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# Fungal Diversity in Deep-Sea Hydrothermal Ecosystems▽†

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**Deep-sea hydrothermal ecosystems are considered oases of life in oceans. Since the discovery of these ecosystems in the late 1970s, many endemic species of *Bacteria*, *Archaea*, and other organisms, such as annelids and crabs, have been described. Considerable knowledge has been acquired about the diversity of (micro)organisms in these ecosystems, but the diversity of fungi has not been studied to date. These organisms are considered key organisms in terrestrial ecosystems because of their ecological functions and especially their ability to degrade organic matter. The lack of knowledge about them in the sea reflects the widely held belief that fungi are terrestrial organisms. The first inventory of such organisms in deep-sea hydrothermal environments was obtained in this study. Fungal diversity was investigated by analyzing the small-subunit rRNA gene sequences amplified by culture-independent PCR using DNA extracts from hydrothermal samples and from a culture collection that was established. Our work revealed an unsuspected diversity of species in three of the five fungal phyla. We found a new branch of *Chytridiomycota* forming an ancient evolutionary lineage. Many of the species identified are unknown, even at higher taxonomic levels in the *Chytridiomycota*, *Ascomycota*, and *Basidiomycota*. This work opens the way to new studies of the diversity, ecology, and physiology of fungi in oceans and might stimulate new prospecting for biomolecules. From an evolutionary point of view, the diversification of fungi in the oceans can no longer be ignored.**

Since the discovery of hydrothermal vent ecosystems 30 years ago, unexpected species diversity has been revealed that has shed light on the functional coupling between the geosphere and the biosphere. When submersibles dive to the seafloor, they bring numerous organisms back to the surface, and this has resulted in the description of nearly two new species per month (10). Deep-sea hydrothermal ecosystems are considered hotspots of microbial diversity on the seafloor. Indeed, they are ecosystems that produce biomass using the wide range of chemical compounds released by the polymetallic sulfite chimneys or "black smokers" that represent the huge quantity of chemical energy that is available (26). The vent fluid, having been heated close to a magma chamber, can have a temperature of 400°C when it is emitted. It is also characterized by a lack of dissolved oxygen, strong acidity (pH 2 to 3), a high concentration of electron donors (i.e., reduced compounds such as methane and hydrogen sulfide), and the presence of heavy metals (36). Continual mixing with the cold ocean water (2 to 4°C) that is rich in electron acceptors creates a dynamic chemical disequilibrium that is a source of energy for microorganisms that control the rates of redox reactions (16).

Each ridge displays varied geochemistry, and the vent fluids differ, even at scales as small as the fractures, pipes, and porosities in the black smokers, creating diverse microhabitats for biota (10, 16, 26). Microbes have colonized these different microsites; an impressive diversity of *Bacteria* and *Archaea* species has been described, and even more diversity has been inferred. Some organisms are chemolithoautotrophic primary producers and are at the base of the trophic web in these habitats. In addition to these key microorganisms, other primary producers, like the photosynthetic green sulfur bacteria, reportedly are able to harvest energy from geothermal radiation (3). The organic compounds produced by the primary producers are consumed by heterotrophic microbes and animals through symbioses, filtering of free-living microorganisms, or grazing of biofilms.

A comparison of hydrothermal vent cycling of carbon with the carbon cycling in terrestrial ecosystems suggests that key organisms in hydrothermal vents, the fungi, have not been studied yet. Contemporary organisms living in land ecosystems are well adapted. However, the first colonizers of land faced a harsh physical environment (29). Thus, it has been suggested that early colonization of land was possible only when there was a macroevolutionary jump (i.e., acquisition of multiple competences during a single evolutionary step) (24, 29). It has been suggested that establishment of eukaryotes on land may have been possible only as a consequence of the association of a fungus and a phototroph (24, 29). The earliest known fossil fungus (460 million years) was thought to form arbuscular mycorrhizae (27). The idea that the colonization of land was made possible by this symbiotic association is supported the findings of Simon et al. (31). Based on molecular clock estimates, some authors have suggested that fungi appeared a long time before Devonian land colonization. The major radiations in the fungi may even have appeared during the Precambrian (1,460 to 960 million years ago) (13), 300 million or more years before colonization of land. These analyses are currently under

