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Exploring and quantifying fungal diversity in freshwater lake ecosystems using rDNA cloning/sequencing and SSU tag pyrosequencing

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Summary

Water samples were collected along transects from the shore to the centre of two French lakes: the deep, volcanic, oligomesotrophic and low allochthonic-impacted Lake Pavin, and the productive and higher allochthonic-impacted Lake Aydat. The biodiversity was analysed using two approaches: the classical approach consisting of cloning/sequencing of the 18S, ITS1, 5.8S, ITS2 and partial 28S region using primers designed for fungus sequences, and the pyrosequencing of 18S rRNA hypervariable V2, V3 and V5 regions using two primer sets (one universal for eukaryotes and one for fungi). The classical approach yielded 146 (Lake Pavin) and 143 (Lake Aydat) sequences, corresponding to 46 and 63 operational taxonomic units (OTUs) respectively. *Fungi* represented half of the OTUs identified in Lake Pavin and 30% in Lake Aydat, and were dominated by sequences from *Chytridiomycota* found throughout Lake Pavin but mostly in the central pelagic zone of Lake Aydat. The pyrosequencing approach yielded 42 064 (Pavin) and 61 371 (Aydat) reads, of which 12–15% and 9–19% reads were assigned to fungi in Lakes Pavin and Aydat respectively. *Chytridiomycota* members were also dominant among these reads, with OTUs displaying up to > 33-fold overrepresentation in the centre compared with the riparian areas of Lake Aydat. Besides fungi, both approaches revealed other major eukaryote groups, with the highest diversity in the central areas of lakes. One of the major findings of our study was that the two lakes displayed contrasting spatial distributions, homogenous for Lake Pavin and heterogeneous for Lake Aydat, which may be related to their peculiarities. This study represents the first unveiling of microbial eukaryote and fungus diversity assessed with two complementary molecular methods, and is considered a major milestone towards understanding the dynamics and ecology of fungi in freshwater lake ecosystems, which are directly link to the abundance and distribution of taxa.

Introduction

Fungi represent one of the last frontiers of the undiscovered biodiversity that challenge the microbial ecology today. They have a crucial role in ecosystem functioning – or are crucial for the maintenance of ecological balance since they influence many environmental processes such as nutrient cycling in the food web. From studies in soil systems, fungi are known to be vital in recycling nutrients through the metabolism of complex organic materials (Treseder, 2005; Watling, 2005). In pelagic environments, we also hypothesize an important role of fungi in many ecosystem processes. However, our ecological knowledge of fungi in pelagic ecosystems is scant. The kingdom *Fungi* was previously subdivided into four main phyla:

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Basidiomycota, *Ascomycota*, *Zygomycota* and *Chytridiomycota* (Alexopoulos *et al*., 1996). But the two latter phyla do not emerge as monophyletic groups in recent phylogenetic analyses (James *et al*., 2000; 2006a). Today, seven phyla are recognized in the phylogenetic classification of *Fungi* (Hibbett *et al*., 2007). The *Chytridiomycota* is retained in a restricted sense, with *Neocallimastigomycota* and *Blastocladiomycota* representing segregate phyla of flagellated *Fungi*. Taxa traditionally placed in *Zygomycota* are distributed among *Glomeromycota* and several subphyla *incertae sedis*, including *Mucoromycotina*, *Entomophtoromycotina*, *Kickxellomycotina* and *Zoopagomycotina* (Hibbett *et al*., 2007). The number of fungi present on earth was estimated to 1.5 million species (Hawksworth, 2001), from which approximately 97 000 have so far been identified (Kirk and Ainsworth, 2008). This suggests a large underestimation of the diversity of this eukaryotic group, because of methodological constraints, but also partly because of the undersampling of pelagic environments, which represent the largest reservoir for life on earth.

In aquatic systems, several culturing assays allowed the isolation of ascomycetous or basidiomycetous yeasts. Freshwater *Ascomycetes* occur in both lentic (Hyde and Goh, 1998a; 1999) and lotic habitats (Hyde and Goh, 1998b,c; 1999) and more than 500 species have been reported so far (Shearer, 2001; Shearer *et al*., 2007). Although ascomycetous fungi, together with bacteria, are efficient decomposers of organic matter, their functional importance in lakes remains unclear (Raja and Shearer, 2008). Basidiomycetous yeasts were retrieved in marine habitats, particularly in deep waters (Bass *et al*., 2007), and from high-altitude cold lakes (Libkind *et al*., 2009).

Fungi are often thought to be present in pelagic systems mainly as allochthonous terrestrial inputs, which is usually not the case for *Chytridiomycota*, i.e. chytrids, which are known to produce zoospores (Wong *et al*., 1998). There are over 1000 species of chytrids (Powell, 1993; Shearer *et al*., 2007); many of them have been characterized as parasites of freshwater algae (Gromov *et al*., 1999a,b; Ibelings *et al*., 2004). One chytrid species (*Batrachochytrium dendrobatidis*) was also described as the causative agent of die-offs and population declines of amphibian species (Berger *et al*., 1998). Previous studies have unveiled a large reservoir of unsuspected fungal diversity in pelagic zone of lakes (Van Donk and Ringelberg, 1983; Holfeld, 1998; Hyde and Goh, 1998a; Richards *et al*., 2005), primarily of chytrids (Lefèvre *et al*., 2007; 2008; Sime-Ngando *et al*., 2011) known as aquatic life forms, as their propagules (i.e. uniflagellated zoospores) have specialized aquatic dispersal abilities. Pelagic freshwater species of chytrids mostly fall within the *Rhizophydiales* order, which contains many host-specific parasitic fungi of various phytoplankton species, primarily diatoms (Jobard *et al*., 2010; Rasconi *et al*., 2011). These organisms are known to often reduce phytoplankton blooms and to play a crucial role in phytoplankton succession (Kagami *et al*., 2004; 2007; Rasconi *et al*., 2011). Several studies have demonstrated epidemic occurrences of chytrids infecting phytoplankton in lakes (Canter and Lund, 1948; 1951; Canter, 1950; Van Donk and Ringelberg, 1983; Kudoh, 1990; Holfeld, 1998).

In the deep-volcanic oligomesotrophic Lake Pavin (France), which is a typical pelagic environment because of the absence of river inflow and low human influences, the occurrence of these parasitic fungi was confirmed from direct microscopic observation of their sporangia (i.e. the infective stage) fixed on various classes of phytoplankton, including diatoms and chlorophytes (Rasconi *et al*., 2009). Chytrids can be characterized by their zoospores that typically have a single, posteriorly directed flagellum (James *et al*., 2000), corresponding to one of the major forms observed directly via epifluorescence microscopy. However, because chytrid life forms are not distinguishable from some small flagellated protists, they may have been miscounted as bacterivorous flagellates in previous studies (Carrias *et al*., 1996; Carrias and Amblard, 1998). Carrias and colleagues (1996) reported that the dominant small unidentified heterotrophic flagellates present in Lake Pavin during a seasonal study were not able to ingest bacteria. Large grazing-resistant diatoms, primarily *Aulacoseira italica* and *Asterionella formosa*, are important components of Lake Pavin, where they typically account for 50–98% of the total phytoplankton biomass production during the spring bloom in the whole water column (Rasconi *et al*., 2009). These diatoms are well known as preferential hosts for chytrids in Lake Pavin. Similarly, filamentous cyanobacterial blooms, often dominated by the highly chytrid-sensible species *Anabaena flosaquae*, are characteristics of autumn in the eutrophic Lake Aydat, France (Rasconi *et al*., 2009). The activity of fungi is suspected to be an important factor in Lake Pavin, Lake Aydat, and other pelagic inland and coastal ecosystems, which regulates phytoplankton community structure and species successions. It has also been shown that the small flagellated zoospores produced by parasitic chytrids are efficiently grazed by zooplankton such as *Daphnia* (Kagami *et al*., 2004). Such considerations suggest that chytrids are important microbial players in pelagic ecosystems, where they are involved both in the decomposition of organic matter from parasitic epidemics or saprotrophy, and in the transfer of matter and energy to higher trophic levels (Lefèvre *et al*., 2008).

However, still little is known about the diversity of fungi in pelagic ecosystems. Most of the related studies are relatively recent (Richards *et al*., 2005), including those in

Lakes Aydat and Pavin (Lefranc *et al*., 2005; Lefèvre *et al*., 2007; 2008). The latter studies were performed initially with the main aim to examine the phylogenetic composition of picoeukaryotes (< 5 μm), and ended up with the unveiling of an important diversity of fungi. For example, in Lakes Aydat and Pavin, studies on the diversity of small heterotrophic eukaryotes based on small subunit (SSU) rRNA gene sequences have shown that *Alveolata*, *Cercozoa*, *Stramenopile* and *Fungi* represented the major part of the total diversity (Lefèvre *et al*., 2007; 2008). However, the molecular approach used in these studies, i.e. polymerase chain reaction (PCR) amplification and cloning of the entire SSU rDNA gene followed by analysis of the positive clones by restriction fragment length polymorphism (RFLP), and sequencing of the selected clones or operational taxonomic units (OTUs), represents a both costly and tedious method, providing only a limited analysis of the diversity associated with the number of clones analysed. Therefore, we have taken a complementary approach, by pyrosequencing of hypervariable regions of the SSU rRNA gene from selected samples. This method, already successfully used for the exploration of deep-sea community composition (Sogin *et al*., 2006; Huse *et al*., 2008), is cost-effective and offers around three orders of magnitude larger SSU rRNA sequencing, compared with classical approaches. In addition, it eliminates cloning bias and allows maximizing the number of organisms sampled in a run (Huse *et al*., 2008).

The aim of this work was: (i) to determine the diversity of microbial eukaryotes, focusing on fungi, in the whole plankton fraction (size class 0.6–150 μm) using two complementary methodological approaches, (ii) to compare this diversity with earlier studies performed from the same lakes, (iii) to estimate the spatial variability in the diversity of pelagic fungi from samples collected along transects from the shore to the centre of the lakes, and (iv) to determine the differences in eukaryote and fungus diversity between two lakes differing by their trophic status. To our knowledge, this study is the first exploration of the spatial dynamics in the diversity of pelagic fungi using a combination of two complementary approaches. The complete sequencing of the SSU rDNA gene, considered as the classical approach, attributed to each sequence a precise taxonomical position and identified potential new clades, based on the use of fungus-designed primers. The second approach, consisting of pyrosequencing a large number of SSU rRNA hypervariable tags, provided a general overview of the eukaryote and fungus diversity, with the use of a universal eukaryote and a fungus-designed set of primers. This second method, referred to as the pyrosequencing approach, unveiled rare species, and gave quantitative information about the OTUs identified by the classical approach. In this study, we have unveiled all the major and minor fungus taxa and their spatial variability (i.e. between and within lakes), which can be considered as the first step towards the understanding of population dynamics and functional role of fungi in pelagic food webs.

