isolated from fresh samples directly on board during oceanographic cruises. However, cryoconserved samples of past cruises enabled isolation of a few strains (12 isolates/55 cryoconserved samples processed). Several strains (MV16, MV18, MV20, MV22) did not grow during the purification step and were finally lost.

Fungi were not found at the same rate in all the studied sites. The greatest part of filamentous fungi was found from MAR at Menez Gwen and Rainbow sites. Isolates from Menez Gwen dominated the filamentous fungi culture collection (27) compared with Rainbow (21), even if the number of samples processed was lower for Menez Gwen (16) than Rainbow (97). This corresponds in fact with the presence of one *B. azoricus* individual that gave a high number of fungal isolates in Menez Gwen. We did not succeeded to isolate any fungal strain at Lucky Strike site. Statistical distribution tests have been performed in order to know the distribution type of fungi in hydrothermal sites. The variance to mean ratio (σ^2/m) was calculated for each site (Cancela da Fonseca, 1966). Significant values corresponds with $(\sigma^2/m) - 1 > 2\{[2n/(n-1)^2]^{1/2}\}$. In all sites studied with sufficient number of isolates, an aggregate distribution ($\sigma^2/m > 1$) was observed: Rainbow (1.75), Elsa (1.71), Menez Gwen (8.12) and Lost City (2). This indicates that fungi were located in specific niches inside the ecosystem (mainly mussels and shrimps). For TAG site, this value was not significant. No fungi were isolated from Lucky Strike and Lau Basin.

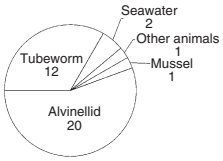
We used a chi-square analysis to test independence of fungal isolation from locations and kind of samples collected. The result was that fungal isolation was very highly dependent ($P < 0001$) on hydrothermal sites ($P = 2.32 \times 10^{-14}$) and kind of samples collected ($P = 2.02 \times 10^{-30}$). Thus, hydrothermal sites and kind of samples had a very highly significant influence on isolation. This very high dependence strongly supports the idea that our isolates were not contaminants. Moreover, when pieces of samples were deposited on Petri dishes, fungal colonies accounting for our collection always grew from those pieces, indicating clearly that fungi were physically associated with samples. Concerning positive enrichments, most frequently, we observed only one fungal colony per isolation Petri dish. However, in some cases, mussel samples (3) yielded to isolation of several colonies (Table 1).

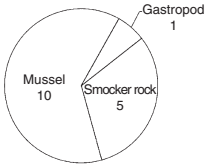
Physiological characterization

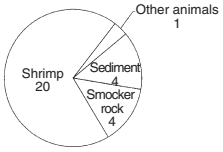
To discriminate individuals (filamentous fungi) for their ability to live in deep-sea hydrothermal ecosystems,

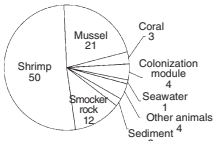
Table 1. Culture collection of filamentous fungi from deep-sea hydrothermal vents.

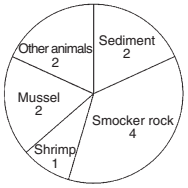
Location (depth)	Strain (culture medium)	Sample processed (type and number)
EPR; 13°N; Elsa site (–2630 m)		
H18E11: *Alvinella pompejana* tube	HE1, HE2 and HE3 (Sab)	
H18E12: *A. pompejana* with bacterial mat	HE4 (Sab)	
H18E09: Seawater surrounding *A. pompejana*	HE5 (Sab)	
H13E04: *Riftia pachyptila* branchial plume	HE6 (GYPS)	
H18E01: *A. pompejana* tube	HE7 (Sab)	
MAR; Menez-Gwen (–860 m)		
DV14E01: *Bathymodiolus azoricus* flesh	DIVA1 (GYPS)	
MV2E01: External face of a shell of *B. azoricus*	MV1 (Ac), MV2 (Lc), MV3 (Xc), MV4 (Xc), MV21 (Cc), MV22 (Pc), MV23 (Pc), MVFS1 (GYPS), MVFS2 (GYPS) and MVFS3 (GYPS)	
MV2E02: *B. azoricus* tissue	MV6 (Ac), MV7 to MV12 (Cc), MV13 (Pc), MV14 (Xc), MV24 (Pc), MV25 (Pc) and MVFS4 (GYPS)	
MV2E03: *B. azoricus* mineral substrate	MV15 (Ac), MV16 (Cc), MV17 (Lc), MV18 (Pc), MV19 (Xc), MV26 (Cc) and MV27 (Xc)	
MAR; TAG (–3650 m)		
Ex13E04: *Rimicaris exoculata*	Ex7 (GYPS)	
MAR; Rainbow (–2300 m)		
Ex6E01: *Rimicaris exoculata*	Ex1 (GYPS)	
Ex8E02: Sediment	Ex2 (GYPS)	
MoPR1: Smoker rocks	Mo1 (GYPS)	
MoPR2 to MoPR5: *Rimicaris exoculata*	Mo2 to Mo13 (GYPS)	
MoPR6: *Bathymodiolus azoricus* byssus	Mo14, Mo15 and Mo18 (GYPS)	
MoPR6: *Bathymodiolus azoricus* tissue	Mo16 (GYPS)	
MoPR6: Commensal worm in *Bathymodiolus azoricus*	Mo17 (GYPS)	
MoPR9: Coral	Mo19 (GYPS)	
MAR; Lost City (–700 m)		
MV5E03: *Bathymodiolus azoricus* tissue	MV20 (Lc)	
Ex18E01: Siliceous sponge	Ex8 and Ex9 (GYPS)	











MAR, Mid-Atlantic Ridge; EPR, East Pacific Rise; H, HERO; DV, DIVANAUT2; MV, MARVEL; Ex, EXOMAR; Mo, MoMARDREAM-Naut; Ac, Potato starch; Cc, Cellobiose; Gc, Glucose; Lc, Arabic gum; Xc, Xylan; Pc, Brain heart; GYPS, Glucose – Yeast extract – Peptone – Starch; Sab, Sabouraud.
Six samples of seaweed (*Sargassum fluitans*) collected at the ocean surface of the TAG site were processed as control and yielded to the isolation of filamentous fungi (Ex3 to Ex6). Twelve strains (HE1, HE2, HE3, HE4, HE5, HE6, HE7, MVFS1, MVFS2, MVFS3, MVFS4 and DIVA1) were isolated from frozen samples.

principal component analysis was carried out on growth data (colony diameters after 14 days of growth) at different temperatures and salinities (see Fig. 1). Axis 1 explained 59% of the total variance and has a significance of growth axis. Axis 2 appeared as a temperature behaviour axis and gathers 20% of the total variance. Seventy-nine per cent of the whole variance is visible on the first plan (Axis 1–Axis 2) that permitted a reliable discussion of the individual positions. On this first plan, we can observe individuals with fast growth on the right side of the figure and other with low growth on the left side distributed depending on their temperature preferendum.