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discussion because of the absence of fossil evidence to support the conclusions and because of possible bias in the molecular clock estimates (34). However, assuming that the protein estimates are correct, it could be hypothesized that fungi emerged and diversified in water before they colonized land. An ancestral characteristic, a flagellated gamete without a wall, is found in old lineages of fungi. This is generally considered an advantage for dispersal and reproduction in water. The loss of flagella in fungi appears to have occurred in a single evolutionary step (20) and has been used as an argument to support the dogma that higher fungi diversified on land before a secondary colonization of water ecosystems, assuming that some terrestrial fungi secondarily invaded salt marshes and mangroves and adapted to life in salt and brackish water. It is a common belief among ecologists, microbiologists, and mycologists that fungi are found (almost) exclusively on land. A recent study showed that the loss of flagella may have occurred at least four times during fungal evolution (15), which suggests an evolutionary paradigm that is more complicated than previously thought. Despite the view that fungi diversified in terrestrial ecosystems, chytrids are considered primarily aquatic fungi. Furthermore, cultural approaches have resulted in the identification of a small, ecologically defined group of marine higher fungi that includes filamentous ascomycetes, their anamorphs, and yeasts (17). We advocate an alternative hypothesis for the loss of flagella. It can be proposed that the loss of motile gametes in fungi was compensated for by the resistance and long-range dispersal of spores. We suggest that this evolutionary innovation in eukaryotes should have led to colonization and long-term persistence in many new environments, including land, even if the primitive transition of fungi to terrestrial life could have occurred without the previous loss of flagellated spores. From the molecular clock predictions (13) and partially in agreement with our computations (P. Vandenkoornhuyse, unpublished data), we suggest that this evolutionary event might have occurred approximately 1,200 to 1,000 million years ago and, therefore, in oceans. If this is true, we would expect to find a large variety of fungi, including fungi producing nonflagellated spores, in oceanic samples even in isolated habitats.

Consequently, the main aim of the present work was to test for the presence of fungi in deep-sea hydrothermal ecosystems by establishing a culture collection and using a culture-independent approach involving direct amplification of the fungal small-subunit (SSU) rRNA gene from environmental samples and then analysis and evaluation of the diversity of the fungi in these environments. The deep-sea hydrothermal ecosystem was an interesting target to test our hypotheses for various reasons. First, it has been suggested that the hydrothermal ecosystem is the "cradle of life" (14, 18, 39), although more recently it has been shown that life emerged first in a mesophilic environment and diversified in thermophilic conditions (5). Second, hydrothermal conditions were very common during the Precambrian (28). Third, use of general eukaryotic primers to perform culture-independent PCRs (ciPCRs) has led to the detection of fungi close to hydrothermal vents previously (21, 22). Fungal sequences have also been detected in deep-sea anoxic sediments (2, 8, 9), and barophilic fungal strains have been isolated from this environment (7).

## MATERIALS AND METHODS

**Studied sites and sampling.** Samples were collected during the following two oceanographic cruises: (i) HERO (30 September 1991 to 4 November 1991) on the East Pacific Rise at the Elsa site (12°48′N, 103°57′W; depth, 2,630 m) and (ii) MARVEL (29 August 1997 to 13 September 1997) on the Mid-Atlantic Ridge at the Menez Gwen site (37°51′N, 31°31′W; depth, 860 m) and Lucky Strike site (37°17′N, 32°16′W; depth, 1,700 m). Deep-sea sampling was performed using DSV *Nautile*. The support research vessels were R/V *Nadir* for the HERO cruise and R/V *Atalante* for the MARVEL cruise (www.ifremer.fr/fleet//index.php). Animals and rocks were collected using sampling boxes, washed on board, disinfected with ethanol, and filled with sterile seawater. All the boxes were waterproof to prevent contamination from the water column during ascent. They were opened for sampling during dives and then closed and placed in the insulated crate of the submersible to maintain a low temperature until subsequent examination on board.

**Sample preparation and fungal cultures.** On board in the lab, sterility was obtained with a Bunsen burner and a vertical laminar flow hood. Solid samples were removed from the container with sterile strips, placed on a sterile petri dish, rinsed well with sterile seawater, and then crushed with a sterile pestle and mortar. After this, the samples were aliquoted and preserved by deep-freeze storage (−80°C) until they were used.

During the HERO cruise, Sabouraud chloramphenicol solid medium (AES Laboratory) was used for aerobic enrichment cultures incubated at 30°C with atmospheric pressure. During the MARVEL cruise, five solid culture media were used. Ac medium contained (per liter) 5 g potato starch (Sigma), 0.5 g yeast extract, 1 g peptone, 30 g sea salts, and 6.05 g piperizine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (Sigma). The starch was replaced by 5 g cellobiose (Sigma) in Cc medium, by 5 g glucose (Sigma) in Gc medium, by 5 g arabic gum and 5 g olive oil in Lc medium, and by 5 g xylan oat spelt in Xc medium. Pc medium contained (per liter) 9 g brain heart infusion (Difco), 23 g NaCl, and 6.05 g PIPES buffer. The pH was adjusted to 7.5 with 4 N NaOH. The five media were supplemented with (per liter) 15 g agar and 500 mg chloramphenicol. Cultures were grown aerobically at 25°C (ambient temperature) and atmospheric pressure. Finally, all strains were able to grow on GYPS medium containing (per liter) 1 g glucose, 1 g yeast extract, 1 g peptone, 1 g starch, and 30 g sea salts. Pure cultures were obtained and kept in a culture collection by continuous culture and then freeze-dried at −80°C. More details about sampling and cultures are available in a recently published paper (6).