Results

Overview of the retrieved diversity

The 'classical approach' yielded a total of 146 (Lake Pavin) and 143 (Lake Aydat) complete SSU rDNA gene sequences, considered an overview of the biodiversity in the pilot lakes at the sampling time. After clustering sequences sharing more than 99% identity, the total number of OTUs increased from 46 OTUs in Lake Pavin (Fig. 1) to 63 OTUs in Lake Aydat (Fig. 2). In Lake Pavin, half of the OTUs identified belonged to *Fungi* with 15, 7 and 1 representatives (i.e. OTUs) from the *Chrytridiomycota*, *Ascomycota* and *Basidiomycota* phyla respectively (Fig. 1, Table S1a). The other represented taxonomical groups comprised SSU rDNA gene OTUs from 11 *Viridiplantae*, 5 *Cryptophyta*, 4 *Alveolata*, 1 *Telonemida*, 1 *Ichtyosporea* and 1 *Stramenopile* (Fig. 1). In Lake Aydat, one-third of the OTUs belonged to *Fungi*, including 10 *Chytridiomycota*, 8 *Ascomycota* and 1 *Basidiomycota* (Fig. 2 and Table S1b). The diversity of chytrids was 50% lower in Lake Aydat compared with Lake Pavin. Similar to the findings in Lake Pavin, other eukaryotic groups were found in Aydat, with SSU rDNA gene OTUs corresponding to 21 *Alveolata*, 9 *Viridiplantae*, 6 *Cryptophyta*, 2 *Ichtyospora* and 2 *Stramenopile* (Fig. 2 and Table S1b). In addition, three *Choanoflagellida* and one *Cercozoan* OTUs were only retrieved from Lake Aydat (Fig. 2 and Table S1b). Within all the identified OTUs, only five were common to both lakes: two *Cryptophyta*, one *Alveolata* and two *Fungi* (one *Ascomycota* and one *Chytridiomycota*).

For both lakes, the rarefaction curves with > 97 and > 99% levels of sequence similarities did not reach saturation (Fig. 3A), indicating biodiversity underestimation using the classical approach. This result justifies the implementation of a complementary pyrosequencing approach, to improve the exploration of the hidden biodiversity, including rare species. With this approach, a total of 23 519 and 18 545 sequences from Lake Pavin, and of 30 337 and 31 034 sequences from Lake Aydat, using eukaryote and fungus-designed primers, were, respectively, obtained. For both lakes and for all sampling stations, the rarefaction curves continued to rise almost linearly with > 99% level of sequence similarity (data not shown), but approaching a plateau if applying > 97%, the apparent diversity being higher when using the fungus set of primers (Fig. 3B).

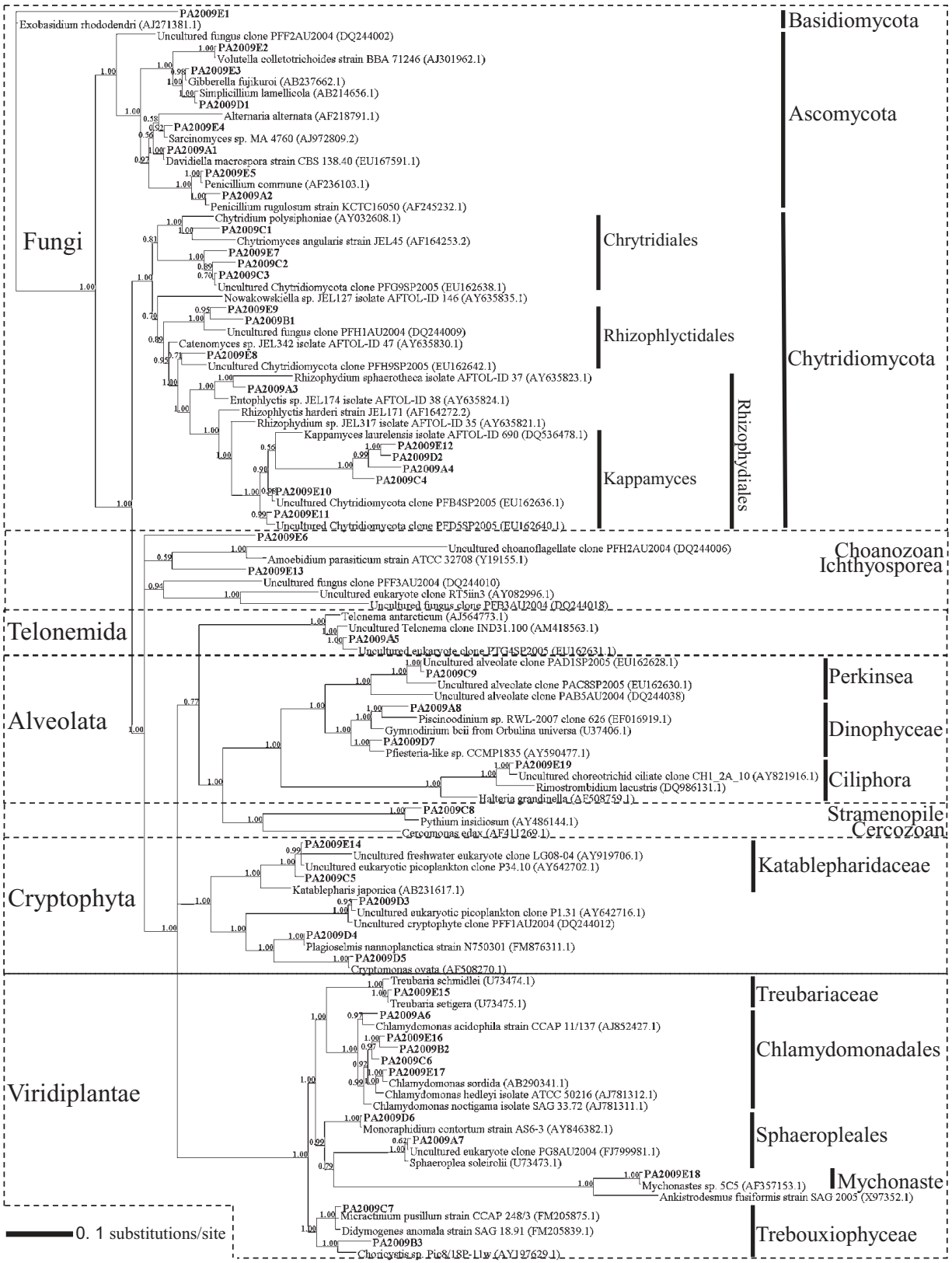
Fungi
Basidiomycota
Ascomycota
Chytridiomycota
Chrytridiales
Rhizophlyctidales
Rhizophydiales
Kappamyces
Choanozoan
Ichthyosporea
Telonemida
Alveolata
Perkinsea
Dinophyceae
Ciliphora
Stramenopile
Cercozoan
Cryptophyta
Katablepharidaceae
Viridiplantae
Treubariaceae
Chlamydomonadales
Sphaeropleales
Mychonaste
Trebouxiophyceae
0. 1 substitutions/site

Fig. 1. Phylogenetic tree based on 18S rRNA gene sequences obtained from the Lake Pavin. The unrooted phylogenetic tree displayed 46 operational taxonomic units (OTUs) inferred from 146 18S rRNA gene sequences sharing less than 99% sequence identity. Representative from each OTU were submitted to a BLAST search (Altschul *et al*., 1990) to determine the closest homologous 18S rRNA gene sequences from the non-redundant nucleotide database (NCBI). The OTUs and reference sequences were aligned using CLUSTALW (Thompson *et al*., 1994), and the construction of the phylogenetic tree was performed using the Bayesian method from MrBayes3 software. The numbers at the nodes correspond to Bayesian posterior probabilities. The boxes (dash lines) showed the different phyla, where each taxonomical subdivisions are delimitated by vertical lines.

With the pyrosequencing approach, an important diversity of fungi was observed in the two investigated environments, but also of many other eukaryotes with the two sets of primer used (eukaryote and fungi). When considering the data obtained with the eukaryote primers, the most represented groups were *Stramenopile* (representing 27% and 3% of the pyrosequence reads in Pavin and Aydat respectively), *Chlorophyta* (19% and 11%), *Metazoa* (15% and 53%), *Fungi* (12% and 9%) and *Alveolata* (10% and 20%) (Fig. 4). The *Cercozoa* and *Telonemida* were also among the major groups in Lake Pavin, accounting for 7% and 8% of the sequences respectively (Figs 4 and 5). When considering the data obtained with the fungus-designed primers, enrichment in fungal sequences (15% and 19% of the pyrosequence reads in Pavin and Aydat respectively) was observed. With these fungus-designed primers, only a few metazoan and *Viridiplantae* sequences were retrieved, while the *Katablepharidophyta* (60% and 10% of the pyrosequence reads in Pavin and Aydat respectively) and *Cryptophyta* (10% and 36%) were among the dominant groups (Fig. 4). Finally, whatever the primer considered, it was not possible to assign a taxonomic group for 1–5% of the sequences from the different samples because of suspicious or ambiguous assignment of the SSU rDNA gene fragments in the database, while less than 1% of the sequences had no hit in the Silva SSU database.

The pyrosequencing method was used to identify dominant taxa and estimate their spatial distribution using data obtained from eukaryote primers for the overall biodiversity, and from fungal primers for the diversity of fungi. In the following sections, we describe the taxonomical groups and species unveiled by the complementary 'classical' and pyrosequencing approaches and we compare the diversity observed in the two pilot lakes, with focus on fungi.

The diversity of fungi

Chytridiomycota. In both lakes, the identified chytrids belonged to the *Chytridiales*, *Rhizophydiales* and *Rhizophlyctidales* orders. The chytrid sequences found here confirmed the high diversity of *Chytridiomycota*-affiliated sequences reported in previous studies from Lake Pavin (Lefèvre *et al*., 2007; 2008) (Figs 1 and 2).

Members of *Rhizophydiales*, which occur in aquatic ecosystems primary as phytoplankton parasites (Letcher *et al*., 2006; James *et al*., 2006b), were the most represented fungi in Lake Pavin with seven OTUs (Fig. 1). Only one *Rhizophydiales* taxon was common to both lakes, as suggested by the 99.58% identity between the OTUs PA2009E11 (Pavin) and AY2009D2 (Aydat) sequences (Figs 1 and 2). These SSU rDNA gene sequences were closely related to PFD5SP2005 sequence previously identified in Lake Pavin and considered as being part of a new clade (Lefèvre *et al*., 2008). These sequences were grouped with *Kappamyces laurelensis* in the *Kappamyces* subclade (Letcher and Powell, 2005). In Lake Pavin, six OTUs were clustered within the *Kappamyces* group (Fig. 1). Interestingly, four of them (PA2009C4, PA2009A4, PA2009E12 and PA2009D2) formed a new sister clade of the main *Kappamyces* group (Fig. 1) which was not found in the pool of sequences obtained from the Lake Aydat.

In both lakes, one OTU (PA2009C1 in Lake Pavin and AY2009B4 in Lake Aydat) belonged to the *Chytridium angularis* clade (James *et al*., 2006b). This clade includes *Chytridium polysiphoniae*, a parasite of the marine brown alga *Pylaiella littoralis*, and *C. angularis* that grow on pollen and heat treated *Oedogonium* (a green algae) baits (Longcore, 1992). Interestingly, in Lake Pavin, three new sequences clustered together to form a sister clade of the *C. angularis* clade (Fig. 1). In Lake Aydat, AY2009C3 and AY2009B3 sequences form an early divergent novel clade within the *Chytridiomycota* (Fig. 2).