When considering colony diameters at 14 days of growth, a majority of strains (35 located in the upper part of the figure) were psychrotrophs, defined as 'those that grow at low temperatures but also above 20°C' (Deacon, 2006). Remaining strains cannot grow at the lower temperature tested in this study (5°C) and thus cannot be defined as psychrotrophs but rather as mesophiles defined as 'those that grow between 10°C and 40°C with an optimal growth temperature of 20–35°C' (Hawksworth *et al.*, 1995; Deacon, 2006). No psychrophiles, defined as 'those having an optimum growth at no more than 16°C and a maximum growth of about 20°C' (Deacon, 2006), were isolated in our study.

A third axis explained 8% of the total variance and is a salinity behaviour axis independent of the two first axes. Strain Ex2 was strictly halophile (no growth without sea salts) and though strongly correlated with this third axis. Four strains (Mo9, MV8, MVFS1 and MVFS4) were inversely correlated with the third axis and defined as non-halophiles (difficulty to grow increasing with sea salts present in the media). Considering colony diameters at 14 days of growth depending on the salinity parameter, four groups were defined:

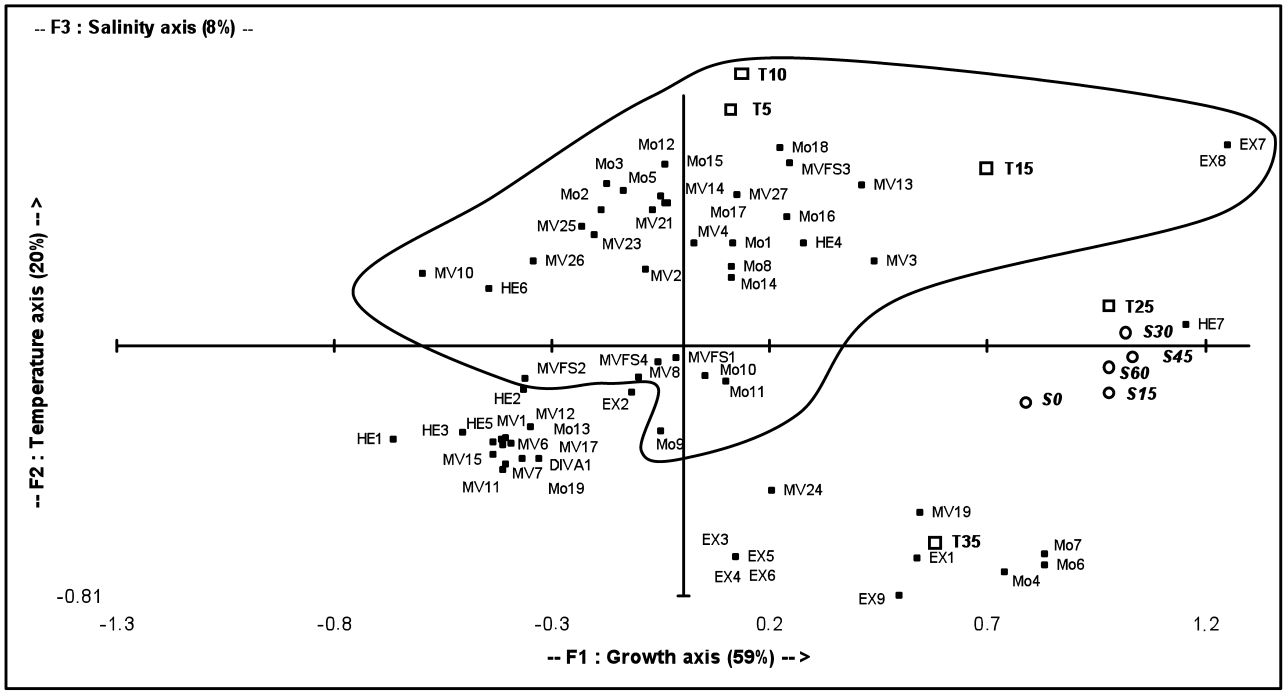


Fig. 1. Physiological analysis of the fungal collection. This figure was obtained from principal component analysis of fungal strains colony diameters (individuals) at different temperatures and salinities (variables). Mycelial colony diameters were measured after 14 days of incubation on GYPS medium depending on growth conditions (four temperatures at 3% sea salts and five salinities at strain optimal temperatures). Strains encircled were psychrotrophs (able to grow at 5°C) and others were mesophiles. Filled squares, empty squares and empty circles corresponds, respectively, to the strains, the temperature and the salinity parameters.

- *Strictly halophile*: Ex2
- *Halophile*: Ex1, Ex7, Ex8, Ex9, Mo4, Mo6, Mo7, Mo12, Mo14, Mo15, Mo16, Mo17, Mo18, Mo19, MV2, MV4, MV10, MV14, MV21, MV23, MV25, MV26, MV27, HE3
- *Halotolerant*: HE2, HE4, HE7, Mo3, Mo5, Mo8, MV13, DIVA1
- *Non-halophile*: Ex3, Ex4, Ex5, Ex6, Mo1, Mo2, Mo9, Mo10, Mo11, Mo13 MV1, MV3, MV6, MV7, MV8, MV9, MV11, MV12, MV15, MV17, MV19, MV24, MVFS1, MVFS2, MVFS3, MVFS4, HE1, HE5, HE6

Phylogenetic diversity

The first goal of this study was to determine whether culturable fungal strains could be isolated from deep-sea hydrothermal ecosystems that were never harvested for such organisms. Based on the gene encoding SSU ribosomal RNA, our purpose was first to identify each fungal strain and, if not, to assess their taxonomic originality based on sequence similarities with closest neighbours in phylogenetic trees. SSU rRNA sequences from culture-independent studies in deep-sea ecosystems were integrated to this phylogenetical analysis. When sequences from our culture collection could not be identified by 18S rRNA, complementary genetic analyses were performed using ITS and 28S rRNA sequences.