**Extraction of DNA.** Each sample was homogenized with sterile glass beads and ground for 28 s at 30 rpm in a bead beater (MM 301; Retsch). Different methods of DNA extraction to obtain amplifiable fungal DNA were tested. The method chosen was an DNeasy Plant mini kit (Qiagen) used according to the manufacturer's recommendations. DNA was extracted from cultivated strains with a Fast DNA Spin kit (MP Biomedicals).

**Cloning and sequencing.** The fungal SSU rRNA genes were amplified by PCR using primers MH2 (5′TTCGATGGTAGGATAGAGG3′) and MH4 (5′GTCTCACTAAGCCATTC3′) (37) for environmental sequences and primers NS1 (5′GTAGTCATATGCTTGTCTC3′) and ITS5R (5′CCTTGTTACGACTTTTACTTCC3′) primers for cultivated strains (40). The AU2/AU4 primer set (38) did not amplify any of the samples analyzed. DNA was amplified by touchdown PCR with the initial step at 94°C for 1.30 min followed by 37 cycles of 94°C for 30 s, 48°C for 1.25 min (with a 0.1°C decrease at each cycle), and 72°C for 1.5 min. The PCR amplification program ended with a final elongation step at 72°C for 10 min. For the samples which did not yield any product we used a primer set modified from the primer set described by Smit et al. (32), EF3 (5′TCCTCTAAATGACCAGTTTG3′) and EF4 (5′GGAAGGGNTGTATTTATTAGAT3′), and the same PCR conditions except for the annealing temperature (53°C instead of 48°C). For these ciPCRs, a dilution of a soil DNA extract and DNA-free water were used as positive and negative controls, respectively. The amplification fragments were purified with a High Pure PCR product purification kit (Roche) and were cloned in the pGEM-T vector (Promega) and DH5α competent cells (Gibco BRL Life Technologies). For each clone library, 16 to 120 randomly selected positive clones were sequenced (ABI-PRISM Dye Terminator cycle sequencing Ready Reaction kit; Perkin Elmer) on both strands using primers T7 and SP6 for environmental sequences and primers NS1, NS3 (5′GCAAGTCTGGTGCCAGCAGCC3′), and ITS5R for the culture sequences (6). The contig of the two sequence strands was calculated using Sequencher 4.6 (GeneCodes), and alignment uncertainties were checked manually. Chimeric artifacts were deleted from the data set using CHIMERA_CHECK 2.7 (http://rdp.cme.msu.edu/html/analyses.html; Ribosomal Database Project).

**rRNA fungal database.** A fungal SSU rRNA gene database was created so that the detected sequences could be analyzed (http://phymycodb.genouest.org/). Al-

gorithms were used to recover SSU rRNA gene sequences for each fungal phylum from the GenBank/EMBL/DDBJ databases. Filters were used to eliminate the sequences that were too short (<1,000 bp), too long (>2,500 bp), or of poor quality or that included at least 10 consecutive undetermined nucleotides (T. Le Calvez, L. Guillot, A. Dufresne, and P. Vandenkoornhuyse, submitted for publication). A multiple-sequence alignment was constructed for each phylum using Clustal X 1.81 with a matrix containing all sequences from our databases and our hydrothermal sequences.

After using this protocol and completing the various analyses, we were able to recover 1,733 sequences from *Basidiomycota*, 215 sequences from *Chytridiomycota*, 4,117 sequences from *Ascomycota*, 292 sequences from *Zygomycota*, and 621 sequences from *Glomeromycota*, which represented all the different branches of fungal phylogeny. Each phylum was then subjected to a multiple-sequence alignment procedure, followed by neighbor-joining analyses. Phylogenetic trees were visualized by using Treeview 1.6.6. The phylogenetic neighbors closest to the environmental sequences analyzed were selected, and then phylogenetic analyses were performed (see below).