Concerning the order *Rhizophlyctidales*, one OTU (PA2009E8 in Lake Pavin and AY2009D3 in Lake Aydat) belonged to the *Rhizophlyctis* clade described by James and colleagues (2006b). Indeed, in this latter study, *Catenomyces persinius*, a cellulose decomposer found in aquatic habitats, currently included in the order *Blastocladiales* (phylum *Blastocladiomycota*), was assigned to the *Rhizophlyctis* clade (including *Rhizophlyctis rosea*), a sister clade to the *Spizellomycetales*. Based on molecular monophyly and zoospore ultrastructure, this *Rhizophlyctis* clade was recently designated as a new order, the *Rhizophlyctidales* (Letcher *et al*., 2006). To note, in each lake, three OTUs constituted a novel clade, which appear to be the sister group of the *Rhizophlyctis* clade (Figs 1 and 2).

The proportion of each fungal OTU (classical approach) was quantified according to the number of matching pyrosequence reads (Table S1a and b). The two approaches consistently displayed the same dominant

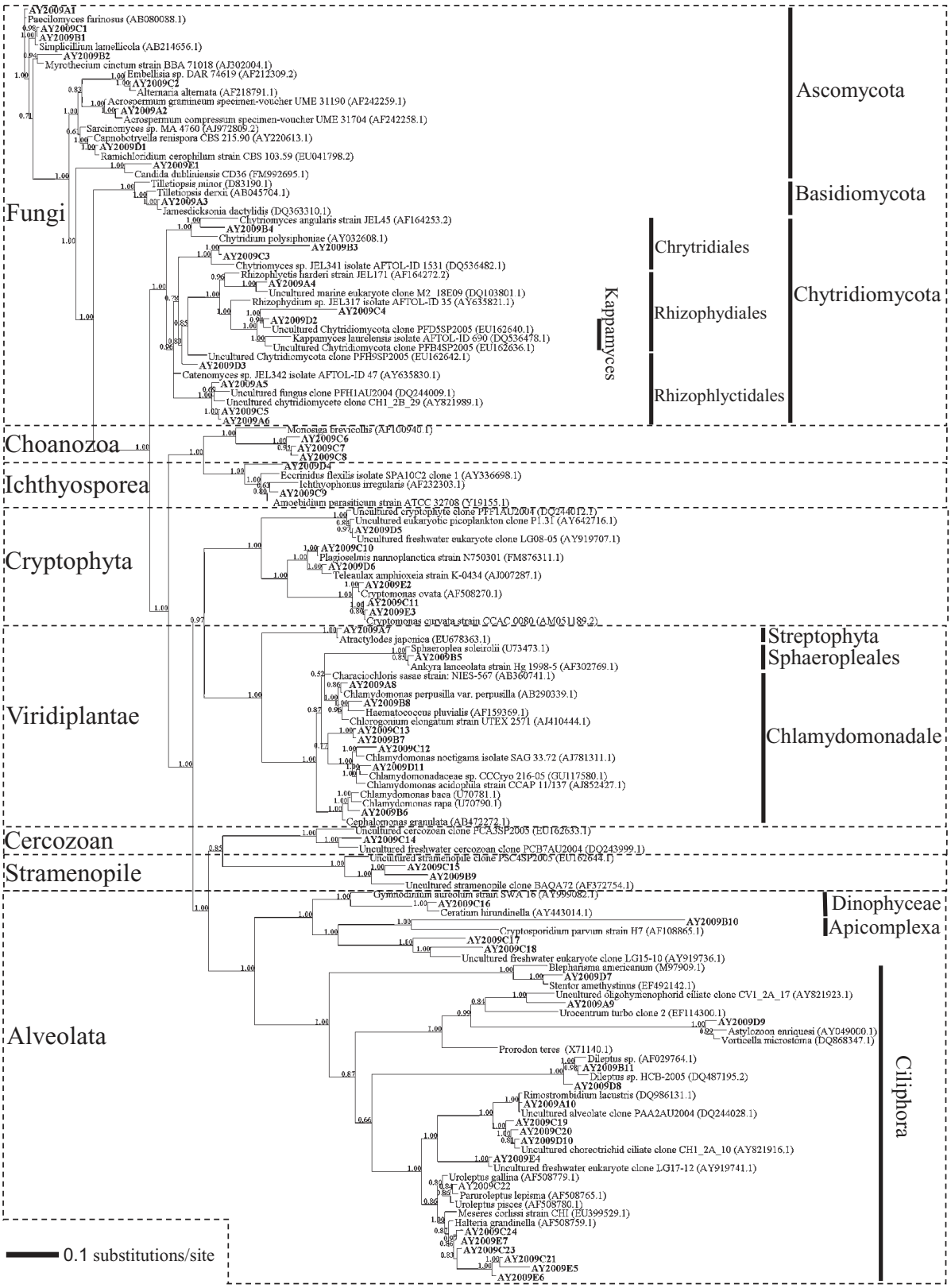

Fig. 2. Phylogenetic tree based on 18S rRNA gene sequences obtained from the Lake Aydat. The unrooted phylogenetic tree, similarly to Fig. 1, displayed 63 OTUs inferred from 143 rDNA gene sequences.

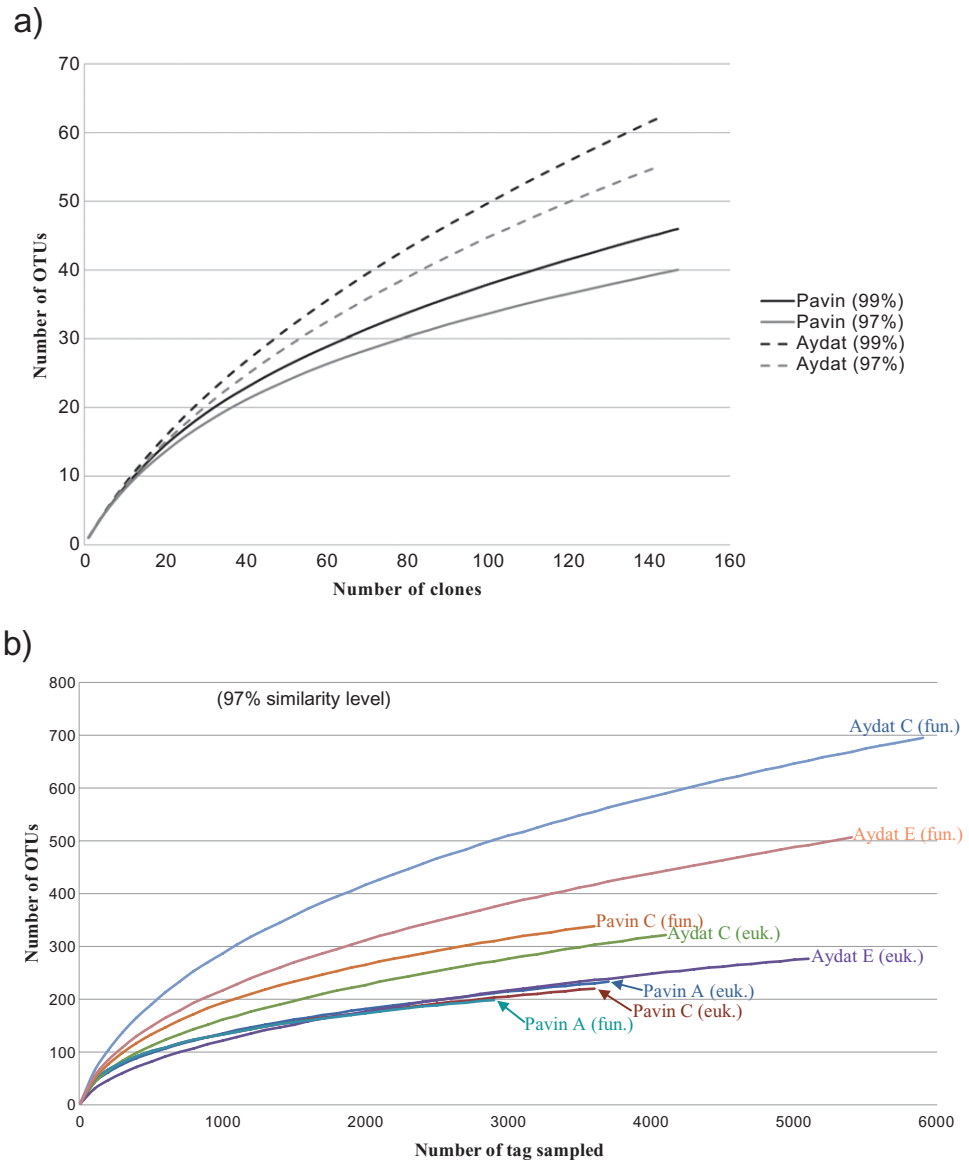

Fig. 3. Rarefaction curves. Rarefaction curves representing the numbers of OTUs versus the number of clones were plotted from sequences obtained with the classical (A) and the pyrosequencing (B) approaches. The curves were calculated using the program Mothur (Schloss *et al.*, 2009), with a cut-off value set to 0.01 and 0.03 (OTUs with differences that do not exceed 1% and 3%) for the analysis.
A. For the classical approach, all the 18S rRNA gene sequences obtained from Lake Pavin (full lines) and Lake Aydat (dash lines) were grouped into OTUs at a level of sequence similarity ≥ 99% (black lines) and ≥ 97% (grey lines).
B. The pyrosequence reads obtained from lakes, sampling stations, and using the two sets of primers [eukaryote (euk.) and fungus (fun.)], were curated (see *Experimental procedures*) and grouped into OTUs at a level of sequence similarity ≥ 97%.

species. In Lake Pavin, pyrosequence data obtained from both primer sets (eukaryotes and fungi) consistently displayed PA2009E8, PA2009E9, PA2009E10 and PA2009E11 as the dominant chytrids' OTUs (Table S1a). In Lake Aydat, the OTU AY2009C4 was consistently found highly dominant among the pyrosequence reads obtained from both sets of primers. This OTU represents 80% of the pyrosequence reads matching a fungal OTU identified from the classical approach (Table S1b), and corresponds to a novel species having only 90.3% similarity with *Rhizophydium* sp. JEL317, its closest relative.

When combining pyrosequence data obtained with the two set of primers, additional sequences were retrieved compared with the sole use of the classical approach. Some *Chytriomyces* sp. JEL341 and representatives of the *Monoblepharidaceae* family were found in Lake Pavin, while several *Rhizophydiales* sp. AF033 sequences were retrieved in Lake Aydat (Figs 5 and 6, and Table S2).

