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Highly Diverse and Seasonally Dynamic Protist Community in a Pristine Peat Bog

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Culture-independent molecular methods based on the amplification, cloning and sequencing of small-subunit ribosomal RNA genes (SSU rDNAs) are powerful tools to study the diversity of microorganisms. Despite so, the eukaryotic microbial diversity of many ecosystems, including peatlands has not yet received much attention. We analysed the eukaryotic diversity by molecular surveys in water from the centre of a pristine *Sphagnum*-dominated peatland in the Jura Mountains of Switzerland during a complete seasonal cycle. The clone libraries constructed from five different temporal samplings revealed a high diversity of protists with representatives of all major eukaryotic phyla. In addition, four sequence types could not be assigned to any known high-level eukaryotic taxon but branched together with a rather good statistic support, raising the possibility of a novel, deep branching eukaryotic clade. The analysis of seasonal patterns of phylotypes showed a clear change in the eukaryotic communities between the warm period (late spring and summer) and the cold period (autumn and winter). Chrysophytes dominated the samples in the cold period while testate amoebae (Arcellinida and Euglyphida) and a few other groups peaked in summer. A few phylotypes (such as a cryptomonad and a perkinsid) were abundant at given sampling times and then almost disappeared, suggesting bloom-like dynamics.

Key words: Environmental diversity; *Sphagnum*-dominated peatland; chrysophytes; new phyla; testate amoebae; seasonal patterns.

Introduction

Culture-independent molecular methods based on the amplification, cloning and sequencing of small-subunit ribosomal RNA genes (SSU rDNAs) are a powerful tool to study the diversity of microorganisms, both prokaryotic and eukaryotic, from environmental samples. In the past few years, progress in the study of microbial eukaryotic phylogeny and diversity has been rapid. As a consequence, both old-established phylogenetic relationships among major eukaryotic lineages (Baldauf 2003) and previous estimates of eukaryotic diversity have been fundamentally questioned (Epstein and López-García 2008). However, only a fraction of the existing ecosystem types have been studied in this way and it is therefore possible that

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some microbial groups remain to be discovered. Peatlands are among the ecosystems that have received little attention until now with respect to eukaryotic microbial diversity.

Peatlands are globally widespread biomes, which occupy up to 3% of the Earth's terrestrial surface (Kivinen and Pakarinen 1981). Among the different types of peatlands, *Sphagnum*-dominated peatlands (bogs and poor fens) are most common in high latitudes (and locally high altitudes), where they can be a dominant feature of the landscape. They are characterised by very low concentrations of mineral nutrients (5 to 50 mg per litre in total), low pH values (sometimes below 4.0) (Dedysh et al. 2006), and high concentration of humic acids which are known to have biocidal properties (Steinberg et al. 2006). Under such environmental conditions, which can be considered relatively extreme (mostly due to the lack of certain nutrients), the degradation rate of organic matter is very low, resulting in an accumulation of organic carbon. At the global scale, this process is of considerable significance: 30% of the world's pool of organic carbon is stored in peat bogs (Gorham 1991). It has been found that they act also as carbon sinks, sequestering 12% of all anthropogenic emissions of CO_2 (Moore 2002).

Despite their importance in the global carbon cycle, it is only recently that the microbial diversity of peatlands has attracted the interest of the scientific community, mainly because of their possible contribution to greenhouse gas emissions and hence global warming (Dedysh et al. 2006). To date, most studies on microbial diversity in peatlands focussed on prokaryotes involved in CH_4 cycling, leading to the discovery of a phylogenetically diverse and mostly unknown prokaryotic community (Dedysh et al. 2006) and also of remarkable adaptations to the harsh environmental conditions (Bräuer et al. 2006). The taxonomic composition of these communities is not fixed, but dynamic in time; increased temperature (as in summer) is paralleled by a proportional increase of methanogenic archaea, which in turn correlates with enhanced methane production (Høj et al. 2008).

In contrast to bacteria and archaea, the study of the diversity of eukaryotic micro-organisms in *Sphagnum*-dominated peatlands is currently limited to morphological observations of the general protist community (Kreutz and Foissner 2006; Strüder-Kypke and Schönborn 1999). Data on the ecology of certain species do exist for certain conspicuous groups, such as fungi (Thormann and Rice 2007 and references therein; Thormann et al. 2007), diatoms (Bertrand et al. 2004; Delluomo 1992) and testate amoebae (e.g. Charman and Warner 1992; Lamentowicz and Mitchell 2005; Mitchell et al. 2008; Tolonen et al. 1992) which make up a high fraction of the total biomass of microorganisms, and also for metazoans (Gilbert et al. 1998a,b; Mitchell et al. 2003). However, to date, the diversity of microbial eukaryotes in the *Sphagnum* peat bog environment has never been investigated with molecular methods. These could be useful to study the small colourless flagellates and naked amoebae which are easily overlooked in microscope-based studies but play a key role in nutrient cycling in many environments, as illustrated in the paradigm of the microbial loop (Azam et al. 1983; Clarholm 1985). In addition, parasitic organisms, difficult to detect solely by observation, may also play a fundamental role in freshwater ecosystems (Lefèvre et al. 2008). Moreover, it can be speculated that strongly selective environments such as *Sphagnum*-dominated peatlands can host an undetected diversity, perhaps with novel clades. Microscopic observation of bog communities has revealed the existence of organisms growing exclusively in these environments (Foissner 1996; Heal 1961). In this work, we have analysed by molecular methods the eukaryotic microbial diversity of a pristine ombrotrophic, (i.e. receiving most of its water and nutrient supply from rain) peat bog in the Jura Mountains (Switzerland), the Praz Rodet bog, along a full seasonal cycle. The aims of our study were (1) to investigate the eukaryotic molecular diversity in the peat bog environment and (2) to assess if this diversity varies seasonally.

Results and Discussion

In order to explore the protist diversity in the Praz-Rodet peat bog and its temporal variability, we analysed the diversity of eukaryotic SSU rRNA genes in five water samples taken during a full seasonal cycle between 2007 and 2008 (March 2007, June 2007, November 2007, January 2008 and May 2008). In addition, original clones obtained from peat-only samples (coded PRS) were also included in the phylogenetic trees as part of the overall peat bog diversity. In the framework of this study we define an operational taxonomic unit (OTU) as the taxonomic entity represented by a group of SSU rRNA gene sequences sharing >97% identity, and it is hence equivalent to phylotypes (environmental sequences) defined on the same criterion. This is a very conservative proxy for the eukaryotic species, since many described eukaryotic species differ by less than 1% at this marker. However, since we are covering the whole eukaryotic diversity, including a

wide variety of phyla, we prefer to use this criterion to facilitate the analyses, although this is likely an underestimation of the real specific diversity.