Our 18S rRNA sequences indicated the presence of *Ascomycota* and *Basidiomycota* in our culture collection (see Fig. 2). The distribution of isolates was unequal between these two phyla as a majority of them were ascomycetes represented by *Pezyzomycotina* subphylum. Only one isolate (HE6) was affiliated to *Basidiomycota* and was close to *Tilletiopsis pallescens* (16 mismatches on 1651 bp).

Within *Ascomycota*, in order of decreasing cluster size, *Helotiales* were dominant (14 strains), 12 strains of which formed cluster Hel2 (MV2, MV4, MV14, MV21, MV27, MVFS3, Mo12, Mo14, Mo15, Mo16, Mo17, Mo18; see Fig. 2). Eleven of those strains were isolated from *B. azoricus* 'microenvironment' (internal and external faces of mussels) and one from *Rimicaris exoculata* (Mo12; see Table 1). Their ITS rRNA gene sequences were similar to *Cadophora malorum* (100% similarity). Hel1 corresponds with two strains (HE3 and DIVA1) closely similar (4 mismatches on 1431 bp for 18S rRNA) isolated, respectively, from *Alvinella pompejana* and *B. azoricus*. Their taxonomic originality was confirmed with 18S, ITS and 28S rRNA gene sequence analyses, respectively, 23 mismatches on 1431 bp (98% similarity) with *Helicodendron paradoxum*, 18 mismatches on 470 bp (96% similarity) with *Phialophora* sp. and 42 mismatches on 1055 bp (95% similarity) with *Hyphodiscus hymeniophilus*.

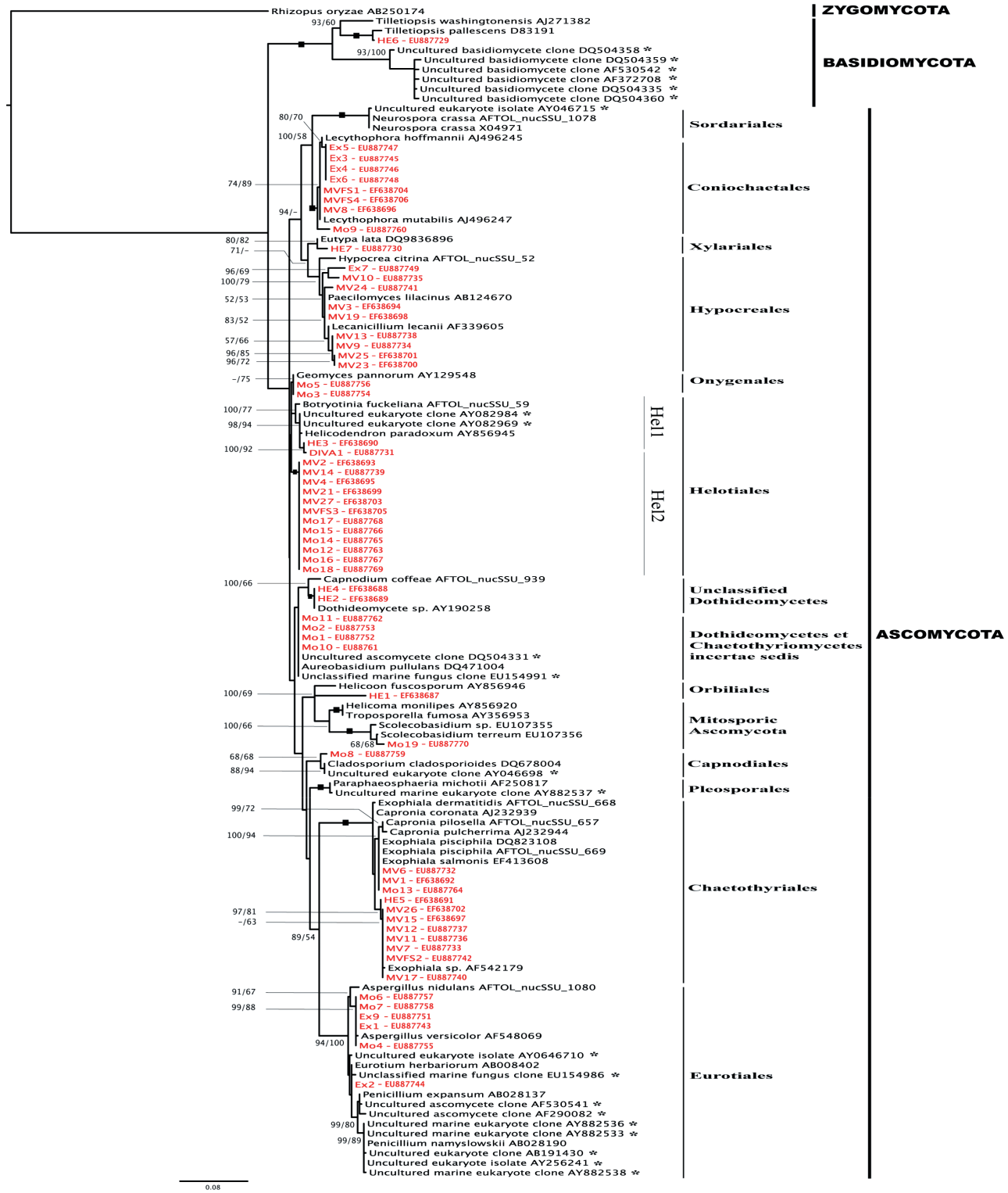


Fig. 2. SSU rRNA phylogenetic positions of deep-sea fungal isolates (coloured terminals). Topology was built using MrBayes v.3.1.2 from a ClustalW 1.83 alignment. Node support values are given in the following order: MrBayes posterior probabilities/PHYML 100 bootstraps. Black squares represent nodes supported by an excess of 0.95 posterior probabilities and 95% bootstraps. Included environmental sequences from deep-sea clones are marked with an asterisk. *Rhizopus oryzae* (AB250174) belonging to the *Zygomoycota* phylum was used as outgroup. All sequences are listed with their GenBank accession numbers.