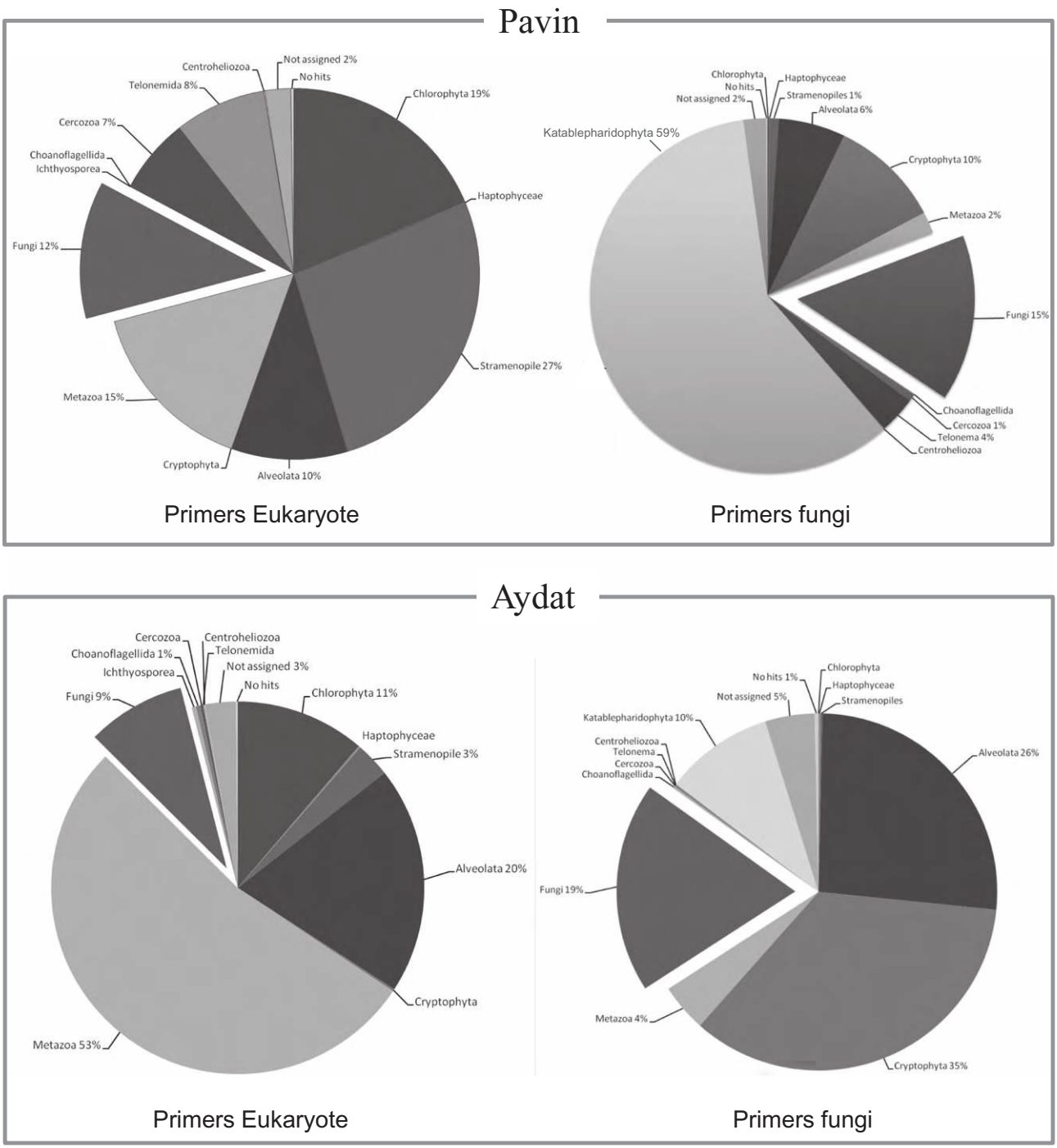

Fig. 4. Proportion of taxonomic groups identified in Lake Pavin and Lake Aydat using pyrosequencing of 18S rRNA gene hypervariable regions. The reads obtained from pyrosequencing of 18S rRNA hypervariable region were subject to BLASTN (Altschul *et al.*, 1990) search against the Silva SSU rRNA database (http://www.arb-silva.de/) to assign a taxonomic group. The pie diagram displayed the proportion of reads, obtained from both lakes sampling stations, and using the two primers sets (eukaryote and fungus), belonging to a particular phylum. 'No hit' corresponds to reads having no homologous sequence in the Silva database (threshold $E = 10^{-5}$). 'Not assigned' corresponds to reads having a match in the Silva database but without a precise taxonomic phylum assignment.

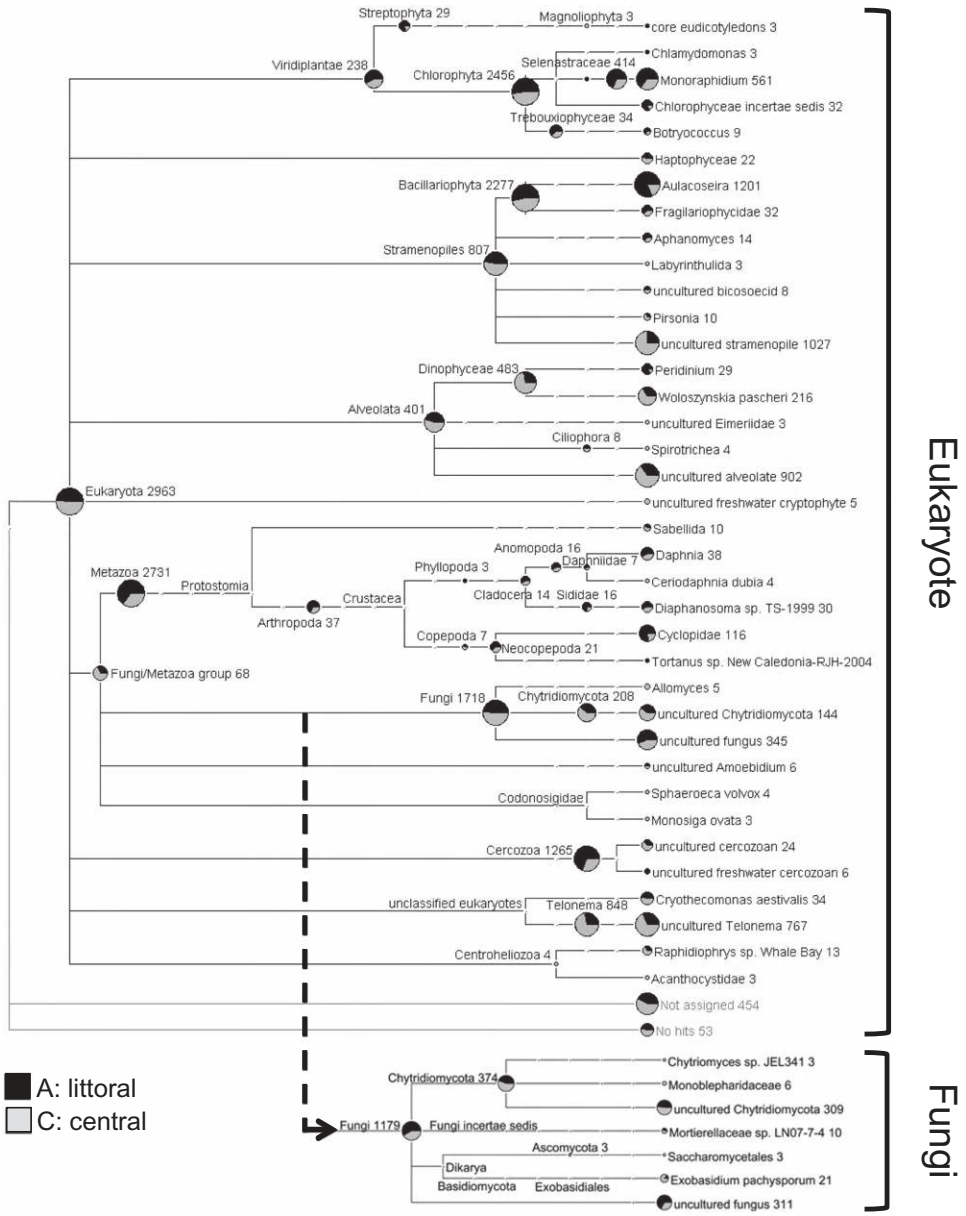

Fig. 5. Taxonomic assignment of the pyrosequences 18S rRNA reads obtained from Lake Pavin. The 18S rRNA hypervariable tag pyrosequences obtained from Lake Pavin were analysed using the software MEGAN, after BLASTN (Altschul *et al*., 1990) search against the Silva SSU rRNA database. The MEGAN software (Huson *et al*., 2007) plots on a schematic phylogenetic tree the number of pyrosequence reads matching a particular taxonomical group. Each taxonomic node is represented by a pie diagram, with littoral sample in black and central samples in grey colour, whose size is proportional to the number of assigned reads (given by the numbers). The tree displays all taxonomic groups identified from the assignment of pyrosequence reads obtained with eukaryote primers, while only the fungal Kingdom is given (on the bottom) for reads obtained using fungus-designed primers.

Other fungi. One *Ascomycota* taxon was common to both lakes and identified as OTUs PA2009D1 (Pavin) and AY2009B1 (Aydat). They indeed shared 99.4% identity, and were similar to *Simplicillium lamellicola*, a pathogen of another fungus, *Agaricus bisporus* (*Basidiomycota*) (Spatafora *et al*., 2007). This pathogen was the dominant *Ascomycota* in both lakes (Table S1a and b). The other *Ascomycota* identified in the two lakes represented minor species according to the low number of corresponding pyrosequence reads. In addition, pyrosequencing yielded *Saccharomycetales* as the sole group of *Ascomycota* in both lakes (Figs 5 and 6). Only one sequence per lake, retrieved from the SSU rDNA clone libraries, clustered within the *Basidiomycota* group, i.e. *Exobasidium rhododendri* in Lake Pavin and *Jamesdicksonia dactylidis* in Lake Aydat. Both of them are putative plant pathogens.

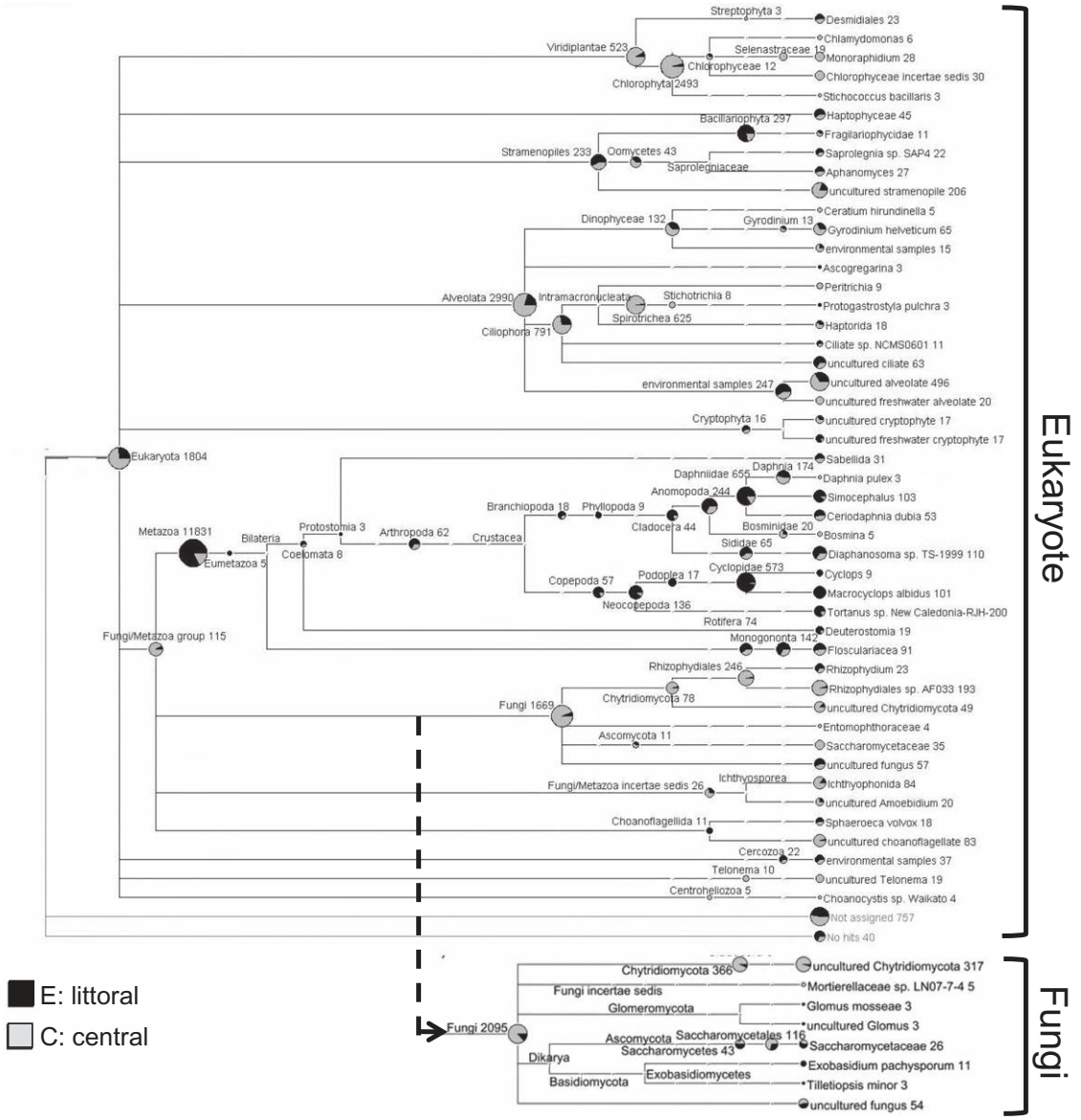

Fig. 6. Taxonomic assignment of the pyrosequences 18S rRNA reads obtained from Lake Aydat. Similarly to Fig. 5, the pyrosequence 18S rRNA assigned reads were displayed on a schematic phylogenetic tree. Pie diagrams, at each taxonomic nod, indicate the proportion of reads obtained from the central (grey colour) and littoral (black colour) sampling stations of Lake Aydat.