Overall Eukaryotic Diversity in Praz-Rodet Peat Bog

Our five clone libraries from peat bog water and three from peat yielded a total of 1070 positive clones (i.e. having incorporated an insert with the expected SSU rDNA fragment amplified), which corresponded to 132 different phylotypes including sequences ranging between 1310 bp (PR3_3E_134) and 2076 bp (PR3_3E_45). These length differences are due to length heterogeneities in the SSU rRNA gene. Clones derived from metazoans (15 phylotypes) or vascular plants (1 phylotype, closest BLAST hit *Sphagnum palustre* Y11370, similarity 99%) were not sequenced at full length because the study of their respective phylogenetic positions was beyond the scope of this paper (taxonomic affiliation of the metazoan sequences is given in Supplementary Table S1). As the clone libraries derived from peat-only samples (directly associated with the decomposing *Sphagnum* biomass) were largely dominated by metazoan sequences (up to more than 90% of all the clones), they were not further analysed, although clones derived from eukaryotic microbes which were exclusively found in these samples were fully sequenced and included in molecular phylogenetic analyses (26 phylotypes). We recovered a total of 745 positive clones corresponding to 90 phylotypes (Supplementary Table S2) from our filtered water clone libraries. These libraries yielded respectively 206, 91, 176, 142 and 130 positive clones and 33, 24, 29, 37 and 23 phylotypes, respectively, in the clone libraries derived from the samples collected in March 2007, June 2007, November 2007, January 2008 and May 2008.

Rarefaction analysis was performed on all clone libraries derived from peatbog water to estimate to what extent the diversity of the samples could be described with the number of clones analysed. Accumulation curves tended to flatten in all samples, indicating that the coverage of the diversity was satisfactory (Supplementary Fig. S1) and that diversity of clone libraries could thus be compared.

Libraries built with two different primer sets at the same sampling time were significantly different between each other with a probability of more than 95% except for January 2008, as indicated by LIBSHUFF (Singleton et al. 2001). A total of 52 and 66 phylotypes, representing respectively 58% and 73% of the total 90 phylotypes from the bog water samples were retrieved using, respectively, the primer combinations 1F/1498R and 82F/1498R. Of these, 28 phylotypes were retrieved using both primer sets (31% of the total) (Supplementary Table S2). This illustrates the advantage of using more than one set of primers to screen environmental eukaryotic molecular diversity (López-García et al. 2001; Von Wintzingerode et al. 1997). The biases appear if the composition of the clone libraries is carefully examined; for instance the clone PR3_4E_61 (an oomycete), which is one of the most frequent of the June 2007 clone library was only amplified with forward primer EK 82F in that sample, and never with primer EK 1F. Conversely, euglyphid sequences were never obtained with primer EK 82F (Supplementary Table S2). For these reasons, an equivalent number of clones were sequenced in each of the clone libraries built for each sample.

Full length sequences from each OTU were then included into different alignments following their phylogenetic affiliation and having taken into account the total number of sequences retrieved for each group: (1) Opisthokonta, (2) Amoebozoa, (3) Alveolata, (4) Heterokonta (=Stramenopiles) + Rhizaria, and (5) Chrysophyceae. Unclassified sequences and other less represented groups were included in a general eukaryotic alignment (6). All these alignments comprised both environmental sequences in GenBank that were closest to our sequences and culture/isolated cell-derived sequences that served as reference and covered a representative diversity of the respective groups. Partial sequences shorter than 2/3 of the total SSUrRNA gene length were excluded from the analysis.

The taxonomic distribution of our sequences was large, since we identified sequences belonging to all major eukaryotic super-groups: Opisthokonta, Heterokonta, Rhizaria, Alveolata, Excavata, Viridiplantae (=Archaeplastida), and Amoebozoa (Supplementary Fig. S2). Other groups of as yet more ambiguous classification were also represented, including the Centrohelida (Sakaguchi et al. 2007), and the Cryptophyta, the latter recently classified within the SAR eukaryotic supergroup (Burki et al. 2008) and proposed to be member of a new super-group, the Hacrobia (Okamoto et al. 2009). In addition, four OTUs (PR2_3E_18, PR4_4E_41, PR3_3E_89 and PR3_3E_134), which represented five different sequences, could not be assigned to any high-level eukaryotic taxon. These sequences branched robustly together in ML analysis (bootstrap value: 80%, see Fig 1); this