Chaetothyriales order pooled 11 strains. Three of which (MV1, MV6 and Mo13) were clearly identified as *Exophiala* sp. and the eight others were close and still belonging to the *Exophiala* clade. Based on their 18S rRNA genes, the closest neighbour for members of *Hypocreales* were *Paecilomyces lilacinus* (MV3, MV19 and MV24) and *Lecanicillium lecanii* (MV9 and MV13). However, five strains (MV10, Ex7, Ex8, MV23 and MV25) were separated from available reference sequences of known species. Eight strains belonged to *Coniochaetales* and were close to *Lecythophora* genus. Interestingly, four of those strains (MV8, MVFS1, MVFS4 and Mo9) were isolated from the deep-sea mussel *B. azoricus* and the deep-sea shrimp *R. exoculata* and the four others (Ex3 to Ex6) from an ocean surface alga (*Sargassum fluitans*). Six strains were affiliated to the *Eurotiales* order. Five were close to *Aspergillus* sp. (Ex1, Ex9, Mo4, Mo6 and Mo7). ITS and 28S rRNA gene sequence analyses have permitted to clearly identifiy *Aspergillus sydowii* (100% similarity). Strain Ex2 was clearly identified as *Eurotium herbariorum* (100% similarity). Four strains (Mo1, Mo2, Mo10 et Mo11) were close to *Aureobasidium pullulans* (99.8% similarity). Those strains were isolated during MoMARDREAM-Naut cruise at Rainbow site from rocks scrapping and *R. exoculata*. Two strains isolated from *R. exoculata* (Mo3 and Mo5) were clearly identified as *Geomyces pannorum* (100% similarity). Representatives of *Dothideomycetes* (HE2 and HE4), *Xylariales* (HE7), *Orbiliales* (HE1), *Capnodiales* (Mo8) were also observed in our study.

Based on SSU rRNA genes, isolates affiliated to *Capnodiales* (Mo8), *Dothideomycetes* et *Chaetothyriomycetes* incertae sedis (Mo1, Mo2, Mo10, Mo11) and *Eurotiales* (Ex2, Ex1, Ex9, Mo4, Mo6, Mo7) clusters were grouped close to environmental clones obtained from deep-sea hydrothermal ecosystems with culture-independent methods. Mo8 grouped closely (99.15% – 8 different nucleotides on 946 bp) to one clone detected in the sulfide and hydrocarbon-rich hydrothermal sediment of the Guaymas Basin at 2000 m deep in the Gulf of California (AY046698). Mo1, Mo2, Mo10 and Mo11 close to *Aureobasidium pullulans* were phylogenetically associated (100% similarity) to clones harvested in the fluid-seawater mix samples processed on MAR in Lost City site at 750–900 m deep and phylogenetically close (99.74% – 3 different nucleotides on 1160 bp) to clones obtained near Bismarck wreck at 4790 m deep (EU154991). Ex2 identified as *E. herbariorum* grouped close (99.60% – 5 different nucleotides on 1257 bp) to one clone isolated from three different deep-sea samples: near Bismarck wrecks and in the drake passage water column at 2000–3000 m and 200–500 m deep (EU154986) and also phylogenetically close (99.43% – 6 nucleotide differences on 1054 bp) to one clone harvested in sediment of the Guyamas Basin (AY046710). Our *Aspergillus* strains (Ex1, Ex9, Mo4, Mo6, Mo7) were also close (98.6% – 16 differences on 1147 bp) to the later (AY046710).

18S rRNA sequences indicated a high heterogeneity of our isolates among *Ascomycota* that grouped within 13 different orders. Several isolates have no similar sequences in the GenBank database. This suggests that novel species/genera were isolated even if we have to consider that fungal 18S rRNA sequence databases could be incomplete.

Discussion

Risk of contamination

When scientists are trying to describe fungal diversity in an environment, they must consider that fungal contaminants could be isolated. But first, definition of a fungal contaminant has to be discussed. Two types of contaminant could occur in deep-sea microbiology depending on contamination origin: (i) anthropogenic contaminant appearing during ROV or submarine sampling or isolation on oceanographic vessel (fungal airborne spores for example); (ii) terrigenous contaminant occurring in deep-sea vents due to natural phenomenons as oceanic currents or sedimentation. These last contaminants are circulators that could survive in deep-sea vents or adapt to hydrothermal conditions.

Deep-sea hydrothermal vents are complex ecosystems, not only on a biological point of view but also for sampling, which is expensive and consequently scarce. This situation limits the use of complex experimental designs to study the risk of contamination. Contamination can occur and have been visualized only once during EXOMAR cruise due to a default of the laminary flow-hood. Control plates led to the obtention of *Penicillium* strains and consequently *Penicillium* strains isolated during EXOMAR with the same morphology have been eliminated. Strains considered as marine are those that have grown directly from hydrothermal substrates. Considering our results, we can observe that 80% of the samples processed were sterile for fungi on the high amount of culture media processed. No fungi were isolated at one hydrothermal site (Lucky Strike) although same methods were employed for sampling. Our chi-squared results have clearly indicated that our isolates were very highly dependent on hydrothermal sites and on type of samples processed. Finally, for 5 clusters, encompassing 38 filamentous fungi isolates (on our 62 strains), samples that led to isolation were processed at different dates during 2 or even 3 different oceanographic cruises. Same strains isolated in the same place after several years strongly support that those fungi were not anthropogenic contaminants or circulators

and had an ecological role at deep-sea ecosystems. All our results suggest that sampling methods and materials used were not a root of anthropogenic contaminations.

Occurrence in hydrothermal vents

To understand the importance of fungi at hydrothermal vents, we needed first to assess their occurrence in these ecosystems. Considering that fungal DNA signatures were previously reported in hydrothermal studies, our goal was to know if fungal strains from such locations were culturable. We succeed in isolating fungi from a relatively low part of the samples collected (20%). Such a scarce degree of presence may indicate that fungi were not widely distributed in deep-sea hydrothermal vents. This statement appears in agreement with previous culture-independent studies that harvested only 18 deep-sea fungal sequences from 239 clones (Bass *et al*., 2007) and only one fungus type from several deep-sea hydrothermal mussels of South-Pacific sites (Van Dover *et al*., 2007). Statistical distribution tests led to visualization of an aggregate distribution in all sites studied with sufficient numbers of isolates. This is concordant with previous study (Van Dover *et al*., 2007) and could explain this moderate fungal presence. Such a degree of presence may also reflect a slow fungal colonization process due to deep-sea conditions. This hypothesis is in agreement with a previous study that did not recovered any fungal sequences from a 15-day colonization module deployment, when significant numbers of sequences were obtained from Titanic and Bismarck wreck areas (Bass *et al*., 2007). Another hypothesis to explain this relatively low isolation rate is the use of culture media rich in organic compounds, which may have selected fungal strains associated with animal tissues, an evident source of organic matter. Oligotrophic strains were likely ignored because of the rich culture media chosen. Looking for oligotrophic fungal strains in the deep-sea should be done in the future using nutrient-free, low-nutrient silica gels or other solidifying agents commonly used for soil samples (Wainwright, 2005).