Compared with the classical SSU rDNA approach, pyrosequencing revealed the presence of *Exobasidium pachysporum* in both lakes and of three sequences affiliated to *Tilletiopsis minor* in Lake Aydat. In both lakes, the *Basidiomycota* sequences retrieved by the two approaches belonged to the class of *Exobasidiomycetidae*. The other fungus sequences, only identified through pyrosequencing, included some *Glomeromycota* (previously known as *Zygomycota*), such as *Mortierellaceae* sp. LN07-7-4 in the two lakes; and *Glomus mosseae* and representatives of the *Entomophthoraceae* family in Lake Aydat (Figs 5 and 6).

The diversity of other eukaryotes

Alveolata. In clone libraries from Lake Pavin, two *Alveolata* OTUs belonged to the *Dinophyceae*, one to the *Perkinsea* and one to the *Ciliophora* group (Fig. 1). In Lake Aydat, one-third of the identified OTUs from the clone libraries corresponded to *Alveolata*, from which 17 belong to the *Ciliophora*, one to the dinoflagellates and one to the *Apicomplexa* group (Fig. 2). One taxon of *Ciliophora* was common to both lakes, i.e. the OTUs PA2009E19 (Pavin) and AY2009D10 (Aydat) sharing 99.7% sequence identity. On their respective phylogenetic trees, these two OTUs

form new clades, supported by Bayesian posterior probabilities of 1.00 and 0.81 respectively. These clades clustered two additional OTUs in Lake Aydat (AY2009C19, AY2009C20), with *Rimostrombidium lacustris* as a sister group. In Lake Aydat, another new clade of *Ciliophora* clustering three OTUs (AY2009E5, AY2009C21 and AY2009E6) was supported by Bayesian posterior probabilities of 0.83. The *Apicomplexa* group was only found in Lake Aydat with one OTU (AY2009B10) having *Cryptosporidium parvum* as closest relative, i.e. a human protozoan parasite that can cause an acute short-term infection and become severe for immunocompromised individuals. Considering the phylogenetic distance between AY2009B10 and *C. parvum*, it was unlikely that Lake Aydat contained this parasite. Therefore, this sequence could represent a new clade or a *Cryptosporidium*-like organism. Consistent with the observations made from the classical approach, pyrosequence data displayed a larger diversity of *Alveolata* in Lake Aydat, compared with Lake Pavin (Figs 5 and 6), which contributed to the apparent overall higher eukaryote diversity in Lake Aydat.

Cryptophyta. Two *Cryptophyta* taxon, identified by the classical approach, were found in both lakes. The first one, corresponding to OTUs PA2009D3 (Pavin) and AY2009D5 (Aydat) sharing 99.6% sequence identity, clustered together with a sequence isolated from Lake Georges (LG08-05) (Richards *et al*., 2005), and a sequence (P1.31) previously obtained from Lake Pavin (Lefranc *et al*., 2005). The second, corresponding to OTUs PA2009D5 (Pavin) and AY2009E2 (Aydat) sharing 99.4% sequence identity, was closely related to *Cryptomonas ovata*. In both lakes, two distinct OTUs (having less than 99% identity) were related to *Plagioselmis nannoplanctica* strain N750301. In Lake Pavin, two OTUs (PA2009C5 and PA2009E14) clustered together in a clade with sequence P34-10 previously isolated from Lake Pavin (Lefranc *et al*., 2005) and sequence LG08-04 from Lake Georges (Richards *et al*., 2005). This clade, named LG-E in the phylogenetic tree based on sequences obtained from the Lake Georges (Richards *et al*., 2005), showed branches that were consistently attracted either to the cryptomonads or to the glaucocystophytes. In fact, this branch derived from the *Katablepharis japonica* group for which SSU rDNA gene sequences were not available at that time, and belongs to the katablepharids, a distant sister group from the *Cryptophyta* (Okamoto and Inouye, 2005). These two OTUs (PA2009C5 and PA2009E14) and their corresponding katablepharids group were matching the highest number of pyrosequence reads obtained when using fungus-designed primers, but were poorly detected when using eukaryote primers (Fig. 4 and Table S1a). Similarly in Lake Aydat, the OTUs AY2009E3 and AY2009C11, related to *Cryptomonas curvata* strain CCAC 0080, and their corresponding *Cryptophyta* group, were displaying the highest number of match with pyrosequence reads obtained from fungus-designed primers, but were also poorly detected using eukaryote primers (Table S1b).

Viridiplantae. This group of organisms was well represented in the clone libraries, accounting for 37% and 21% of the total numbers of sequences in Lakes Pavin and Aydat respectively. Representatives of the *Sphaeropleales* and *Chlamydomonadales* were found in both lakes, with the addition of the *Treubariaceae*, *Trebouxiophyceae* and *Mychonaste* groups in Lake Pavin (Figs 1 and 2). Two distinct new clades within the *Chlamydomonadale* group were identified in the two lakes, including the OTUs PA2009E16 and PA2009B2 in Lake Pavin (Fig. 3) and AY2009C13 and AY2009B7 in Lake Aydat (Fig. 4). Reads corresponding to *Viridiplantae* 18S rRNA genes were only retrieved from pyrosequencing when using eukaryote primers.

Minor groups. Stramenopiles were poorly identified in both lakes' clone libraries (Figs 1 and 2), but abundantly in Lake Pavin according to the pyrosequence data (Fig. 4). Through the classical approach, the *Telonemida* group was only identified in Lake Pavin, with the OTU PA2009A5. The pyrosequencing approach confirmed the abundance of *Telonemida* in Lake Pavin, and their rarity in Lake Aydat. On the opposite, *Choanozoa* sequences were only retrieved in Lake Aydat, both with the classical approach (i.e. OTUs, AY2009C6, AY2009C7 and AY2009C8, clustering together with *Monosiga ovata* and more distantly with *Monosiga brevicollis*) (Fig. 2) and with the pyrosequencing approach (Fig. 6). Finally, in addition to taxa identified with the classical approach, pyrosequencing using eukaryote primers allowed the retrieval of SSU rDNA sequences from numerous *Arthropoda* ($n = 312$ in Lake Pavin and 2502 in Lake Aydat), *Rhizaria* ($n = 1329$ and 59), a few *Haptophyceae* ($n = 22$ and 45) and *Centroheliozoa* ($n = 20$ and 9) (Figs 5 and 6). Overall, the pyrosequencing of 18S rRNA hypervariable regions confirmed the higher eukaryote diversity in Lake Aydat, compared with Lake Pavin (Figs 5 and 6).

Within-lake spatial variability

Lake Pavin. Both the classical and the pyrosequencing approaches showed a relatively homogenous spatial distribution of eukaryotes in Lake Pavin (Fig. 5 and Table S1a). The classical approach yielded 23 OTUs in the centre of the lake and 15, 18, 18 and 19 OTUs for stations A, B, D and E respectively (Fig. 1, Table S1a). The pyrosequence data, independently of the set of

primer used (eukaryote or fungi), displayed a similar number of reads corresponding to the main groups of taxa retrieved at the two sampling stations (Fig. 5). The number of pyrosequence reads corresponding to fungi decreased from the shore station A to the central station C, with a consistent number of reads related to uncultured fungi (i.e. having not clear taxonomical assignment) on the littoral zone (Fig. 5 and Table S2).

Chytridiomycota was the most identified group of fungi among the pyrosequence reads obtained using fungus-designed primers. Chytrid sequences increased from 25.8% ($n = 313$) of all the fungal reads in the riparian station A to 37.7% ($n = 379$) in the centre station C (Table S2). A similar result was observed when using eukaryote primers, but with about two times less chytrid reads retrieved from pyrosequencing. The dominant chytrids OTUs were equally found located on the littoral and central areas of the lake (Table S1a). The *Ascomycota* were consistently more frequently retrieved from the shore station by both approaches (classical and pyrosequencing) (Fig. 5 and Table S1a). The dominant *Ascomycota* (OTU PA2009D1) was almost exclusively located in the shore station (Table S1a). Finally, the *Basidiomycota* OTU (PA2009E1) retrieved with the classical approach was mostly found at the littoral station of Lake Pavin (Fig. 1, Table S1a). This contrasts with the general within-lake distribution of the other *Basidiomycota*, which were mostly retrieved in the central ($n = 18$) compared with the littoral ($n = 3$) stations of the lake according to pyrosequence data (Fig. 5 and Table S2).

The spatial patterns for other eukaryote species could also be estimated using data from pyrosequencing. In this respect, most of the eukaryotic groups displayed a homogeneous localization between the riparian and central sampling stations. Some differences were observed for *Cercozoa* which were mostly sampled in the littoral waters while, in contrast, *Telonemida* were preferentially sampled in the pelagic central waters of Lake Pavin. Finally, the *Cryptophyta* group, only unveiled when using fungus-designed primers, was threefold more abundant in the centre ($n = 1135$) compared with the littoral ($n = 347$) areas of the lake, according to the pyrosequencing (Table S2).

Lake Aydat. Both methods (classical and pyrosequencing) showed a more heterogeneous spatial distribution of species in Lake Aydat (Fig. 6 and Table S1b), compared with Lake Pavin. The classical approach yielded 24 OTUs in the central area, and 19, 19, 22 and 20 OTUs at stations A, B, D and E respectively (Fig. 2 and Table S1b). Among the eukaryotic groups, fungi displayed a decreasing proportion from station A (45%), B (36%), C (22%), D (25%) to station E (12%) relative to the total number of sequences retrieved by the classical approach. Fungi thus displayed a clear spatial pattern from station E to A, following the up–downstream flow of the river. With the pyrosequencing approach and independently of the set of primers used, fungi represented around 15% of all the pyrosequence reads at the central station C, but only 1–3% at the littoral station E (Fig. 6 and Table S2).