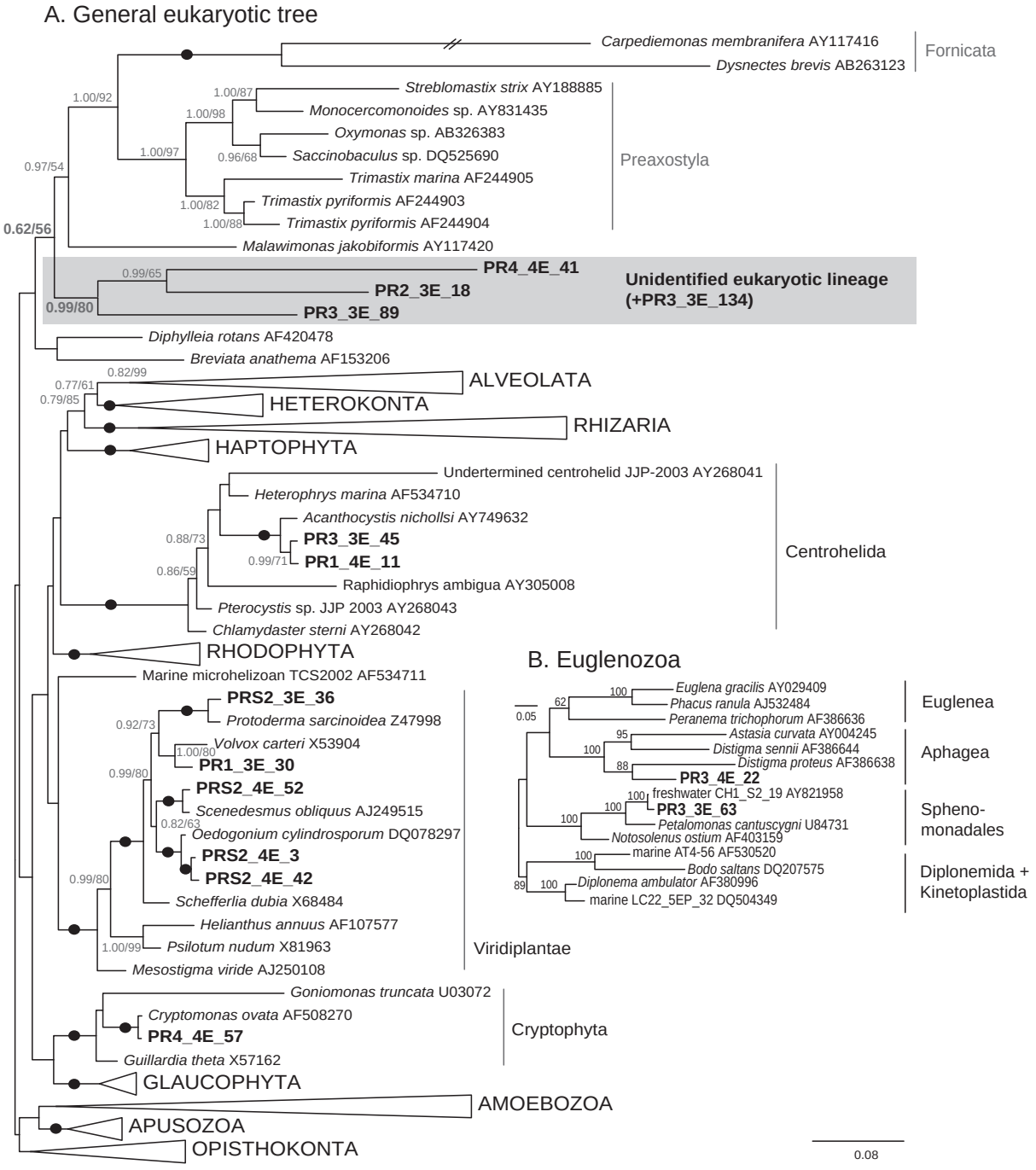

Figure 1. A: Bayesian phylogenetic tree of a broad sampling of eukaryotes, illustrating the position of several unidentified eukaryotic sequences found in the Praz-Rodet peat bog, and also of some clones belonging to groups which presented lower diversity (Centrohelida, Cryptophyta and Viridiplantae).The tree was based upon an alignment of 108 sequences and 1090 characters. Numbers indicate respectively Bayesian posterior probabilities (PP) and maximum likelihood bootstrap values (BV). Only values above 0.75 and 50% are shown here, except for the nodes at the base of the novel eukaryotic group and of the Fornicata + Preaxostyla + *Malawimonas* group. The root is placed between unikonts and bikonts. **B**: ML phylogenetic tree representing sequences from euglenids encountered in this study. Alignment comprised 15 sequences and 1188 characters. Numbers at nodes represent bootstrap values; all values below 50% were omitted. Clones obtained in this study are shown in bold.

was confirmed by Bayesian analysis (PP = 0.99). This clade branched within a group composed of *Malawimonas* + Fornicata + Preaxostyla (Excavata) in the ML and the Bayesian analyses. However, the statistical values which support this branching are very weak (PP = 0.62; BV = 56); similar results were obtained with or without the long-branch forming sequence PR3_3E_134. Its removal from the analysis did not change the topology of the tree (Fig. 1). Thus, we consider this group as an unidentified lineage, potentially belonging to excavates, which could also possibly be a novel, deep branching eukaryotic clade. Further work is needed to confirm these observations and to characterise this clade morphologically. The rest of the clone sequences branched robustly within described eukaryotic taxa; they are discussed here in reference to their phylogenetic affiliation.

Opisthokonta

The opisthokonts (excluding metazoans) appeared to be the most diverse eukaryotic high-level taxon, with 33 different OTUs of which 28 were affiliated to Fungi (Fig. 2 and Supplementary Table S2). Many clones derived from fungi were closely related to species typically isolated and cultured from peat bogs such as *Mortierella* sp., *Umbelopsis* sp., *Rhizophidium* sp., *Blittyomyces helicus*, *Cryptococcus* sp., *Candida* sp., *Mrakia* sp., *Penicillium purpurogenum*, and *Botrytis cinerea* (Thormann and Rice 2007; Thormann et al. 2003). In addition, other sequences were probably derived from symbiotic species, such as PR1_4E_47, which is closely related to *Symbiotaphrina* spp., a genus of arthropod symbionts of uncertain affiliation within the Ascomycota (Noda and Kodama 1996).

We also encountered sequences associated to a clade that may represent the deepest branching group of fungi. This highly diverse clade, from which the only isolate-derived sequences belong to the genus *Rozella*, has been tentatively named "Rozellida" (Lara et al. 2010)

In addition to Fungi, other opisthokont sequences corresponded to free-living organisms such as nucleariids and a choanoflagellate, and also organisms that were most likely parasites, such as the mesomycetozoans PR2_4E_07 and PR4_4E_73. The latter, as a member of the Eccrinales, was likely to be an arthropod parasite (Marshall et al. 2008).

Amoebozoa

Seven OTUs belonged to the Amoebozoa (Fig. 3 and Supplementary Table S2). Here again, some of our sequences were derived from organisms considered as typical inhabitants of peat bogs. The clone sequences PR3_4E_134 and PR4_4E_85 shared more than 99% similarity with the sequences of, respectively, *Hyalosphenia papilio* (EU392153) and *Nebela carinata* (EU392144). These two species of arcellinid testate amoebae are considered as indicators for the most oligotrophic conditions in peat bogs; when these conditions are met, they can be extremely common (Mitchell 2004; Mitchell et al. 2000). Microscopic observations confirmed the presence of these two species in the samples. A third arcellinid clone sequence, PR3_4E_25, branched with *Argynnia dentistoma.* Its position in the arcellinid tree (Fig. 4) shows that, unlike PR3_4E_134 and PR4_4E_85, it does not belong to the "core Nebelas", the only group of arcellinids which has been extensively characterised at the SSU rRNA gene level (Lara et al. 2008).

Two other amoebozoan clone sequences from peat (PRS2_3E_84 and PRS2_4E_19) branched robustly with the flagellated form *Phalansterium solitarium*. Members of genus *Phalansterium* and the morphologically related *Rhipidodendron* are also considered as characteristic inhabitants of oligotrophic peat bogs (Kreutz and Foissner 2006; Strüder-Kypke and Schönborn 1999).

Alveolata

Ciliates were the most diverse group within the alveolates, with 16 OTUs, including representatives from numerous classes such as Oligohymenophorea, Colpodea, Nassophorea and Spirotrichea (Fig. 5). Such a high diversity of ciliates is an expected result in freshwater and soil environments (Lara et al. 2007b; Šlapeta et al. 2005). Clone PR2_3E_66, closely related to *Uroleptus pisces*, could correspond to *U. caudatus*, described as one of the most characteristic species of acidic and oligotrophic peat bogs, reaching its highest density in the submerged *Sphagnum* habitat (Grolière 1977; Strüder-Kypke and Schönborn 1999). As in the case of *Rozella* spp., the joint analysis of environmental and isolate-derived SSU rRNA gene sequences helped to give a taxonomic identity to a clade represented only by environmental sequences; the environmental LKM63 clade (Lara et al. 2007b; Van Hannen et al. 1999) was identified as corresponding to the Cyrtolophosida as described in Dunthorn et al. (2008). Clone PR5_3E_39 branched within a clade that has been erroneously claimed to correspond to freshwater representatives of the marine parasitic alveolate clade Syndiniales (Di Giuseppe and Dini 2004;