**Diversity and phylogenetic analyses.** Rarefaction curves were computed for each sample. The number of species was determined for 100 random combinations of 1 to *n* sequences by using 100 bootstrap pseudoreplicates (http://viceroy.eeb.uconn.edu/EstimateS) implemented in EstimateS. A multiple-sequence alignment procedure was performed using CLUSTALX 1.81 (35), and the alignment was refined by eye. This analysis included our environmental sequences, sequences from the fungal cultures isolated, and the most relevant representative SSU rRNA gene sequences of the *Chytridiomycota*, *Basidiomycota*, and *Ascomycota* (93 sequences, including 18 representative environmental sequences and 26 sequences from fungal cultures). The alignment was performed with 1,145 nucleotides as described above. Other multiple-sequence alignment methods tested gave similar results (not shown). Phylogenetic analyses were performed as follows. CLUSTALX 1.81 was used to obtain neighbor-joining (NJ-K2P) phylogenies with distance correction, gap omission, and 1,000 bootstrap pseudoreplicates. PAUP 4.0β10 was used for maximum parsimony analysis using a heuristic tree search with 500 random-addition replicates and with tree bisection and reconnection as the branching algorithm, with each of the 500 bootstrap iterations utilizing 10 random-addition replicates and tree bisection and reconnection branch swapping. In addition, the data were analyzed using the ML-GTR + I + G procedure with 100 iterations. Modeltest 3.7 software (25) was used to select the model.

After these analyses were performed, phylotypes were specified using a cutoff of 98% (pairwise distance computed using PAUP 4.0β10).

**Quantitative PCR (qPCR) assays.** PCRs were performed with 10-μl (final volume) mixtures using iQ SYBR green Supermix (Bio-Rad), which contained SYBR green PCR buffer, 2.7 μM dATP, 2.7 μM dTTP, 2.7 μM dGTP, 2.7 μM dCTP, and 0.42 U of iTaq DNA polymerase (Bio-Rad). Then 0.35 μM primer MH2 (5′TTCGATGGTAGGATAG3′) and 0.35 μM primer FungqPCR1 (5′TGTCGGGATTGGGTAATTT3′) were added to the mixture.

All reactions were performed in optical tubes (Bio-Rad); 8.5 μl of the master mixture was added first, followed by 1.5 μl of template. The tubes were sealed with microseal film (Bio-Rad). All reactions were performed with a Chromo 4 thermocycler (MJ Research), using an initial denaturation at 94°C for 3 min to activate the enzyme, followed by 35 or 40 cycles of denaturation at 94°C for 30 s and annealing-extension at 48°C for 45 s and then by plate reading. The dissociation curve for temperatures from 65°C to 95°C was measured after the last qPCR cycle. All data were analyzed using Opticon Monitor 3 (MJ Research).

This approach was used to study six samples in triplicate in two independent runs. The three samples which exhibited the strongest signals in two preliminary runs were compared in a final run. Seven different plasmid concentrations ($10^9$ to $10^3$ copies in 1.5 μl) were used to construct a standard curve for absolute quantification. The numbers of copies in the standards were calculated using the following formula: molecules/μl = $a$/(plasmid length × 660) × ($6.022 \times 10^{23}$), where $a$ is the plasmid concentration (in μg/μl), 660 is the average molecular weight of one base pair, and $6.022 \times 10^{23}$ is the molar constant.

**Nucleotide sequence accession numbers.** The sequences reported in this paper have been deposited in the GenBank database under accession numbers EF638466 to EF638515 for sample MV2E1, EF638516 to EF638564 for sample H18E12, EF638565 to EF638614 for sample MV2E2, EF638615 to EF638636 for sample MV2E3, EF638637 to EF638654 for sample MV5E1, EF638655 to EF638686 for sample MV5E2, and EF638687 to EF638706 for cultivable strains. The matrix is available upon request from the corresponding author.

## RESULTS

**Diversity of the culture collection.** Cultivation and isolation of microbes (*Bacteria* and *Archaea*) from deep-sea hydrothermal samples are difficult, and the procedure includes many constraining manipulations. Nevertheless, we were able to establish a fungal collection with different samples from several sites without using complex culture procedures, such as pressurized fermentors (see Materials and Methods). The fungi were isolated using a classic medium supplemented with sea salt at a pressure of 1 atm and 25°C. Physiological analyses demonstrated that the strains were able to grow in deep-sea salinity (3% [wt/vol] NaCl) and at low temperatures (6). The SSU rRNA gene sequence analyses revealed that all 21 isolates in the collection belonged to the phylum *Ascomycota*, as did previously isolated marine strains (15). A subset of the cultivated *Ascomycota* belonged to the classes *Dothideomycetes* (Fig. 1, phylotypes 14 to 16) and *Sordariomycetes* (Fig. 1, phylotypes 17, 18, and 20), and at least one new undescribed phylotype belonging to each of these groups was discovered. Black yeasts were also detected, including two phylotypes of *Exophiala* (order *Chaetothyriales*) (Fig. 1, phylotypes 10 to 13), a group that includes pathogens and saprophytes. In a recent study, black yeasts were identified in mussel tissues present in hydrothermal samples from the Fidji Basin, where they appeared to be mussel parasites, possibly regulating the trophic chain (37). Finally, one phylotype was obtained from cultures that clustered with the mitosporic *Ascomycota* (Fig. 1, phylotype 19), which have been described as "common" pathogenic fungi in tidal seawater (4, 30). As expected based on previous information, the spores produced by the cultivated isolates were not flagellated, even though some isolates appeared to be specific to the oceanic habitat (for details about the culture collection, physiology, and morphology, see reference 6).