Chytrids spatial distribution, estimated from pyrosequencing and confirmed by the use of both primer sets (eukaryote and fungi), showed a preferential localization in the centre pelagial of Lake Aydat (Table S2). The classical approach showed that the OTU AY2009C4, representative of a novel clade within the *Rhizophydiales* order, was particularly abundant in the centre of the lake. When searching corresponding reads for AY2009C4 in the pyrosequence database obtained with fungus-designed primers, it appeared that matching reads were 33 times more abundant in samples taken from the central station C ($n = 701$) compared with the shore station E ($n = 21$) (Table S1b). This was confirmed by the pyrosequence data obtained from eukaryote primers for the OTU AY2009C4 which was 40 times more abundant in the central than in littoral stations. Two others OTUs, AY2009B3 and AY2009C3, clustering together with *Chytriomyces* sp. JEL341, were two- to threefold more represented in the central than in the littoral areas of the lake, according to pyrosequence data and independently of the set of the primers used (Table S1b).

The classical approach showed that the number of OTUs and of sequences composing OTUs belonging to *Ascomycota* decreased from the littoral sampling stations A and B (located close to the within-lake islands) to the rest of the lake (Table S1b). This gradient was confirmed by the pyrosequence data, with an overall higher number of *Saccharomycetales* at the station C ($n = 115$) compared with station E ($n = 70$) (Fig. 6 and Table S2). A low number of *Ascomycota* OTUs, identified by the classical method (Fig. 4), were matching pyrosequence reads at station E (Table S1b), thus reinforcing our hypothesis of a decreasing gradient from stations A to E for *Ascomycota* in Lake Aydat. *Basidiomycota* sequences were mainly retrieved in the riparian areas of this lake, by the two approaches. The AY2009A3 OTU, closely related to *Jamesdicksonia dactylidis*, was only retrieved by the classical method at station A (Table S1b). The other *Basidiomycota*, *E. pachysporum* ($n = 11$) and *T. minor* ($n = 3$), were exclusively retrieved by pyrosequencing at the sampling station E. These results are in contrast with those obtained in Lake Pavin, where sequences corresponding to *Basidiomycota* were mainly found in the centre of the lake. Other groups of fungi were identified solely by pyrosequencing, among which *Glomeromycota* were exclusively located in the littoral area ($n = 6$), while *Mortierellaceae* sp. LN07-7-4 ($n = 5$) was exclusively found in the centre of the lake (Fig. 6, Table S2).

Besides fungi, the spatial pattern for other eukaryotes was mainly estimated from the pyrosequence data obtained with eukaryote primers. The major groups of species displaying spatial distribution included *Viridiplantae* and *Alveolata* which were preferentially localized in the centre of the lake, while *Stramenopile* and *Arthropoda* were mainly located in the riparian area. The minor taxonomic groups, *Choanoflagellida*, *Centroheliozoa* and *Telonemida*, were mostly or exclusively found in the centre of Lake Aydat (Fig. 6, Tables S1b and S2). Finally, the kathablepharids group, only detected with the fungus-designed primers, was almost exclusively found in the centre ($n = 1559$) compared with the littoral ($n = 47$) areas of the lake (Table S2). The heterogeneous spatial distribution of the kathablepharid group in Lake Aydat contrasts with its homogenous distribution in Lake Pavin. In contrast to the observation made in Lake Pavin, *Cryptophyta* were homogeneously located in the central ($n = 3476$) and in the peripheric ($n = 2219$) areas of Lake Aydat. However, two *Cryptophyta* OTUs (AY2009C11 and AY2009E3) clustering with *C. curvata* strain CCAC 0080 were twofold more represented in the littoral than in the central areas of Lake Aydat (Table S1b).

Discussion

General and methodological considerations

This study's aim was to identify the diversity of microbial eukaryotes within two freshwater environments, Lakes Pavin and Aydat, Massif Central, France, which contrasted in terms of the influences of allochthonous terrestrial inputs. These two lakes indeed differ by their surface, altitude, depth, trophic status, geological origin, water flux (river, drainage basin) and anthropogenic activities (agricultural and industrial impact). Considering all these differences, a homogenous distribution of species was expected in Lake Pavin where typical pelagic organisms are characteristics of the whole water body. Conversely, we anticipated Lake Aydat would reveal a heterogeneous spatial variation of species, due to its exposition to enhanced allochthonous materials from terrestrial inputs. Few studies have investigated the diversity of picoeukaryotes in these two lakes (Lefranc *et al*., 2005; Lefèvre *et al*., 2007; 2008), and highlighted the importance and ecological significance of chytrids (Lefèvre *et al*., 2008). Our work intended to complete these studies by identifying new fungi and describe their quantitative importance. This goal was achieved through two complementary methods to beneficiate from their respective advantages: a qualitative taxonomic identification of each species, and of new clades, by the 'classical cloning/sequencing approach', and the corresponding dominant and rare species by the pyrosequencing of the 18S rRNA gene hypervariable regions. In addition, the large number of reads obtained with the pyrosequencing was a powerful tool to evaluate the distribution of species between the littoral areas versus the central pelagial ones. While one set of primers for fungi was used for the classical approach (White *et al*., 1990), two sets were utilized for pyrosequencing, i.e. a universal eukaryote set of primers (Casamayor *et al*., 2002; Lopez-Garcia *et al*., 2003; Lepere *et al*., 2006), and a set of fungus-designed primers (Borneman and Hartin, 2000). Although the latter primers were not specific for the 18S rDNA genes of the sole fungi, they allowed a significant fungal enrichment of our database (compared with eukaryote primers), up to 2.6-fold for total fungi and 3.5-fold for chytrids. The fungal primers did not amplify 18S rDNA genes from the abundant groups of *Arthropoda* and *Viridiplantae*, leading to the enrichment in 18S rDNA gene sequences from other eukaryotes, primarily from fungi, i.e. compared with the eukaryote primers. The concurrent use of fungal primers was therefore justified. In addition, the fungus-designed primers allowed the identification of an increased number of fungi taxa compared with universal eukaryote primers (Figs 5 and 6). Nevertheless, designing primers specific for pelagic fungus rRNA genes is one of the major bottlenecks that need to be resolved for future studies. In this regard, the ITS1, 5.8S and ITS2 sequences that were generated with the classical approach used in this study will be very helpful to design new tools to follow the dynamics of particular fungal species in natural waters.

The diversity in Lake Aydat was greater than that observed in Lake Pavin, which is likely to be linked to the differences in the trophic status of theses lakes. It is interesting to observe that the two lakes shared only fives taxa in common (two *Cryptophyta*, one *Alveolata*, one *Ascomycota* and one *Chytridiomycota*), highlighting the difference between the two lakes and providing evidence that aspects of eukaryote microbial diversity are specific to certain aquatic environments. The rarefaction curves constructed from the 146 sequences in Lake Pavin and 142 sequences in Lake Aydat displayed a linear pattern, indicating a still undersampling of the total diversity. Consistently with this result, the same groups of organisms were identified, and confirmed the existence of new clades known from previous studies (Lefèvre *et al*., 2007; 2008), in addition to the unveiling of many new rDNA gene sequences that were not previously retrieved. Finally, this low-throughput approach allows the retrieval of SSU rDNA sequences from species that were probably the dominant ones in the lakes. In contrast, the high-throughput pyrosequencing of 18S rRNA gene hypervariable regions was initiated in order to increase the sampling effort of rDNA gene sequences in the target lakes and to unveil a much extended eukary-

ote diversity, with a focus on fungi. Rarefaction curves computed from pyrosequence data displayed linear pattern if applying 99% sequence identity threshold, while it is approaching a plateau if applying 97% identity threshold (Fig. 3B). A 97% identity threshold is better adapted to computer rarefaction curves from deep pyrosequencing of rRNA reads (Kunin *et al*., 2010). These patterns of rarefaction curves suggest a good sampling of the water samples taken from the lakes and showed higher apparent species richness when using fungus-designed primers compared with eukaryote primers. The variability within different regions of the SSU rRNA molecule and the length of the amplicon have a great effect on the apparent species richness, as it was shown when tracking the microbial diversity of the termite hindgut (Engelbrektson *et al*., 2010). Species evenness and richness should not be directly compared between different regions of the rRNA molecules (Engelbrektson *et al*., 2010). In addition, richness should also be temperate by some technological limits (alignments of large sets of sequences, pyrosequencing errors, statistical tools etc.) (Kunin *et al*., 2010) and by the unknown variability of the multiple copy of rDNA operon from the same organism, as it was demonstrated for bacteria belonging to the genus *Vibrio* (Moreno *et al*., 2002).

Pyrosequencing of rRNA gene hypervariable region reveal much greater eukaryote diversity in the studied lakes than in the previous studies (Lefranc *et al*., 2005; Lefèvre *et al*., 2007; 2008). The reason is that only the classical approach was used in these studies, unveiling dominant but not minor species. Rare species, corresponding to species that are maintained at a low number [arbitrarily chosen below 0.05–1% of the total number of pyrosequence reads (Sogin *et al*., 2006; Huse *et al*., 2008)], may play an important role in lakes by becoming dominant in response to environmental changes, in addition to represent a nearly inexhaustible source of genomic innovation (Sogin *et al*., 2006). They indeed represent a hypothetically important reservoir of ecological redundancies that can buffer the effects of dramatic environmental shifts and, perhaps, insure the maintenance of basic biogeochemical processes in natural ecosystems (Sime-Ngando and Niquil, 2011). However, rare microbial eukaryotes are largely underexplored in aquatic systems, primarily in freshwaters. All studies on the 'rare biosphere' are indeed restricted to marine prokaryotes, with only one theoretical attempt considering microbial eukaryotes (Caron and Countway, 2009). The improved unveiling of eukaryote diversity, such as in the present study, thus opens great perspectives for future identification of novel 'species', genes and metabolic pathways in lakes by complete deep sequencing of complex community, similar to what was done in marine ecosystems (Venter *et al*., 2004; Delong *et al*., 2006).

Diversity patterns

A greater diversity of fungi and chytrids was observed in Lake Pavin compared with Lake Aydat. The classical approach showed that fungi represent half of the OTUs identified in Lake Pavin, and one-third in Lake Aydat. Both approaches used confirmed that Lake Pavin contains around 20% more fungal diversity than Lake Aydat, suggesting a preference or the capacity of most fungi unveiled to adapt to a pelagic lifestyle. In both lakes, the four phyla of the main fungal divisions were represented in our sequences. The presence of *Glomeromycota* 18S rDNA gene sequences in the littoral area of Lake Aydat is consistent with their distribution in terrestrial rather than aquatic habitats and further implies a contribution of specimens in our samples from allochthonous inputs. The low allochthonous water input thus probably explained their absence in Lake Pavin, which have a water retention time about 10-fold longer than Lake Aydat (Camus *et al*., 1993; Sarazin *et al*., 1995). It is often difficult to compare results obtained from pyrosequencing and classical approaches, because of the use of different primers. Despite the lack of replicates, a good correlation was observed between the two methods for the identification of dominant fungi: the highest number of pyrosequence reads corresponds to the fungus OTUs with the highest number of sequences in the classical approach (Table S1a and b). Still, the quantitative information given by pyrosequencing data should be interpreted with care, because of the inherent bias of the PCR technique and also because no information is available on the number of copies of rRNA operons per fungal cells. Yet, such comparison revealed that the OTUs identified by the classical approach corresponded to around 13.8% (Station A, Pavin), 9.3% (C, Pavin), 19.2% (C, Aydat) and 10.4% (E, Aydat) of the pyrosequence reads obtained with fungus-designed primers. This result suggests a realistic representation of the fungal complete 18S rDNA gene sequences obtained with the classical approach. Several dominant chytrids were specific to each lake. The chytrids OTUs PA2009E8 and PA200911were dominant in Lake Pavin, while the OTU AY2009C4 corresponds to the main chytrid sequence isolated from Lake Aydat. These dominant chytrids may play an important role in the lakes, but further characterization of these species is needed to better understand their dynamics and functional importance.