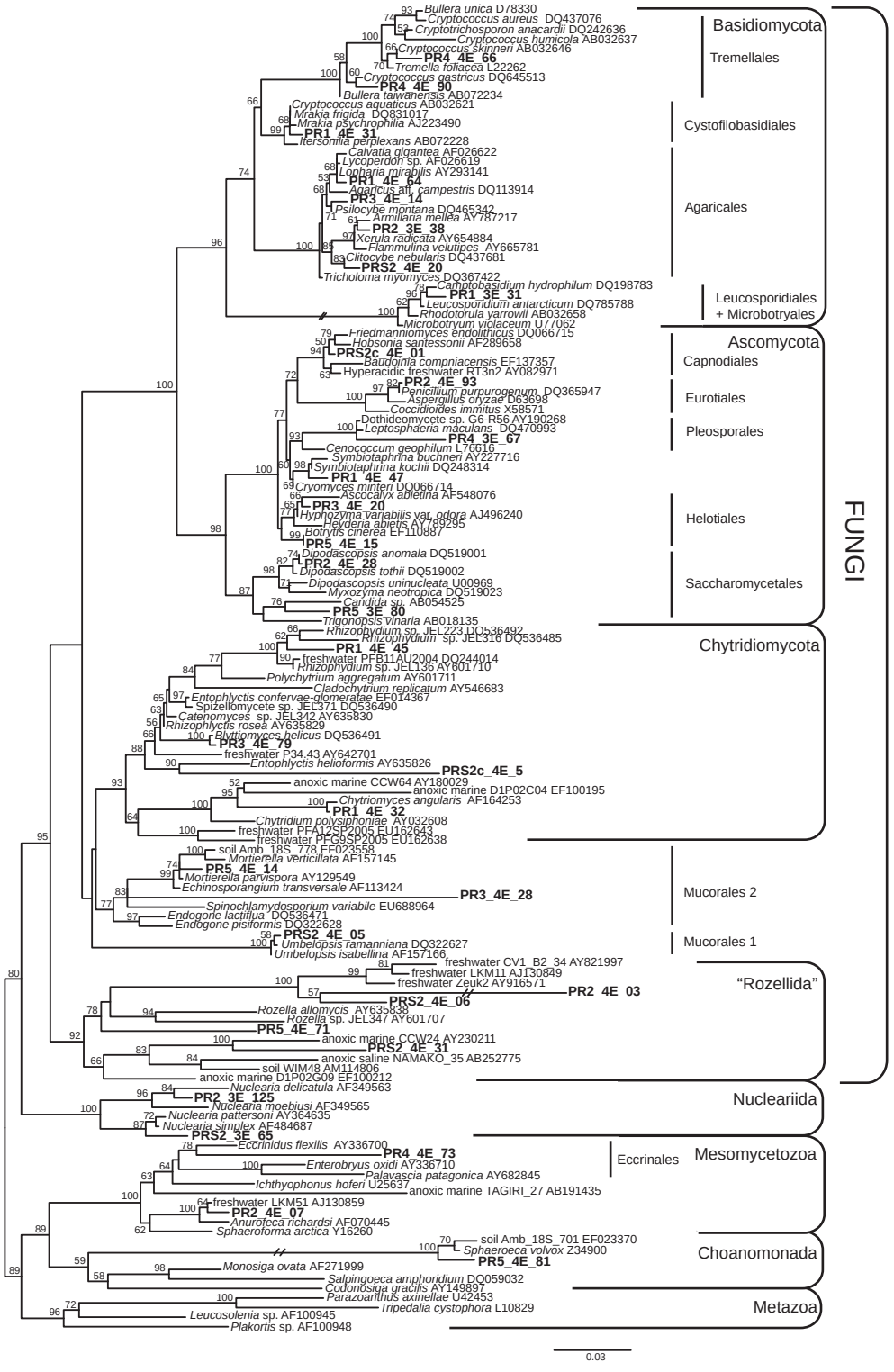

Figure 2. ML phylogenetic tree representing the position of the opisthokont sequences (excluding Metazoa) from Praz-Rodet, based on an alignment of 139 taxa and 1195 characters. Root is placed between Metazoa + Choanomonada + Mesomycetozoa and Fungi + "Rozellida" + Nucleariida. Numbers at nodes represent bootstrap values; all values below 50% were omitted. Clones obtained in this study are shown in bold. Branches presenting a double barred symbol are reduced to half for clarity.