We have isolated filamentous fungi from all sites sampled excepted Lau Basin (frozen samples) and Lucky Strike (fresh samples). A previous study reported prevalence higher than 58% of fungal epizootic diseases on mussels of Mussel Hill hydrothermal site in the North Fiji Basin (Van Dover *et al*., 2007). Our negative results are in agreement with this work that simultaneously did not recover any fungal infected samples from Lau Basin. Consequently, our absence of isolation for Lau Basin does not seem abnormal even if we have to consider that frozen samples may had have a negative impact on culturability. Absence of fungal isolates at Lucky Strike appears more surprising because clones were harvested in a previous study (Lopez-Garcia *et al*., 2007). It could be the result of a too low sampling effort on this site (only six samples) and/or because of the presence of uncultivated strains.

Only one fungal strain was isolated from sediment, indicating a low abundance of culturable fungi in this kind of substrate. Other sediment samples from Guaymas Basin were studied for fungi (data not shown). Using 35 frozen sediment samples from 0 to 40 cm depth for cultivation, no fungi were isolated. Those data are concordant with previous studies on deep-sea sediments that led to obtention of only eight fungal sequences from 515 clones (Edgcomb *et al*., 2002; Bass *et al*., 2007).

Our first results appear encouraging as more than 60 filamentous fungal strains were isolated. Filamentous fungi appear associated to animal samples as mussels and shrimps (when considering absolute frequencies). Filamentous fungi were associated to mussels especially inside animal (from flesh) and outside (from shell and byssi). Further studies should be useful to assess their role as symbionts, parasites or saprotrophs.

Endemic versus ubiquist

Successful isolation of culturable fungi led to the outcoming question whether strains were endemic (autochtonous) or ubiquist (allochtonous). Considering growth depending on temperature and salinity, our culture collection was divided in two groups. First, psychrotrophs that could be common deep-sea marine fungi adapted to low temperatures conditions in such environments. We can hypothesize that those psychrotrophs are real deep-sea endemic strains. Second, mesophiles that did not show specific adaptation to live in dominant cold deep-sea conditions. But, considering their physiology, those strains were able to survive and maybe to use specific niches at deep-sea vents. So, we can here hypothesize that those mesophiles could be ubiquist fungi present at hydrothermal vents due to sedimentation or currents. Those hypotheses are in agreement with the cold *in situ* temperatures measured in shrimp swarms at Rainbow site: 13.2 $\pm$ 5.5°C (Desbruyères *et al*., 2001) and 8.7 $\pm$ 2.3°C (Zbinden *et al*., 2004) and in *B. azoricus* mussel beds: 6.0 $\pm$ 3.9°C at Rainbow and 10.1 $\pm$ 0.5°C at Menez Gwen (Desbruyères *et al*., 2001).

Some strains have shown difficulties to grow under our cultivation conditions that were not necessarily optimal. Hydrostatic pressure, whose effect was not studied in this study, could be an important parameter to control for their growth. Indeed, although previous works have dealt with the effective growth of marine fungi (Raghukumar and Raghukumar, 1998; Damare *et al*., 2006) and marine yeasts (Lorenz and Molitoris, 1997) under elevated

hydrostatic pressure, no true piezophilic fungi have been reported to date.

Adaptation or natural selection of fungal strains to deep-sea vent conditions may occur. Eight filamentous fungi belonging to *Lecythophora* genus were isolated from different substrates: four strains were isolated from *Sargassum fluitans* from the ocean surface used as control and four from *B. azoricus* shell and flesh and *R. exoculata* (Table 1). Principal component analysis and crude values from physiological analyses indicated that the four strains from seasurface were mesophiles (not able to grow at 5°C) and the four strains from vents were psychrotrophs and therefore able to grow at 5°C (Fig. 1). Considering the Raghukumar hypothesis (see next part), sinking organic materials might bring fungal strains in deep-sea that could adapt to cold deep-sea conditions.

Diversity and potential role in deep-sea ecosystems

Our cultures showed the presence of five strains (Ex1, Ex9, Mo4, Mo6 and Mo7) belonging to *Aspergillus* sp. (*Eurotiales* order) isolated during two different oceanographic cruises (EXOMAR and MoMARDREAM-Naut) from Rainbow and Lost City sites. Complete analyses based on rRNA Internal Transcribed Spacer (ITS1-5.8S-ITS2) sequencing revealed the species *A. sydowii*. This species is a common terrestrial fungus isolated from diverse habitats and causing an epizooty among sea fan corals (Alker *et al*., 2001). Recent study reported *A. sydowii* in deep-sea sediments (Raghukumar *et al*., 2004). Our five strains were described as mesophiles in physiological analysis (Fig. 1) and so not specifically adapted to usual deep-sea marine conditions. A first possibility could be that these ubiquitous strains are contaminants incoming from ocean surface or transported by currents. Their presence in marine ecosystems is congruent with the fact that all *Aspergillus* isolated were halophiles. But, in our case, these five strains were isolated at two different dates, persons, oceanographic cruises and sites. Moreover, our strains grouped close to environmental sequences from deep-sea environments: AY046710 (Edgcomb *et al*., 2002) and EU154986 (Bass *et al*., 2007). These data suggested that our *Aspergillus* strains were naturally existent at deep-sea. *Aspergillus* may have a role in deep-sea environments even if they are not indigenous and even if they are not in optimal growth conditions. Occurrence of fungi in deep-sea sediments from the Chagos Trench (Indian Ocean) at 5000 m depth was reported (Raghukumar *et al*., 2004; Damare *et al*., 2006; Damare and Raghukumar, 2008). Their study yielded almost exclusively the mitosporic fungus *Aspergillus sydowii* present at core depths of 160–370 cm. Spores of *A. sydowii* from their core samples germinated and grew at elevated hydrostatic pressures and low temperatures. They hypothesized that 'such spores might eventually sink to the deep-sea surficial sediments, undergo natural selection mechanisms with time and acquire capabilities to grow and multiply in the presence of suitable nutrient sources' (Raghukumar *et al*., 2004). Our paper reveals the first presence of *A. sydowii* at deep-sea hydrothermal vents, where such microorganism may grow and reproduce thanks to temperature gradients and to high and diverse nutrient sources in this particular ecosystem.