The classical approach reveals the presence of species belonging to the group of *Viridiplantae* in both lakes, and a large diversity of *Alveolata* in Lake Aydat. Among the *Alveolata*, one rDNA gene sequence was corresponding to a *C. parvum*-like organism. Additional studies and analysis are required to characterize this parasite-like organism and to determine, if it is of concern to human

safety. Within the *Alveolata* group, some *Perkinsus* rDNA sequences were identified, which have not been yet characterized earlier by traditional microscope observations because these organisms do not display distinctive morphological characteristics (Burreson *et al*., 2005; Gestal *et al*., 2006). In recent molecular studies, a growing number of *Perkinsus* have been identified from freshwater environments (Brate *et al*., 2010), indicating that more efforts are needed to culture and characterize members of this lineage. Only one cercozoan sequence (AY2009C14) was found in Lake Aydat. This was surprising since many cercozoans were isolated from both lakes in previous studies (Lefranc *et al*., 2005; Lefèvre *et al*., 2007; 2008). It may be suggested that cercozoans were poorly represented in the two lakes at the time of our sampling.

The pyrosequencing of the 18S rDNA gene hypervariable regions led to the identification of many other eukaryotes. Among them, the *Cryptophyta*/*Katablepharidophyta* was the most represented group, accounting for more than 50% of the total pyrosequencing reads when using fungus-designed primers, but was not detected with eukaryote primers. On the contrary, the groups of *Viridiplantae*, *Stramenopile* and *Arthropoda*, were only retrieved when using eukaryote primers. These two examples highlight the importance of using a combination of primers sets, to accurately determine environmental biodiversity. Few metazoan rDNA sequences were retrieved by the pyrosequencing approach. Since most metazoan could not pass through the filtration process, these sequences may come from larva, decomposed dead cells or free DNA molecules. In this study, we consider ourselves to have unveiled the most exhaustive catalogue of eukaryotes, and particularly that of fungi, identified from freshwater environments, by taking advantage of the complementarities between the classical and pyrosequencing approach, and the specificity of different primers.

Spatial distribution

The spatial distribution of taxa within the two lakes, estimated from our data, should be interpreted with care since no biological repeats were performed. Nevertheless, some general patterns can be observed such as a more heterogeneous distribution of taxa in Lake Aydat compared with Lake Pavin (Figs 5 and 6, Table S1a and b). This observation may be linked to the specific characteristics of both lakes, including the oligomesotrophic homogenous Lake Pavin compared with the heterogeneous eutrophic status of Lake Aydat. In addition, the deep-crater Lake Pavin characterized by a low drainage basin, the absence of an influent river and a 10-fold longer retention time, compared with Lake Aydat, establishes a typical pelagic environment in the whole water body of the lake. These characteristics may explain why chytrids, which are typical pelagic organisms, were located throughout Lake Pavin, and preferentially in the central water column of Lake Aydat, which is subject to a higher level of water flux and of terrestrial input. As an example, the Lake Aydat chytrid OTU AY2009C4, belonging to the *Rhizophydiales* order, was at least 33 times more represented in the centre compared with the littoral area of the lake, a spatial pattern that is apparently consistent with the typical pelagic lifestyle of chytrids. The *Arthropoda* were mostly located in the littoral areas of both lakes; it is particularly true for Lake Aydat. This localization can be explained by the food source of these metazoans that might be more located in littoral areas. Finally, pyrosequence data obtained using fungus-designed primers revealed a preferential central localization of *Cryptophyta* in Lake Pavin, and for *Katablepharidophyta*, its sister group, in Lake Aydat. In general, in both lakes, the greatest diversity of eukaryotes was observed at the central point.

Conclusions

Very few species were shared between Lakes Pavin and Aydat, which indicates a different community composition in the same regional area. Geographical, physical and chemical factors of the biotope influence the species community structure and spatial variability. The characterization of the biodiversity is the first, but perhaps not the most important step to understand the functioning of an ecosystem. Matching sequences to organisms represents one of the last frontiers of the undiscovered biodiversity that challenge pelagic microbial ecology today (Sime-Ngando and Niquil, 2011). This study has to be completed by further characterization of the species, at least for the dominant ones, from their mode of nutrition (autotrophy, heterotrophy, mixotrophy), to their interactions between each other (food web, saprotrophism, parasitism, commensalism etc.). In order to do that, we first need to learn how to grow them in the laboratory. Chytrids play a major role in lake ecology, primarily as parasites of inedible phytoplankton, and by transferring these matter and energy (in the form of zoospores) to higher trophic levels (Lefèvre *et al*., 2008). Sequencing the genome of chytrids, developing molecular tools (mutagenesis) and performing phenotypic analysis will lead to a major breakthrough in the understanding of the functioning of lake ecosystems. This knowledge could be integrated into mathematical models to predict ecosystem functioning. In this respect, Lake Pavin, because of its species composition homogeneity, is an ideal model for this type of study. Although the data presented in this article are only a snapshot of the whole lake's biodiversity, the massive parallel pyrosequencing of the 18S rRNA gene hypervari-

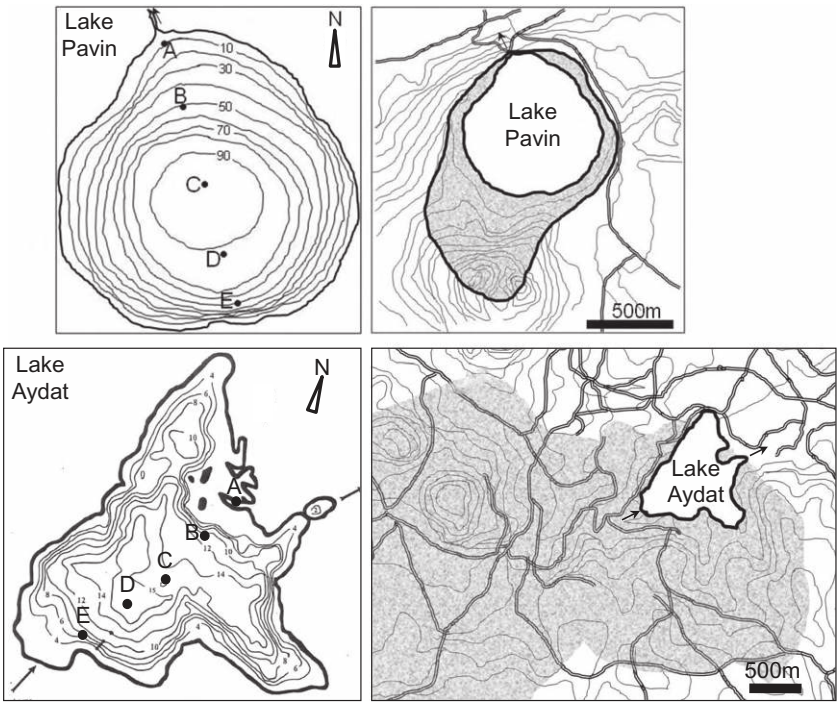

Fig. 7. Bathymetric maps (left) and water catchment area (right) from Lake Pavin and Lake Aydat. The bathymetric maps of Lake Pavin (from Delbecque, 1898) and Lake Aydat (from Rabette and Lair, 1999) display the location of the five sampling stations: two littoral (A and E), one central (C) and two intermediaries (B and D). On the bathymetric maps, the arrows show the flux of water and the numbers indicate the depth in metre. The two water catchment areas of Lake Pavin (from Lair, 1978) and Lake Aydat (this study) show the larger size of Lake Aydat drainage basin (grey shade) compared with Lake Pavin. Arrows indicate the flux of water, lines show the ground topography and roads are indicated by double lines.

able regions allows the assessment of the impact of space, time and complex environments on microbial communities. A timeline survey of the lake's biodiversity, with a regular sampling over several years, is needed. This will give crucial information to understand the succession of stable and unstable distribution of eukaryote community, the dynamics, interactions and successive blooms of species, and the changes of the eukaryote biodiversity due to climate change or anthropogenic activities.

Experimental procedures

Sampling procedure and DNA extraction

Lake Pavin (45°30′N, 2°53′′W) is a meromictic, dimictic, oligomesotrophic lake situated in the Massif Central of France. It is a deep volcanic mountain lake (Z_{max} = 92 m), characterized by a permanently anoxic monimolimnion from a 60 m depth downwards. This site offers a unique environment with low human influences, characterized by a small surface area (44 ha), about equal to the drainage basin area (50 ha), with no river inflow (Fig. 7). The water retention time in the mixolimnion is approximately 10 years, with an average water turnover rate of 0.09 per year. In the monimolimnion, this water turnover rate decrease to 0.005 per year (Camus *et al*., 1993). Lake Aydat (45°39′N, 2°59′′W) is a small (Z_{max} = 15 m, surface area = 60 ha) dimictic, eutrophic lake, also located in the Massif Central of France. It was formed when a lava flow dammed the small river Veyre. Compared with the surface of the lake, the catchment area (2.5×10^3 ha) is very large and contains intensive agricultural lands (Fig. 7). The water retention time of Lake Aydat is approximately 1 year (Sarazin *et al*., 1995).

For each of the two lakes, five samples were collected along a transect across the lake, including two littoral (points A and E), two intermediary (point B and D) and one central site (point C) located in the deepest area of the lake (Fig. 7). For Lake Pavin, station A is located near the lake outlet, while on the other side of the lake, station E is influenced by the catchment effects (Fig. 7). For Lake Aydat, the stations A and B were located near the Veyre River outlet and the within-lake islands, while station E was located next to the lake 'estuary' (Fig. 7). Samples were collected during the end of the thermal stratification period, on 16 July 2008 for Lake Pavin and on 21 July 2008 for Lake Aydat. In Lake Pavin, the temperature of surface waters fluctuated between 17.5°C and 17.8°C, and pH values were at 8.33, 8.30, 8.27, 7.94 and 8.28 for the sampling points A, B, C, D and E respectively. In Lake Aydat, the temperature fluctuated between 19.6°C and 19.5°C and pH values were at 9.70, 9.74, 9.81, 9.85 and 9.83 for sampling points A, B, C, D and E respectively.

The whole euphotic water column [determined from Secchi disk measurements, $Z_{eu} = 1.7 \times Z_s$ (Reynolds, 1984)] was collected at each sampling point using a flexible plastic tube (diameter 4 cm) provided by a rope connecting the ballasted bottom of the tube with a surface manipulator. The euphotic depths were at 4.5 m and 20 m in Aydat and Pavin respectively. Samples were immediately pre-filtered through 150-μm-pore-size nylon filter (i.e. to eliminate larger metazoans), poured into clean recipients previously washed with the lake water, and returned back to the laboratory for immediate processing. Subsamples of 150 and 300 ml from Aydat and Pavin, respectively, were filtered onto 0.6-μm-pore-size polycarbonate filters (47 mm diameter) using a vacuum pump

(pressure < 100 mbar) to collect all planktonic microorganisms. The filters were stored frozen at −80°C until DNA extraction.