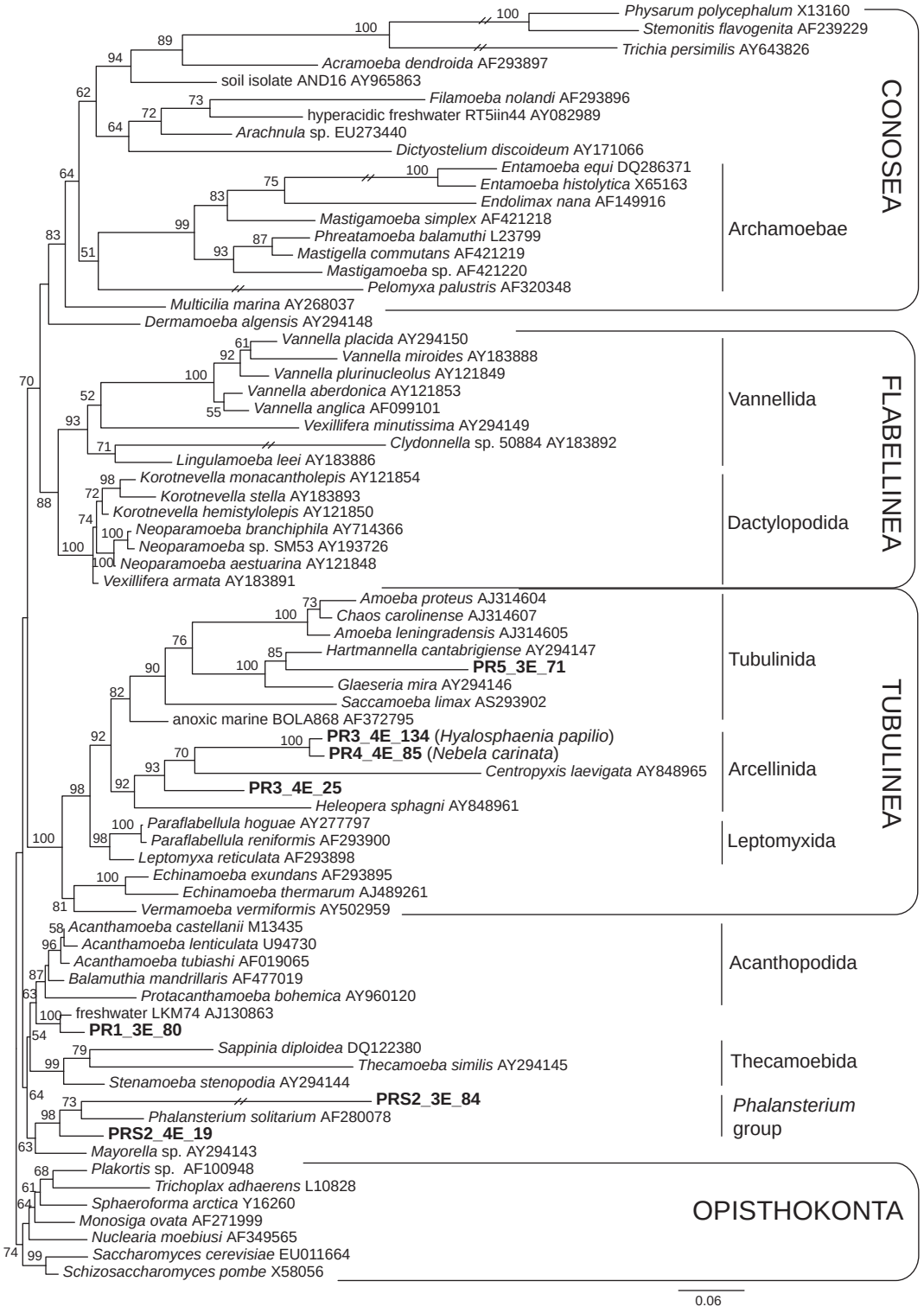

Figure 3. ML phylogenetic tree representing the position of the amoebozoan sequences from Praz-Rodet. The tree was constructed using an alignment of 74 sequences and 939 positions and was rooted with 7 representative opisthokont sequences. Numbers at nodes represent bootstrap values; all values below 50% were omitted. Clones obtained in this study are shown in bold. Branches presenting a double barred symbol are reduced to half length for clarity.

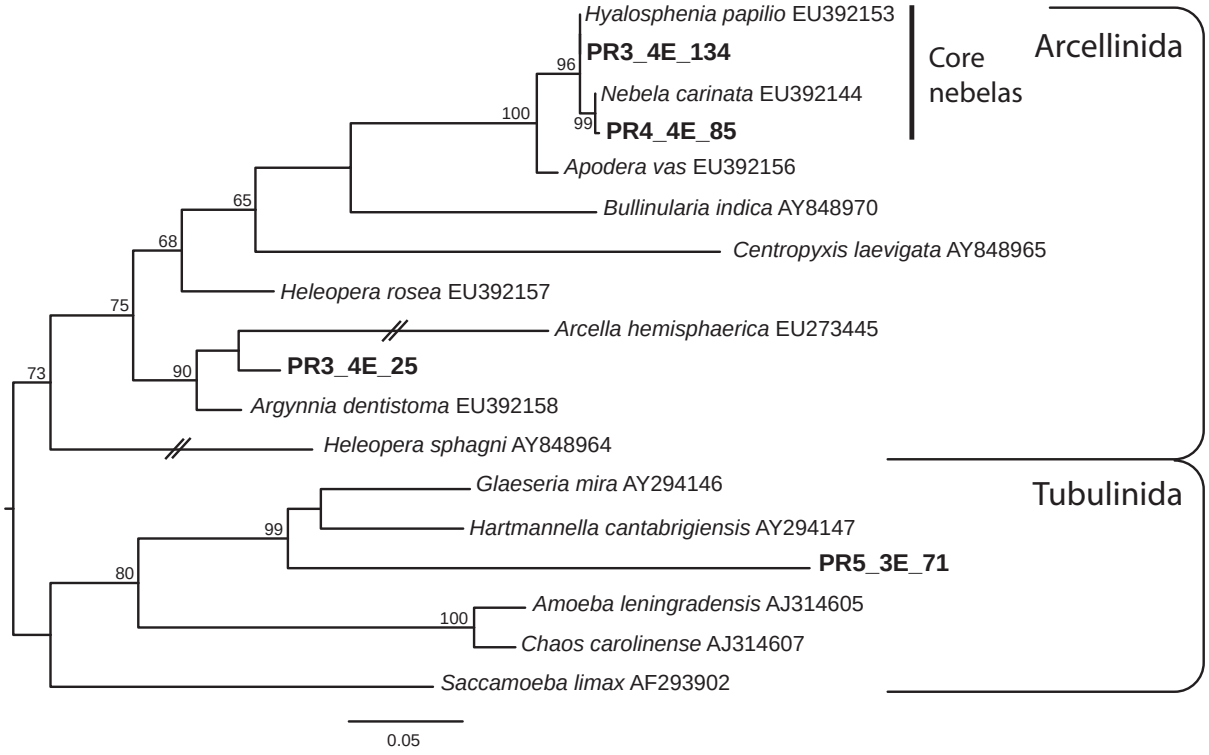

Figure 4. ML phylogenetic tree representing the position of the arcellinid sequences from this study. The tree was derived from an alignment of partial SSU rRNA sequences (18 sequences, 668 positions) and was rooted with 6 representative Tubulinida sequences. Numbers at nodes represent bootstrap values; all values below 50% were omitted. Clones obtained in this study are shown in bold.

Lefèvre et al. 2008). Our analysis showed that these sequences are related to *Cryptocaryon irritans*, a prostomatean ciliate ectoparasite of marine fishes (Wright and Colorni 2002), in agreement with Guillou et al. (2008). Moreover, we showed here that the species *Balanion masanensis* (Kim et al. 2007), a marine free-living species is also included within this clade (Fig. 5). This illustrates the wide ecological range of this ciliate group which includes both free-living and parasitic species, encountered from acidic and mineral-poor freshwater to marine environments. Despite the ecological width of the group, our clone was included in a very well supported clade of environmental sequences deriving exclusively from freshwater environments (Fig. 5).

We also identified a dinoflagellate clone that was closely related to the species *Gloeodinium montanum*, a freshwater species of uncertain affiliation, but which might be a representative of a larger undersampled freshwater clade (Logares et al. 2007). Clones affiliated to alveolate parasitic taxa were also found in our survey, namely one Perkinsea OTU (PR2_3E_17), two Coccidiasina (PR3_4E_33 and PR2_3E_116) and one Gregarinasina (PR1_4E_51). Perkinsea are regularly found in freshwater systems, where their flagellated free-living stage is part of the picoplankton assemblages (Lefèvre et al. 2008; Richards et al. 2005). One alveolate (PR2_3E_74) did not cluster with any previously known group (Fig. 5).