Other mesophilic strains were isolated (MV19 and MV24) and clearly identified as *P. lilacinus*. They were isolated from *B. azoricus* samples. Their real development in deep-sea is less evident because those strains were clearly non-halophiles.

One strain in our collection (Ex2) was strictly halophile and characterized as *E. herbariorum*. Our results from cultures were well correlated with those from culture-independent methods: AY046710 (Edgcomb *et al*., 2002) and EU154986 (Bass *et al*., 2007). Sequences close to our organism have been detected in several deep-sea environments and also in the water column. Moreover, *E. herbariorum* has been isolated in other extreme environments: hypersaline waters of solar salterns (Butinar *et al*., 2005) and the Dead Sea waters (Jin *et al*., 2005). In these environments, *E. herbariorum* was described as the most common species isolated and thus characterized as indigenous. In our case, Ex2 was isolated from a sediment core sample where this strain may have a role in decomposition of organic matter in deep-sea floor.

Helotiales order gathered 14 strains in our collection that were isolated during different oceanographic cruises. Twelve strains were identified as *C. malorum* and described physiologically as psychrotrophs and also mainly halophiles (except MVFS3 that was non-halophile). These last 12 strains corresponding to a single fungal species (identical 18S, ITS and 28S rRNA sequences) were isolated from two different hydrothermal sites: Menez-Gwen (6) and Rainbow (6) and thus support the ecological significance of this species in the deep-sea vents. *Cadophora malorum* has been reported largely in Antarctica on mosses (Azmi and Seppelt, 1998; Tosi *et al*., 2002), which demonstrated their wide distribution on the Antarctic continent. A recent study has physiologically demonstrated that *Cadophora* sp. strains isolated in Antarctic environments were psychrotrophs. Scientists have speculated that the *Cadophora* sp. studied (*containing C. malorum*) were endemic in Antarctica (Duncan, 2007). *Geomyces pannorum* was also frequently isolated as psychrotroph in Antarctic soils and speculated as indigenous in Antarctica (Duncan, 2007). Moreover, *G. pannorum* was isolated from Arctic cryopegs and marine deposits (Kochkina *et al*., 2007). Two of our strains were clearly identified as *G. pannorum* and were characterized as psychrotrophs and also halotolerant. Such psy-

chrotrophic strains from our collection harvested in cold deep-sea vents could be the result of exchanges with polar environments. This hypothesis is concordant with previous study on deep-sea and Antarctic isolates of *Psychrobacter*, reporting that these deep-sea microorganisms probably originate in polar regions and disseminate with the global deep ocean circulation (Maruyama *et al*., 2000). Eleven *C. malorum* from our collection were isolated from *B. azoricus*: 10 from mussels and 1 from a *Polynoidae* scale-worm associated to one mussel sampled. Some scale-worms seem to be able to leave their host mollusks to infest new ones (Britayev *et al*., 2007). Thus, fungi may be disseminated by scale-worms in mussel communities.

Occurrence of fungi inducing diseases in deep-sea vents animals has been reported only once. An emergent epizootic event was reported in the deep-sea mussels *Bathymodiolus brevior* at the Mussel Hill hydrothermal vent in Fiji Basin in which fungi were the causal agent (Van Dover *et al*., 2007). A *Capronia*-like fungus (order *Chaetothyriales*) elicited a host immune response in mussels and was associated with tissue deterioration (using histological observations, genetic analyses, Fluorescent *in situ* hybridization technique but not culture-dependent methods). Eleven isolates of our collection were also affiliated to the *Chaetothyriales* order. Ten strains were characterized as low growing strains and non-halophiles, and only one was psychrotroph and halophile (MV26). They were mostly isolated from *B. azoricus* mussels (> 80%). This set of results suggests that fungi of the *Chaetothyriales* order were predominant in deep-sea mussels of different species (*B. brevior* and *B. azoricus*) and of different locations (South-west Pacific and MAR sites) and can sometimes be associated with pathologic situation.

Our results proved that those fungi isolated from deep-sea mussels are not symbionts because a large amount of living samples were processed and only a few led to the visualization of a fungal presence. Our mussel samples looked apparently healthy with a normal creamy coloration without necrosis or lesions and confirmed the Van Dover hypothesis that 'identification of fungi in otherwise healthy individuals makes it clear that the fungus is not a strict saprophyte' (Van Dover *et al*., 2007). It suggests that fungi may be facultative parasites or opportunistic pathogens of deep-sea animals. This hypothesis is consistent with the fact that we only processed living animal samples. This could support the hypothesis that hydrothermal vents host a unique array of animal parasites (Moreira and López-Garcia, 2003). Four strains isolated during MoMARDREAM cruise at Rainbow site and identified as *Aureobasidium pullulans* were observed for the first time in hydrothermal vents. This yeast-like fungus was previously isolated from deep-sea sediments of the Pacific Ocean (Li *et al*., 2007). These strains were all psychrotrophic. Three isolates (Mo2, Mo10 and Mo11) were obtained from *R. exoculata* and one from a chimney rock scrapping (Mo1). Food intake of *R. exoculata* is composed of rock particles (Segonzac *et al*., 1993). Our study may indicate that a transfer could occur between fungi present on chimney rock and organisms scrapping it. Those culture-dependent results are concordant with previous culture-independent works. Indeed, our strain sequences match with environmental sequences obtained from deep-sea environments: EU154991 and DQ504331 (see Fig. 2). Even if those strains were characterized as non-halophiles, these results indicated that *A. pullulans* is frequently observed in Atlantic deep-sea environments at different depths, from wrecks (4790 m) to hydrothermal sites (750–900 and 2300 m).

Occurrence of fungi in healthy and diseased corals has been widely reported in shallow waters. Fungi are described as skeletal-components of healthy, partially dead and diseased corals (Ravindran *et al*., 2001). One fungal parasite was found to cause necrotic patches on five different corals (*Porites lutea*, *Porites lichen*, *Montipora tuberculosa*, *Goniopora* sp. and *Goniastra* sp.). Infections were due to *Scolecobasidium* sp. (Raghukumar and Raghukumar, 1991). The same genus was found in deep-sea corals (Mo19), near Rainbow hydrothermal vent site (Fig. 2), indicating that this halophilic fungus also occurred in corals at several thousand meters depth and could be implicated in coral diseases. This is the first description of such an association in the whole deep-sea.