**Fungal diversity determined by a culture-independent approach.** We investigated the fungal SSU rRNA genes from environmental samples (Table 1) directly amplified by ciPCRs using fungus-specific primers. A customized database was constructed to analyze in depth the SSU rRNA gene sequences detected. This database contained all available fungal SSU rRNA sequences that passed filters for length and quality (144 *Chytridiomycota* sequences, 266 *Zygomycota* sequences, 418 *Glomeromycota* sequences, 2,327 *Basidiomycota* sequences, and 4,270 *Ascomycota* sequences), and there was at least one representative of each known fungal genus in the GenBank database. All of these 7,425 sequences, which represent the whole fungal tree of life, were used to determine, with high confidence, the phylogenetic affinities of the new sequences. A secondary matrix of sequences was then constructed using these computations along with BLASTn analyses (1), which included the closest relatives of the environmental SSU rRNA sequences analyzed (for each phylum, neighbor-joining phylogenetic trees obtained using the entire data sets are shown in Fig. S1, S2, S3, and S4 in the supplemental material). A total of 20 distinct phylotypes were detected using the ciPCR results and the fungal culture collection (Fig. 1). The ciPCR results revealed previously unsuspected diversity of fungal phylotypes in the vent ecosystems; there were nine distinct phylotypes, five of which were new at the genus level or a higher taxonomic level in the *Chytridiomycota* (Fig. 1, phylotypes 1 and 2; see Fig.