Heterokonta and Rhizaria

The largest diversity of heterokonts was found in the Chrysophyceae clade, with 13 different OTUs widely distributed in the tree (Fig. 6), and therefore one of the most diversified taxa in our clone libraries. Such a result is in agreement with similar findings in an environmental DNA survey of another freshwater system, the oligotrophic, dimictic Lake George (Richards et al. 2005). However, other less oligotrophic freshwater environments did not seem to host such great chrysophyte diversity (Berney et al. 2004; Lefèvre et al. 2008; Šlapeta et al. 2005). Chrysophyceae comprise both photosynthetic/mixotrophic and phagotrophic forms; it is difficult to predict the physiology of most of the organisms from which our clone sequences derived, because the photosynthetic ability has

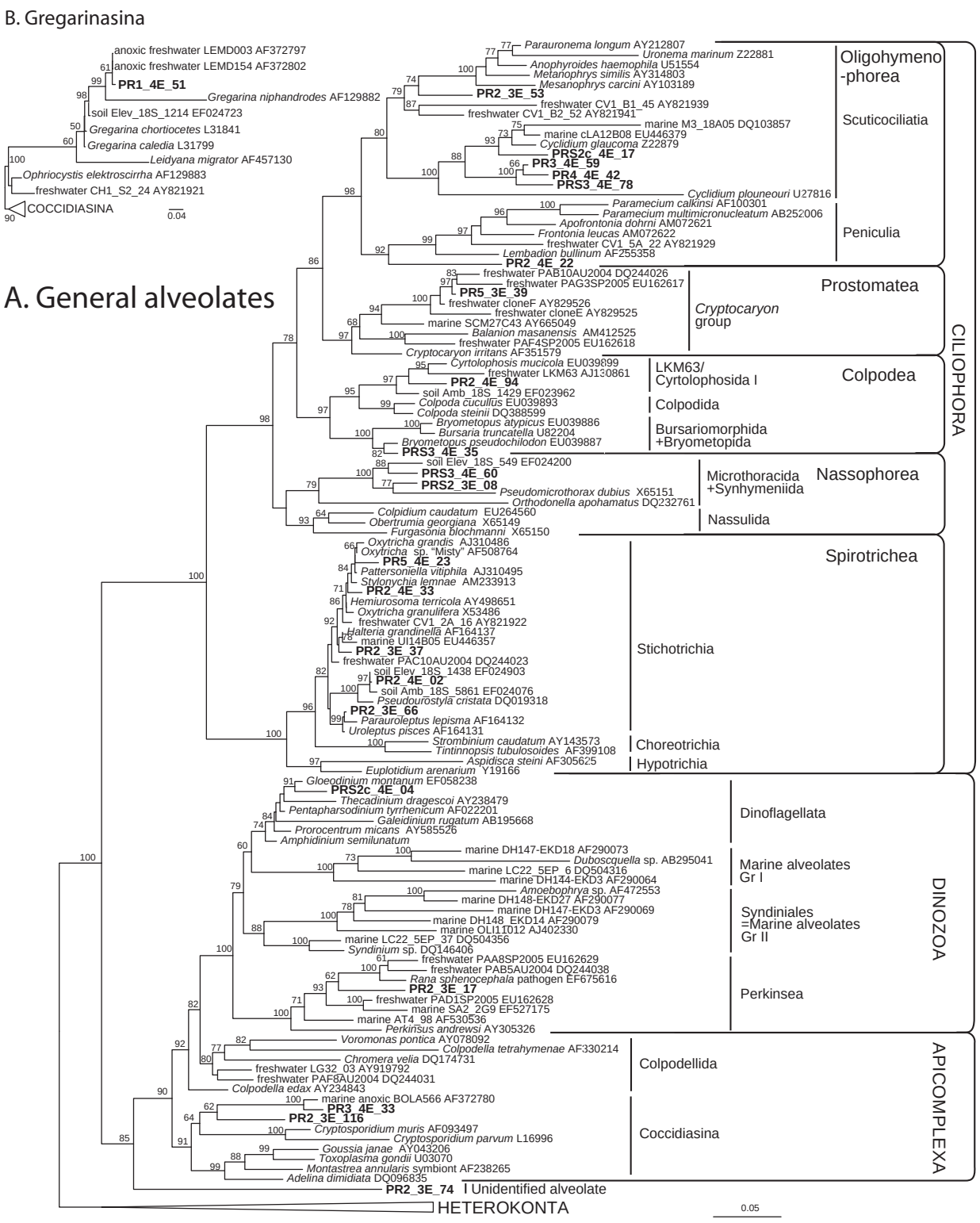

Figure 5. ML phylogenetic tree representing the position of A. general alveolate sequences from this study, based on an alignment of 130 taxa and 1233 characters, rooted with 13 heterokonts. B. the position of PR1_4E_51 within gregarines, based on an alignment of partial sequences (14 taxa, 798 characters) and rooted with 4 Coccidiasina. Clones obtained in this study are shown in bold.