HE7 was characterized as an halotolerant fast growing fungus phylogenetically close to *Eutypa lata*, a genus mostly isolated from mangrove woods (Alias *et al*., 1995; Sarma *et al*., 2001). HE2 and HE4 were also halotolerant and close to *Dothideomycete* sp., belonging to an ascomycetous class mainly composed of plant pathogens. HE6 was non-halophile and close to *Tilletiopsis pallescens*, also described as plant pathogen. Wood fungi recovered in deep-sea hydrothermal vents may be explained by sunken wood presence in deep-sea (Palacios *et al*., 2006).

Our study revealed a non-exhaustive culturable fungal diversity until now never harvested in deep-sea environments. The preventive measures taken (sterile sampling equipments, laminary flow-hood on board, etc.) permit to control contamination. Moreover, physiological analyses confirmed that fungi isolated from several deep-sea vents mostly gathered likely indigenous species. Further works concerning the effect of hydrostatic pressure on growth (use of pressure vessels), metabolic activities and the use of diverse media are needed to assess more their adaptation to deep-sea conditions. *In situ* observations could also permit to approach the ecological importance of such organisms and to know whether they form dense or scarce communities.

Experimental procedures

Environmental sampling

Hydrothermal vents. 210 hydrothermal samples were collected during six oceanographic cruises: (i) BIOLAU in the Lau Basin, South-west Pacific (12/05/1989–27/05/1989; 20°3.0′S, 176°7.8′W; −2620 m); (ii) DIVANAUT2 (19/06/1994–01/07/1994) on the MAR at Menez Gwen (37°51′N, 31°31′W; −900 m) and Lucky Strike (37°17′N, 32°16′W; −1650 m) sites; (iii) HERO on the EPR at Elsa site (30/09/1991–04/11/1991; 12°48′N, 103°57′W; −2630 m); (iv) MARVEL (29/08/1997–13/09/1997) on the MAR at Menez Gwen (37°51′N, 31°31′W; −900 m) and Lucky Strike (37°17′N, 32°16′W; −1650 m) sites; (v) EXOMAR (25/07/2005–28/08/2005) on the MAR at Rainbow (36°08′N, 34°00′W, −2300 m), TAG (26°02′N, 44°54′W, −3630 m) and Lost City (30°04′N, 42°12′W, −900 m) sites; (vi) MoMARDREAM-Naut (08/07/2007–19/07/2007) on the MAR at Rainbow site (36°08′N, 34°00′W, −2300 m).

Surface. Samples of *Sargassum fluitans* were found floating at ocean surface in the TAG area during EXOMAR. Individuals were sampled with surrounding seawater for comparison with deep-sea hydrothermal samples using sterile strip and tubes.

Deep-sea sampling was performed using the Deep Submergence Vehicle Nautile for BIOLAU, HERO, DIVANAUT2 MARVEL and MoMARDREAM-Naut (http://www.interridge.org/en/node/4897) cruises and the ROV 'Victor 6000' for EXOMAR cruise. Support research vessels were 'Nadir' for HERO, BIOLAU and DIVANAUT2 cruises, 'Atalante' for MARVEL and EXOMAR cruises and 'Pourquoi Pas ?' for MoMARDREAM-Naut cruise (http://www.ifremer.fr/fleet//index.php). Deep-sea waters were collected with titanium syringes, sediments with a sediment sampler, shrimps with a fauna aspirator, alvinellids, mussels, sponges and minerals using insulated boxes. After washing, those equipments were sterilized (sediment sampler) or ethanol disinfected (titanium syringes, aspirator containers and pipes, sampling boxes) and filled with sterile seawater.

All samples were processed directly after the Nautile or ROV recovery. On board, in the lab, sterility was obtained with Bunsen burner or using a vertical laminary flow hood. Solid samples were taken in their container with sterile strips, laid on a sterile Petri dish and abundantly rinsed with sterile seawater before crushing in a mortar with a pestle, both autoclaved previously. Sterile seawater was added for crushing when necessary. Sterile seawater contained (per litre) 30 g of Sea salts (Sigma) diluted in 1 l of distilled water filtered on 0.22 µ and autoclaved to obtain sterility. Collected seawaters, sediments and crushed samples were used to inoculate culture media and frozen in cryotubes at −70°C with DMSO as cryoprotectant (5% v/v) for further use. During MoMARDREAM-Naut cruise, dissections were realised on animal samples in order to analyse precisely fungal location.

Enrichment conditions

During HERO cruise, Sabouraud (Sab) chloramphenicol solid medium (AES Laboratoire) was used for aerobic enrichment cultures at 30°C and atmospheric pressure. During MARVEL cruise, five solid culture media were used. Ac medium contained per litre: potato starch (Sigma) 5 g, yeast extract 0.5 g, peptone 1 g, sea salts 30 g and PIPES buffer (Sigma) 6.05 g. Starch was replaced by cellobiose (Sigma) 5 g in Cc medium, by glucose (Sigma) 5 g in Gc medium, by arabic gum 5 g and olive oil 5 g in Lc medium, by xylan oat spelts 5 g in Xc medium. Pc medium contained per litre: brain heart infusion (Difco) 9 g, NaCl 23 g and PIPES buffer 6.05 g pH was adjusted to 7.5 with NaOH 4 N. The five media were supplemented per litre with agar 15 g and chloramphenicol 500 mg. Cultures were done aerobically at 25°C (ambiant temperature) and atmospheric pressure. During EXOMAR and MoMARDREAM-Naut cruises, GYPS medium was used and contained per litre: glucose 1 g, yeast extract 1 g, peptone 1 g, starch 1 g, sea salts 30 g. This medium was supplemented per litre with agar 15 g and chloramphenicol 500 mg pH was also adjusted to 7.5. Cultures were done aerobically at 4°C, 15°C, 25°C (ambient temperature), 45°C (only during EXOMAR) at atmospheric pressure. Pure cultures were obtained by streaking and central picking on their respective enrichment media and kept in culture collection by continuous culture on GYPS medium supplemented per litre with agar 15 g.