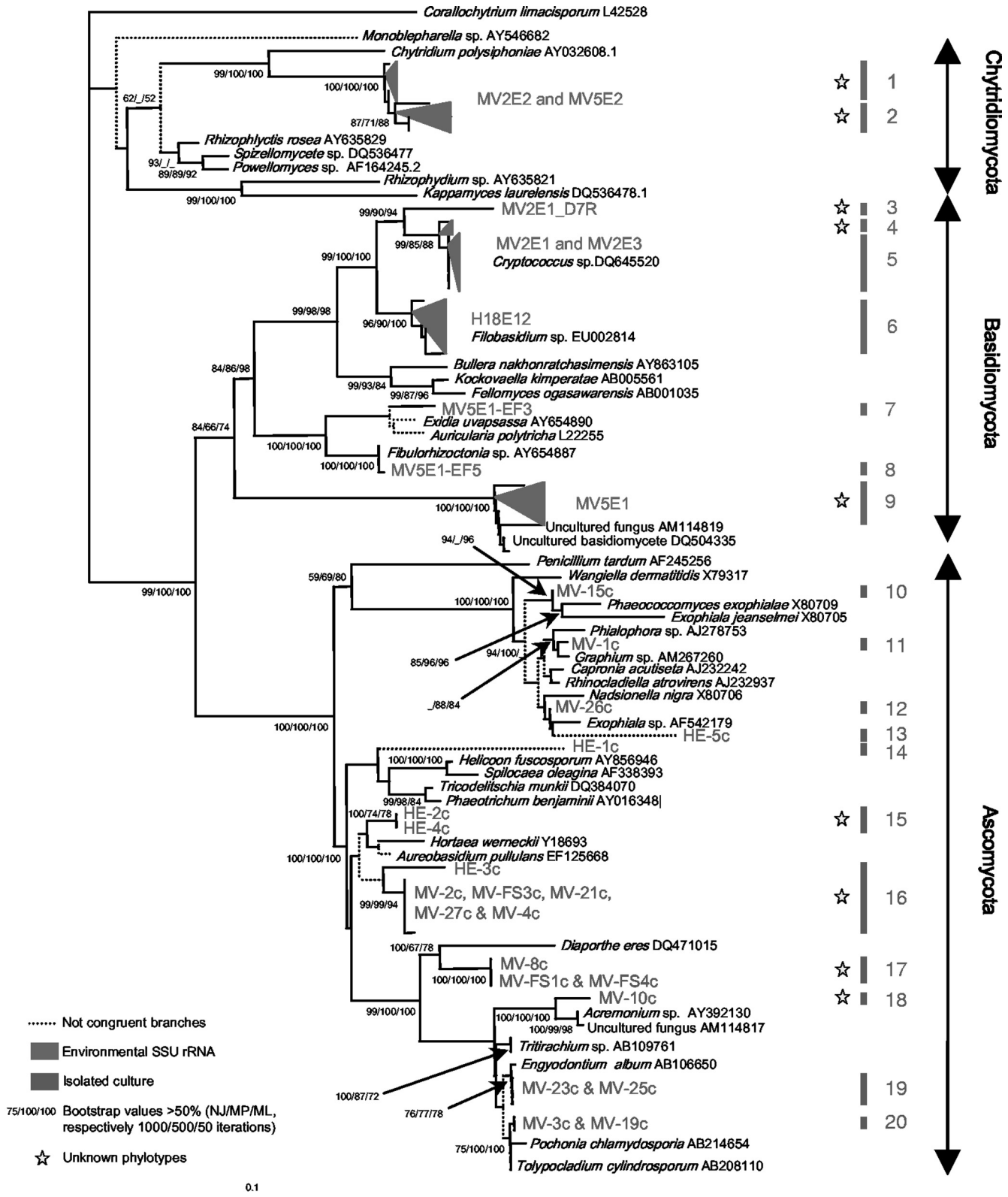


FIG. 1. Phylogenetic positions of deep-sea hydrothermal fungi. This consensus tree includes environmental SSU rRNA sequences isolated from environmental ecosystems (phylotypes 1 to 9) and isolated cultures from the same samples (phylotypes 10 to 20), along with the closest known related SSU rRNA fungal sequences. The tree was constructed using the neighbor-joining algorithm. Bootstrap values of >50% are indicated at the nodes (estimated using 1,000, 500, and 50 iterations for the neighbor-joining [NJ], maximum parsimony [MP], and maximum likelihood [ML] analyses, respectively). Stars indicate the phylotypes considered new phylotypes. The scale bar indicates 0.1 change per position computed using

TABLE 1. Samples analyzed in this study

| Sample | Source | Location | Depth (m) | No. of phylotypes | No. of SSU rRNA sequences | No. of copies of rRNA per μg of genomic DNA[a] |
|---|---|---|---|---|---|---|
| MV2E1 | Exterior of *B. azoricus* shell | Menez Gwen, Mid-Atlantic Ridge | 860 | 8 | 59 | $1.91 \times 10^5$ |
| MV2E2 | *B. azoricus* (body) | Menez Gwen, Mid-Atlantic Ridge | 860 | 5 | 47 | $1.483 \times 10^6$ |
| MV2E3 | Surrounding friable rocks | Menez Gwen, Mid-Atlantic Ridge | 860 | 6 | 25 | $3.01 \times 10^6$ |
| MV5E1 | Sulfide of surrounding rocks | Lucky Strike, Mid-Atlantic Ridge | 1,700 | 3 | 18 | NA[b] |
| MV5E2 | Exterior of *B. azoricus* | Lucky Strike, Mid-Atlantic Ridge | 1,700 | 2 | 32 | $1.35 \times 10^7$ |
| H18E12 | *A. pompejana* shell (epiderm) | Elsa, East Pacific Rise | 2,630 | 2 | 49 | NA |

[a] Determined by qPCR.
[b] NA, not applicable.

S4 in the supplemental material) and *Basidiomycota* (Fig. 1, phylotypes 3, 4, and 9).

On the one hand, the *Basidiomycota* phylotypes detected belonged to the *Agaricomycotina* (Fig. 1, phylotypes 3 to 8), mostly to the genus *Cryptococcus*, a phylotype previously detected in another hydrothermal area (33), and the *Filobasidium* (East Pacific Rise) anamorphs, which have economic, agricultural, and medical importance. Phylotype 9 (Fig. 1), a close relative of uncultured fungi, was detected previously in deep-sea and hydrothermal sediments (2) and fluids (22). Moreover, homobasidiomycete yeasts belonging to the order *Auriculariales* (Fig. 1, phylotype 7) were also found. On the other hand, the *Chytridiomycota* phylotypes retrieved belonged to species that have not been described previously (Fig. 1, phylotypes 1 and 2; see Fig. S4 in the supplemental material) and, in particular, to an apparently ancient evolutionary lineage in the order *Chytridiales*. These sequences shared only 95.7% of similarity with their closest relative, *Chytridium polysiphoniae* (Fig. 1). The phylogenetic position of *C. polysiphoniae* is uncertain because it is a pathogenic fungus (19) and such fungi tend to show a long-branch attraction bias (Fig. 1). When *C. polysiphoniae* was removed along with the other ambiguously placed pathogenic *Chytridiomycota*, the new group of species formed one of the most ancient branches of the fungal kingdom (see Fig S4 in the supplemental material). It is very probable that the phylogenetic position of these newly discovered fungi was determined correctly because the branches in this group were short, a result that would not be expected if the terminal organisms were pathogens. This result is also supported by a Bayesian phylogenetic reconstruction (result not shown).