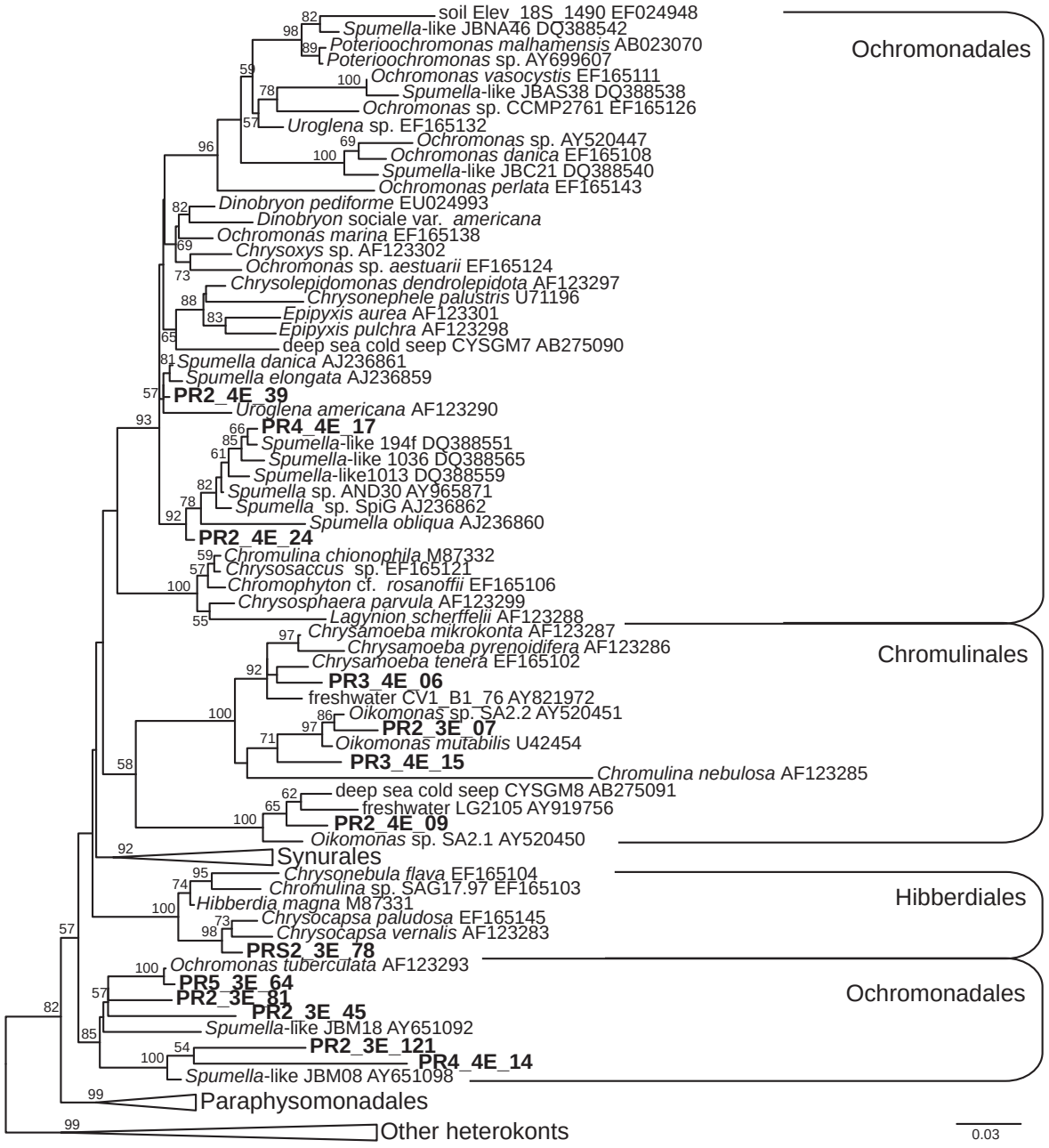

Figure 6. ML phylogenetic tree representing the position of the chrysophyte clones from this study (86 taxa, 1261 characters), rooted with 6 other heterokonts. Clones obtained in this study are shown in bold.

been lost independently several times in many lineages (Boenigk et al. 2005). Other photosynthetic heterokont groups were represented by two eustigmatophytes (PRS2_4E_40 and PRS2_3E_43) and one diatom (PR4_4E_05). The latter belonged to genus *Eunotia*, a genus typical of acidic environments (Flower 1986) (Fig. 7).

Non-photosynthetic groups were represented by Oomycetes (3 OTUs), uncultured MAST-12 clade (1 OTU), bicosoecids (1 OTU), and labyrinthulids (2 OTUs). The first group comprises sequences from saprotrophic or parasitic organisms. They have complex life cycles that include a motile flagellated stage (zoospore), which is planktonic. MAST-12 is a clade of uncultured heterokonts whose sequences were first identified in marine oxygen-depleted environments (Massana et al. 2004). These organisms were identified as small

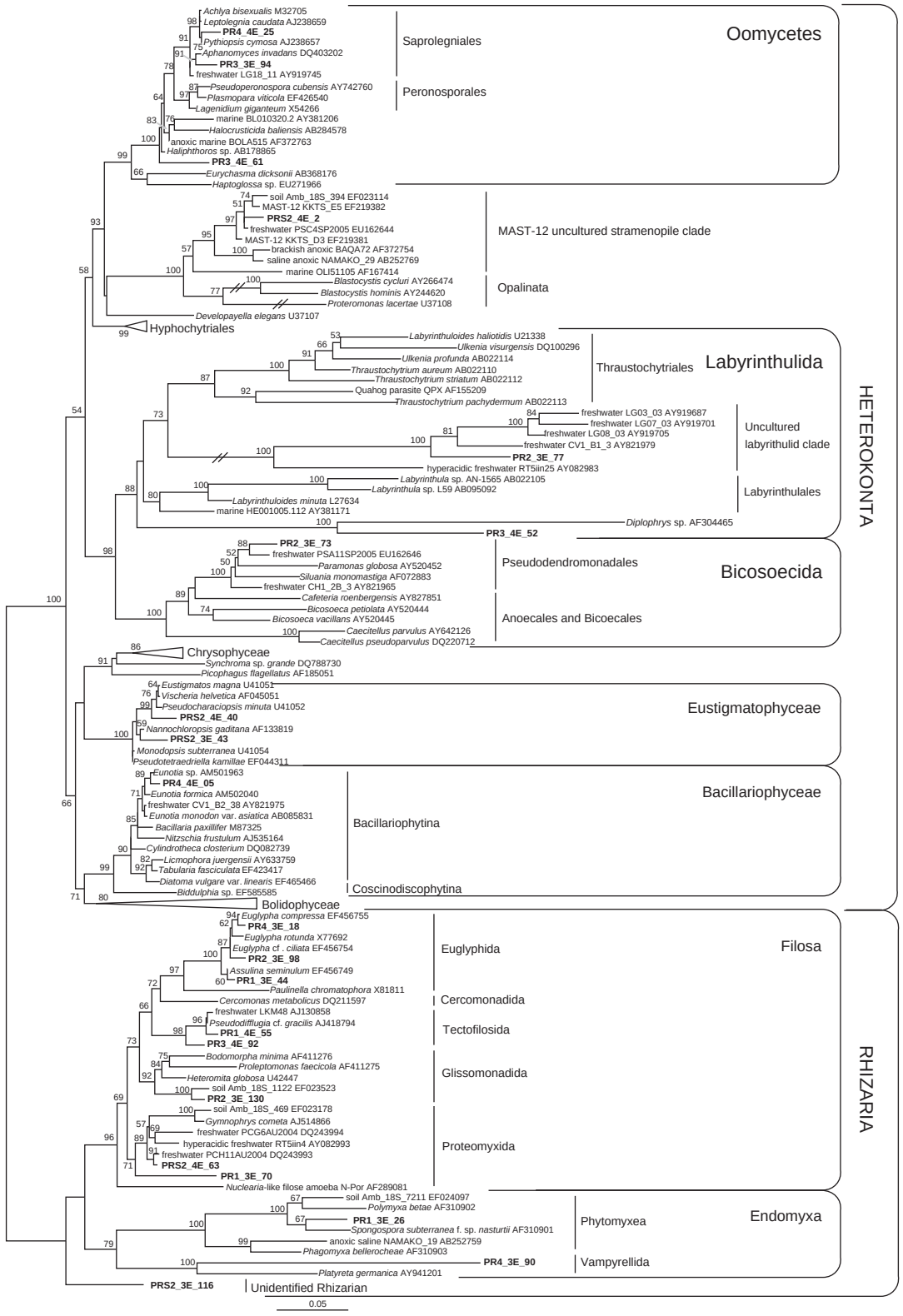

Figure 7. ML phylogenetic tree representing the position of the heterokont and rhizarian clones from this study (146 taxa, 1161 characters), with the root placed between these two groups. Clones obtained in this study are shown in bold. Branches presenting a double barred symbol are reduced to a quarter for clarity.

phagotrophic flagellates by combining fluorescent in situ hybridisation and scanning electron microscopy (Kolodziej and Stoeck 2007). In the meanwhile, other related sequences have been found in freshwater (Lefèvre et al. 2008) and soil, though being misidentified as a member of the apicomplexan parasitic group Eimeriidae (Lesaulnier et al. 2008). In our analysis, all MAST-12 clones from freshwater and soil form together a robust clade to which our clone belongs. It seems that there is only little overlap between freshwater/soil and marine genotypes, which suggests that the barrier imposed by salinity has been crossed relatively rarely during the evolution of this clade. A similar situation has been observed for many other protists such as cryptophytes (Shalchian-Tabrizi et al. 2008), dinoflagellates (Logares et al. 2007) and euglyphid testate amoebae (Heger et al. 2010). Bicosoecids are common members of the freshwater picoplankton; the low diversity observed in our samples contrasts with observations in another oligotrophic, though very different, freshwater system (Lake George), where a wide diversity was found (Richards et al. 2005). Labyrinthulids were represented by one OTU (PR2_3E_77) branching within a clade constituted only by environmental sequences, commonly found in freshwater systems (Amaral Zettler et al. 2002; Richards et al. 2005; Šlapeta et al. 2005). The other one clustered robustly with the enigmatic long branch-forming *Diplophrys* sp., confirming its position within labyrinthulids as suggested by Cavalier-Smith and Chao (2006).