DNA from each sampling point was extracted using the kit NucleoSpin® Plant DNA extraction Kit (Macherey-Nagel, Düren, Germany) adapted for fungal material. The initial step aimed at digesting fungal chitin wall. Filters were then incubated overnight at 30°C with 400 Units of lyticase enzyme (Sigma, NSW, Australia) in a 500 µl sorbitol based buffer (Karakousis *et al*., 2006). Then sodium dodecyl sulfate (final concentration 1%) and proteinase K (final concentration, 0.1 mg ml^{-1}) were added and incubated 1 h at 37°C. All subsequent DNA extraction steps were conducted following the manufacturer's instructions.

The classical approach: PCR, cloning/sequencing and phylogenetic analyses

The primers sense NS1 (GTAGTCATATGCTTGTCTC) and antisense ITS4 (TTCCTCCGCTTATTGATATGC) known to preferentially amplify fungal DNA (White *et al*., 1990) were used to amplify the complete 18S rDNA, ITS1 (Internal Transcribed Spacer region), 5.8S rDNA, ITS2 and partial 28S rDNA region (Fig. S1). Only the 18S rDNA gene sequences were used in the phylogenetic analyses. PCRs were carried out in 50 µl volume according to standard conditions for *Taq* DNA polymerase (Bioline) with 50 ng of environmental DNA as template. After the denaturation step at 95°C for 3 min, 34 cycles of amplification were performed with a GeneAmp PCR System Apparatus (Applied Biosystems) as follows: 30 s at 95°C, 30 s at 59°C and 3 min at 72°C. The reaction was completed with an extension step at 72°C for 10 min. The products were separated on agarose gel electrophoresis in order to confirm the presence of bands of the expected size (around 2600 bp). PCR products were precipitated by sodium acetate/ethanol and titrated for cloning. For each sampling point, a genetic library was constructed. An aliquot of PCR product was cloned using the TOPO-TA cloning kit (Invitrogen) following the manufacturer's recommendations. The presence of the insert in the colonies was checked by PCR amplification using M13 forward and reverse universal primers. PCR products of the correct size were analysed by RFLP using the restriction enzyme HaeIII (Invitrogen). Clones showing the same RFLP patterns were grouped into a single OTU and one representative of each OTUs was sequenced from minipreparations of plasmid DNA using the Nucleospin kit (Macherey-Nagel). A total of 146 and 143 SSU rDNA gene sequences for Lake Pavin and Lake Aydat, respectively, were obtained and aligned using CLUSTALW (Thompson *et al*., 1994). Sequences sharing more than 99% identity were grouped into OTUs. A total of 46 OTUs in Lake Pavin and 63 OTUs in Lake Aydat were identified. One representative sequence of each OTU was submitted to a BLAST search (Altschul *et al*., 1990) on the non-redundant nucleotide database (NCBI) for an approximate phylogenetic affiliation and in order to select representative taxa for tree constructions. The resulting alignments including representative OTU sequences and reference sequences were corrected manually and regions of ambiguous alignment were removed using the Bioedit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic trees were built using both the Neighbour-Joining (NJ) method from the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html) and the Bayesian method from MrBayes3 software (http://mrbayes.csit.fsu.edu/index.php) (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Topologies of the phylogenetic trees generated by the two methods were similar. Only the tree generated by the Bayesian method is displayed since this method was considered to give more accurate estimates (Alfaro *et al*., 2003). The Bayesian posterior probabilities are showed at each node.

The pyrosequencing approach

DNAs extracted from the sampling points A and C of Lake Pavin, and C and E of Lake Aydat were used in a pyrosequencing approach. Two set of primers were used in this study (Fig. S1). The first set of primers, 18S-82F (GAAACT GCGAATGGCTC) and Ek-516r (ACCAGACTTGCCCTCC) (Casamayor *et al*., 2002; Lopez-Garcia *et al*., 2003; Lepere *et al*., 2006), was designed to amplify a 480 bp region containing the complete V2 and V3 domains of all eukaryote SSU rDNA gene. We will refer to this universal set of primers as the eukaryote primers. The second set, composed of primers nu-SSU-0817-5′ (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1196-3′ (TCTGGACCTGGTGAGTTTCC), was previously designed by Borneman and Hartin (2000) to preferentially amplify an around 400 bp region containing the V4 (partial) and V5 (complete) variable domains of the SSU rDNA gene from all four major phyla of fungi. We will refer to this set of primers as the fungus-designed primers. A 10 bp tag specific to each sample, a 4 bp TCAG key and a 26 bp adapter for the GsFLX technology were added to the sequences of the primers. PCRs were carried out according to standard conditions for *Taq* DNA polymerase with 20 ng of environmental DNA as template. After the denaturation step at 95°C for 3 min, 20 cycles of amplification were performed with a GeneAmp PCR System Apparatus (Applied Biosystems) as follows: 30 s at 95°C, 30 s at 60°C and 1 min at 72°C. The pyrosequencing project was performed by the Genoscreen company (Institut Pasteur de Lille, France). The library and the 454 titanium (Roche) pyrosequencing run were prepared following the manufacturer's recommendations. A total of 103 435 sequences with 53 856 and 49 579 sequences from the eukaryote and fungus-designed primers, respectively, were obtained. The reads were classified according to the tag sequence corresponding to each of the four samples of interest. With the eukaryote primers, 12 133 and 11 386 sequences from the sampling stations A and C in Lake Pavin, and 15 259 and 15 078 sequences from the sampling stations C and E in Lake Aydat, were, respectively, obtained. Using the fungus-designed primers, a total of 8018 and 10 527 sequences from the sampling stations A and C in Lake Pavin, and 16 672 and 14 362 sequences from the sampling stations C and E in Lake Aydat, were, respectively, obtained. Primers, tag and key fragments were subsequently removed before analysing the sequences. Globally, around two-thirds of the reads showed a length above 200 bp and one-third of the reads had a length above 400 bp.

For identification, the resulting sequences were compared with the Silva SSU rRNA database (http://www.arb-silva.de/) release 102 (updated on February 15, 2010) comprising

1 246 462 18S SSU rRNA sequences (including 134 351 eukaryotic sequences) using the BLASTN software (Altschul *et al.*, 1990). BLAST results (with 10^{-5} *E*-value threshold) were visualized using the metagenomic software MEGAN (Huson *et al.*, 2007). This software allows exploring the taxonomic content of the samples based on the NCBI taxonomy using the option 'import BLASTN'. The program uses several thresholds to generate sequence–taxon matches. The 'min-score' filter, corresponding to a bit score cut-off value, was set at 35. The «top-percent» filter used to retain hits, whose scores lie within a given percentage of the highest bit score, was set at ten. The «min-support core» filter, used to set a threshold for the minimum number of sequences that must be assigned to a taxon, was set to three. These stringent parameters result in the 'safe' assignment of many sequences to lower branch (with less precision) of the taxonomic tree. Distribution of the sequences was schematically represented by trees and pie diagrams.

Comparison between the classical and the pyrosequencing approaches

The pyrosequences from the SSU hypervariable regions were compared with the full-length 18S rDNA gene sequences obtained with the classical approach. This comparison allowed the identification of the dominant species, the determination of the within-lake spatial variation of species, and an evaluation of the correlation and complementarities between the two approaches. The 18S rDNA gene pyrosequences were submitted to a BLAST analysis (with 10^{-23} *E*-value threshold for BLASTN) against databases containing the full-length 18S rDNA gene sequences from each lake obtained by the classical approach. This analysis was performed independently for both lakes. Several criteria were applied to select relevant BLAST results, including a minimum query sequence of 60 bp and a minimum of 99% similarity over 80% of the sequence length with match starting, at least, from the third nucleotide of the query sequence. BLAST results were manually checked in order to remove duplicates, i.e. a pyrosequence read that was matching several times with the same full-length 18S rDNA gene sequence (i.e. from the classical approach) on different sites. However, 18S rDNA gene pyrosequences could still match with several full-length 18S rDNA gene sequences from the classical approach, when their lengths are too short for a precise identification.

Rarefaction curves

The quality of the sampling effort was assessed through the calculation of rarefaction curves, i.e. the number of OTUs versus the number of clones (Hughes *et al.*, 2001). Rarefaction curves for sequences obtained from the classical and the pyrosequencing approaches were done independently but following the same procedure. For the classical approach, all the obtained 18S rDNA gene sequences were considered for analysis. For the pyrosequencing approach, all the obtained 18S rDNA gene sequences with a length over 400 bp were considered. This size limit was set in order to include in the analysis the entire variable V2 (obtained with the eukaryote primers) and V5 (obtained with the fungus-designed primers) regions of the 18S rDNA gene sequence. With the eukaryote primers, a total of 3786 and 3684 sequences for stations A and C in Lake Pavin, and 4182 and 5141 sequences for stations C and E in Lake Aydat, were, respectively, analysed. With the fungus-designed primers, a total of 2948 and 3691 sequences for stations A and C in Lake Pavin, and 5935 and 5444 sequences for stations C and E in Lake Aydat, respectively, were analysed. The sequences considered for both approaches were aligned using the program MUSCLE (Edgar, 2004) (with parameters –diags and –maxiters 2) and were manually corrected using the Bioedit software to remove ambiguous terminal region of the alignment. The resulting alignment was used as input for the Mothur program (Schloss *et al.*, 2009), with a cut-off value set to 0.01 and 0.03 (i.e. OTUs with differences that do not exceed 1% and 3%) for the analysis.

Accession numbers

Nucleotide sequences obtained from the complete 18S, ITS1, 5.8S, ITS2 and partial 28S sequences were deposited in GenBank under the Accession No. HQ191282–HQ191427 for Lake Pavin samples and HQ219333–HQ219474 for Lake Aydat samples. The pyrosequences were deposited in GenBank-SRA under the accession numbers SRA012393.5.

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Supporting information

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

Fig. S1. Schematic representation of genomic rDNA region and localization of the primers used for the classical and the pyrosequencing approaches of this study. This figure shows a schematic representation of the genomic rDNA region (18S, ITS1, 5.8S, ITS2, 28S, IGS1, 5S and IGS2) with the localization of the primers and resulting amplicons that were obtained by the classical and pyrosequencing approaches. The hypervariable regions of the 18S rRNA gene are displayed by black boxes numbered from V1 to V9. The sequence of each primer is given between brackets.

Table S1. Number of sequences composing OTUs in the clone libraries, phylogenetic affiliation of the representative OTUs and corresponding numbers of matching read in the pyrosequence data. These tables summarize the number of clones obtained from each OTUs from Lake Pavin (S1a) and Lake Aydat (S1b) clone libraries. For each OTU, the taxon, the closest relative with its percentage of sequence identity, the number of clones composing OTU from the classical approach and their corresponding sampling stations localization (see Fig. 1), and the number and percentage of matching reads from the pyrosequencing approach (both using fungi and eukaryote primers) are given. The '–' symbol, for sequence PA2009E6, indicates that no closest relative sequence and it corresponding percentage could be determined.

Table S2. Number of pyrosequencing reads assigned to each taxonomic group. This table displays the number of pyrosequencing reads, obtained from the two lakes, sampling stations and primer sets (fungi and eukaryotes), which were assigned to a taxonomic group after a BLASTN search. 'No hit' corresponds to reads having no homologous sequence in the Silva database (threshold $E = 10^{-5}$), and 'Not assigned' corresponds to reads having a match in the Silva database but with an unclear taxonomic assignment.

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