Different fungal communities were discovered when the fungal diversities of the Mid-Atlantic Ridge and the East Pacific Rise were compared; these two ridges are known to have distinct endemic animal communities (40) (*Bathymodiolus azoricus* and *Alvinella pompejana* samples, respectively). However, *Agaricomycotina* (Fig. 1, phylotypes 3 to 8) and *Dothideomycetes* (Fig. 1, phylotypes 14 to 16) sequences were found in both ecosystems. *Agaromycotina* fungi seem to be widely distributed because they were also found in deep-sea sediments (11, 23) and methane seeps (33).

**Estimation of fungal SSU rRNA gene copy number in environmental samples.** qPCR analyses were performed to indirectly assess the fungal SSU rRNA gene copy numbers in environmental samples obtained from sites on the Mid-Atlantic Ridge and East Pacific Rise to test the possibility of contamination of the environmental samples by exogenous fungi. In these samples the numbers of fungal SSU rRNA gene copies would be expected to be low. Therefore, a set of specific fungal primers was designed on the basis of the diversity analyses, as described in Materials and Methods. Strikingly, the number of SSU rRNA gene copies ranged from $1.91 \times 10^5$ to $1.35 \times 10^7$ per μg of genomic DNA. Thus, this analysis clearly allowed us to reject the hypothesis that of all the fungi had an exogenous origin (i.e., that there was contamination of the samples by the water column or during sampling and conditioning). As the highest level of fungi was not observed in the bodies of animals (*B. azoricus* or *A. pompejana*) but on the exterior of the shell (*B. azoricus*) and the surrounding rock (Table 1), we hypothesized that most of these fungi were not biotrophic.

## DISCUSSION

This study reports the first inventory of fungal diversity in deep-sea hydrothermal environments. Unsuspected diversity, including new species in three fungal phyla, was found. One of the main results of the study described here is the evidence of an old *Chytridiomycota* lineage, unknown either at the genus level or at a higher taxonomic level (Fig. 1; see Fig. S4 in the supplemental material). As protein clock analyses (13) suggested previously that fungi emerged in oceans approximately 1 billion years ago during the Proterozoic era of the Precambrian, deep branches, such as this unknown phylotype, were expected based on our working hypotheses. It is thus possible

the NJ-K2P model. The dotted lines indicate branches not recovered in all three analyses. Designations that begin with MV followed by a hyphen indicate isolates from MARVEL cruise samples (Mid-Atlantic Ridge), and designations that begin with HE followed by a hyphen indicate isolates from "HERO" cruise samples (East Pacific Rise) (Table 1). The cultures obtained from sample MV2E1 are MV-1c to MV-4c, MV-21c, MV-23c, MV-FS1c, and MV-FS3c; the cultures obtained from sample MV2E2 are MV-8c, MV-10c, MV-25c, and MV-FS4c; the cultures obtained from sample MV2E3 are MV-15c, MV-19c, MV-26c, and MV-27c; the culture obtained from sample H18E9 is HE-5c; the cultures obtained from sample H18E11 are HE-1c to HE-3c; and the culture obtained from sample H18E12 is HE-4c. In the *Ascomycota*, the terminal HE-1c and HE-3c cultures were not defined as new phylotypes since their phylogenetic affinities were unclear.