Nutrient plates exposed under laminary flow hood during each isolation were processed as control. No fungal colonies yielded on control plates, indicating no occurrence of aerial contaminations during isolation. Exception made for the laminary flow hood used during EXOMAR cruise that led to isolation of *Penicillium* strains on control plates. All *Penicillium* strains with identical morphology isolated during this cruise were eliminated.

Each purified strain from our collection has been integrated to the 'Souchothèque de Bretagne' (http://www.ifremer.fr/souchotheque/internet/htdocs/generique.php?pagebody=catalogue.php) and are available with an LMSA number associated to their GenBank number.

Physiological characterization

All experiments were done in triplicate. Fungal strains were grown on solid and yeast in liquid GYPS medium. The effect of temperature was determined at 5°C, 15°C, 25°C and 35°C. The effect of salinity was determined in modifying sea salts concentrations in GYPS medium: 0, 15, 30, 45 and 60 g l^{-1}.

Growth measurement and statistical analyses

After central picking on Petri dishes, mycelium diameter was measured each 7 days for a maximum of 5 weeks to characterize fungal growth. Principal component analysis used to analyse physiological results was done with 'Statbox 6.6′ software (Grimmersoft).

DNA extraction and purification

DNA extraction was performed by FastDNA Spin Kit (MP Biomedicals) that is specific for fungi and yeasts following the manufacturer's recommendations.

DNA amplification

18S rRNA gene sequences were amplified with NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and SR6R (5′-AAGTAGAA

GTCGTAACAAGG-3′) primers (White *et al*., 1990) for genetic identification of fungi (Vandenkoornhuyse *et al*., 2002). When SR6R was unefficient, 1020r (5′-AAACATCCTTGGRAATGC TTT-3′) or 1055r (5′-AAGAACGGCCATGCACCAC) (Atkins, 2000) primers were used. Internal Transcribed Spacer and rRNA 28S (partial) were amplified using ITS5 (5′-GGAA GTAAAAGTCGTAACAAGG-3′) and LR6 (5′-CGCCAGTTC TGCTTACC-3′) primers (respectively White *et al*., 1990; Vilgalys and Hester, 1990). All PCR reactions were performed in 20 μl reaction volumes containing 1× Taq Titanium Buffer (Ozyme), 2 mM of each of the four dNTPs (Promega), 0.08 μM of each primer (Proligo), 1 U of Taq Titanium DNA polymerase (Ozyme) and 1 μl of genomic DNA. Reaction mixtures were incubated in a PTC-200 thermal cycler (MJ Research). PCR temperature profile consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C and 2 min at 72°C and a final extension step of 2 min at 72°C before a conservation at 4°C. A negative control in which DNA was replaced by sterile water was included. PCR products were controlled by electrophoresis in 0.8% (w/v) agarose gel (Promega) in 0.5× Tris-Borate EDTA (TBE) buffer at 90 V for 90 min and stained with ethidium bromide. Molecular size marker was Lambda DNA/EcoR1 + HindIII Markers (Promega). DNA banding patterns were visualized under UV transillumination and picture files were generated using Gel-Doc 2000 (Bio-Rad).

Phylogenetic analyses

Fungal amplicons were sequenced with NS1, NS3 (5′-GCAAGTCTGGTGCCAGCAGCC-3′), NS5 (5′-AACTTAAA GGAATTGACGGAA-3′) (White *et al*., 1990) and SR6R primers for SSU rRNA gene sequences. For ITS and LSU rRNA, ITS5 and LR6 primers were used. Sequences were obtained by 'Big Dye Terminator' technology (Applied Biosystems). This work was done at 'Ouest Génopole' sequencing facility in the 'Station Biologique de Roscoff' (http://www.sb-roscoff.fr/SG/).

Chromatograms obtained were translated in nucleotidic sequences with DNA Baser v.2.10 (http://www.dnabaser.com). After cleaning, sequences were imported to MEGA 4.0 software (Tamura *et al*., 2007). Each sequence was analysed in order to find GenBank sequences with close BLAST-N hits (Altschul *et al*., 1990). Additional 18S rRNA sequences from clones harvested during environmental deep-sea studies were included in our analysis (Edgcomb *et al*., 2002; Lopez-Garcia *et al*., 2003; 2007; Takishita *et al*., 2005; Bass *et al*., 2007). Similarities between sequences were assessed using pairwise distance calculation with MEGA 4.0. Finally, sequences from the Assembling the Fungal Tree of Life project have been added to our phylogenetic tree (James *et al*., 2006). Sequences were trimmed to ensure that all sequences had the same start and end-point.

All SSU rRNA sequences were aligned using ClutalW v.1.83 (Thompson *et al*., 1994), one of the most widely used packages, and other packages described as faster and more accurate: MAFFT v.5 (Katoh *et al*., 2005) and MUSCLE v.3.6 (Edgar, 2004). After visual checking and manual curation, each alignment was analysed for the Bayesian estimation of phylogeny using MrBayes v.3.1.2 software (Ronquist and Huelsenbeck, 2003). Three million generations option has been set to run the Metropolis-coupled Monte Carlo Markov Chain method (*mcmc*). After generation 3 000 000, the average standard deviations of split frequencies were in increasing values: $P = 0.01373$ (ClustalW v.1.83), $P = 0.02190$ (MAFFT v.5) and $P = 0.02199$ (MUSCLE v.3.6). For all alignment softwares, a P-value of < 0.05 shows that convergence occurred. Software generating the smallest frequency (ClustalW v.1.83) has been chosen in order to produce an evolutionary tree that would be accurate and robust. Chosen alignment was analysed using MODELTEST v.3.7 (Posada and Crandall, 1998), in order to obtain the more realistic evolutionary model used for phylogenetic analyses (TrN + I + G model; gamma-distribution shape parameter = 0.6470). Phylogeny was then evaluated using two different methods: (i) Bayesian inference with MrBayes v.3.1.2 analysis using 3 000 000 generations and the *mcmc* method. The tree search included two *mcmc* searches with four chains (setting default temperature for heating the chains) and a sampling frequency of 100 generations. A 'burnin' of 7500 (25% of the 3 000 000 generations/100 sample frequency) was set in order to exclude the first 7500 trees generated. (ii) Maximum likelihood with 100 bootstrap iterations using PHYML (Guindon *et al*., 2005) and the parameters obtained with MODELTEST v.3.7. Final phylogenetic tree topology was realized using MrBayes v.3.1.2 analysis results. Nodes in the tree show Bayesian posterior probabilities and ML bootstraps respectively.

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