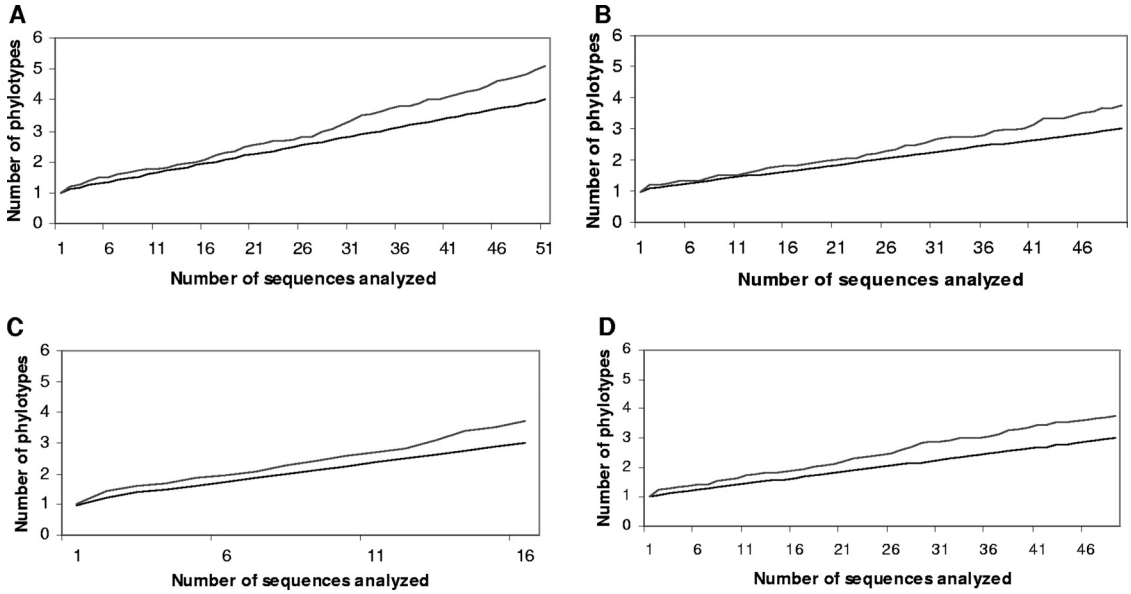


FIG. 2. Estimates of fungal community diversity as a function of sampling effort for environmental samples. Rarefaction curves were computed using 100 bootstrap replicates (upper line), and the expected richness function (lower line) is the number of phylotypes estimated (random sampling without replacement) from our sequence data set generated using ciPCRs. (A) MV2E2; (B) H18E12; (C) MV2E1; (D) MV2E3. Plots for MV5E1 and MV5E2 were not drawn since only one phylotype was found in each sample when sequence analyses of the ciPCR products obtained using primers MH2 and MH4 were performed.

that the emergence and initial diversification of fungi occurred in a marine environment. This study supports this hypothesis, but the data are not conclusive. The loss of flagella and therefore motility in fungal gametes, which likely occurred more than once (15), does not invalidate our conclusion since this loss could be compensated for in terms of fitness by the resistance and long-range dispersal of both mitotic and meiotic spores in aquatic environments. If the molecular clock estimates are correct, this hypothesis implies that fungi diversified in oceans before they colonized the land during the late Silurian or early Devonian period (i.e., approximately 400 million years ago). In this study we found a variety of fungi belonging to three of the five fungal phyla, which supports our assumption even if the possibility of secondary colonizations from land to marine ecosystems cannot be excluded. This conclusion contradicts the widely accepted hypothesis that the diversification of fungi occurred on land. The possible early colonization of hydrothermal habitats and the ecological function of the deeply branching *Chytridiomycota* (Fig. 1; see Fig. S4 in the supplemental material) are being addressed in an ongoing metagenomic analysis of the fungi living in deep-sea hydrothermal ecosystems.

**Fungal diversity in marine hydrothermal ecosystems.** Despite the effectiveness of the primer set used here for amplification of *Ascomycota* (37), we did not find any *Ascomycota* when ciPCR was used. Rarefaction curves resulting from resampling and pseudoresampling (bootstrap) procedures indicated that the number of sequences analyzed might have been insufficient to represent the entire fungal diversity in all samples. One or two additional phylotypes might be discovered (Fig. 2) by doubling the sequencing effort. We suggest that the abundance of *Ascomycota* in the environmental samples was extremely low and that cultivation resulted in amplification of these rare organisms. This could explain why ciPCR and subsequent sequencing failed to detect them. Should this be the case, it is likely that the fungal diversity described here has been underestimated.

The observation that a specific fungal community was found for each sample might be explained by the diversity of the surrounding physicochemical conditions (i.e., gradients of temperature, pH, etc.). Low redundancy of phylotypes was observed in the samples analyzed. This allows us to argue that the diversity of fungi in hydrothermal ecosystems is high and that the recently reported numbers of living fungal species (12) are substantial underestimates. Given the high fungal SSU rRNA gene copy number obtained for a given sample (up to $1.91 \times 10^5$ copies/$\mu$g), it can also be suggested that the fungi might be involved in important ecological functions. Moreover, three of the phylotypes found in this work were detected by ciPCR in previous studies (2, 22). This also argues for rejection of the hypothesis that these sequences originated from contamination.

As we have no data on how the fungi identified live, harvest energy, and interact, their roles in the hydrothermal ecosystems remain unclear. Fungi are considered key organisms in land ecosystems, and they could be as important in oceanic ecosystems, especially at the bottom of oceans. We need more information about the fungal diversity and functions in deep-sea hydrothermal habitats and, more generally, in oceans. The numerous uncharacterized fungi might be a new source of drugs and biotechnological discoveries.

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