Several OTU affiliated to Euglyphida were found in our study, in agreement with previous observations of peat bog environments based on light microscopy (Kreutz and Foissner 2006; Strüder-Kypke and Schönborn 1999). Clone PR4_3E_18 branched robustly (bootstrap 94%) with the species *Euglypha compressa*, which belongs to a cluster of moss-inhabiting testate amoebae (Lara et al. 2007a). Another clone (PRS2_4E_63) was related to the newly described rhizarian family Limnofilidae, a group of freshwater filamentous protists (Bass et al. 2009). In addition to Filosa, a sequence of a Phytomyxea (a clade of obligate plant parasites) was retrieved, as well as a vampyrellid (a group which contains some apparently exclusive eukaryote predators). Moreover, one clone from the peat (PRS2_3E_116) did not cluster with any known rhizarian group. Its affiliation to Rhizaria is nevertheless justified, as it branched robustly with other rhizarians in the general eukaryotic tree (BV = 100%; Fig. 1).

Other Taxa

One cryptophyte OTU (PR4_4E_57), closely related to *Cryptomonas ovata*, a species already reported in peat bogs (Kreutz and Foissner 2006), appeared in our survey. Three identical sequences of *Cryptomonas* nucleomorph were also obtained (not shown). Viridiplantae were represented in particular by sequences related to the genera *Oedogonium* (PRS2_4E_3 and PRS2_4E_42) and *Scenedesmus* (PRS2_4E_52), two ubiquitous freshwater genera. In addition, two centrohelid sequences related to genus *Acanthocystis*, and two euglenids also appeared. All these clones, which belong to groups whose diversity was lower in our study, are represented in Figure 1.

Relationship Between Protist Communities and Environmental Variables

The different physico-chemical variables measured (Supplementary Table S3) were typical for ombrotrophic *Sphagnum*-dominated peatlands: high DOC (>30 mg/l on average) and values for both total nitrogen (tot-N) (<1.25 mg/l on average) and NH_4^+ (<0.13 mg/l on average) were comparable to those observed in other European ombrotrophic peat bogs (Hoosbeek et al. 2002). High DOC concentration in peatlands is mainly due to the presence of recalcitrant humic acids while low N content is due to the fact that these ecosystems are fed only by atmospheric deposition and are therefore nutrient-poor.

Total nitrogen values were higher in the summer and fall samples (late June 2007, November 2007 and May 2008) than in winter and early spring samples. In contrast, NH_4^+ values were highest in early spring at snowmelt (March 2007), remained low in June 2007, November 2007 and January 2008 and increased again in May 2008. In contrast, total nitrogen was higher in the June 2007 and November 2007 samples. Such trends are in accordance with previous observation in other bog environments (Kilroy et al. 2008; Vitt et al. 1995). DOC has its lowest value in June 2007, and then increases gradually to reach its highest value in January 2008. March 2007 and May 2008 show intermediate values. TOC followed almost exactly these values.

As a measure of the biological variables that could be associated to eukaryotic dynamics, we counted prokaryotic cells from all the water samples. These counts showed lower values in January 2008 and comparable values for the other four

samples; these numbers increased with higher temperatures, being maximal in late spring and summer (Supplementary Table S3). Our prokaryotic counts were roughly one order of magnitude below the results of Gilbert et al., in other peat-bog systems (1998a). However, the environment they investigated (squeezed *Sphagnum fallax*) was a poor-fen, which is less oligotrophic than the Praz-Rodet peat bog and thus would be expected to contain a higher density of bacteria.

Seasonal Patterns of Microbial Eukaryotic Communities

We carried out a detrended correspondence analysis (DCA) in which the water chemistry variables and the prokaryotic cell counts were passively projected in the ordination space defined by the clone data (abundance of phylotypes in each sample). In the DCA ordination (Fig. 8) samples which appear close together in the ordination space have similar community structure while samples which are distant have different community structure. The environmental variables were then projected on this multivariate space. The length of the vectors representing environmental variables indicates how well the variable fits the multivariate ordination space. Thus Bacteria + Archaea counts and DOC are less correlated to seasonal changes in community structure than total N, NH_4^+ and temperature. The fall and winter samples clustered together in the ordination space (Fig. 8) while the May and June samples are quite different from this group and also from each other. Separate analyses on different subsets of the data showed that this pattern was mostly due to the 16 most abundant species, which each accounted for at least 5% of the total clones (data not shown).

Although a detailed assessment of the ecology and seasonal pattern of each taxonomic group is beyond the goals of this study, the following observations can be made from the position of clones and samples in the DCA ordination. Fungi and chrysophytes were the two most diverse taxa within the clones selected for this analysis (Supplementary Table 2) with respectively 14 and 11 different OTUs. Therefore an analysis of seasonal patterns of diversity may be pertinent for those two groups. Interestingly, for both groups the highest diversity was not observed during the summer period but in January. Chrysophyte diversity then declined during the spring and summer and increased again in November. The abundance of chrysophyte sequences represented 62% of all sequences retrieved in January, while they represented only 10% and 4%, of the total number of sequences in May and June, respectively (Supplementary Fig. S2). In the DCA ordination, they appear linked to low temperatures and low bacterial numbers (Fig. 8), which might be perceived as a contradiction with the bacterivorous/mixotrophic lifestyle of most of these organisms. A possible explanation could be the predation pressure during the most productive periods. Interestingly, the only two chrysomonad sequences which had a peak of abundance in the summer samples were most likely phototrophic: PR3_4E_06 branched within the genus *Chrysamoeba*, a very distinctive genus of amoeboid organisms which comprises only phototrophs (Preisig and Andersen 2002) and PR5_3E_64 sequence shared >99% identity to *Ochromonas tuberculata*, another photosynthetic chrysomonad (Fig. 6).

Fungal diversity (excluding “Rozellida”) was lowest in March and did not vary much the rest of the year. Clone PR1_4E_41 branched within the *Mrakia* clade, a known group of psychrophilic yeasts whose relatives have been found in glaciers and polar environments (De García et al. 2007). Interestingly, this clone was only found in the January sample. Sequences from multicellular fungi can derive from various sources, such as spores or mycelium fragments. Therefore, their relative abundance in the different libraries did not necessarily reflect their true abundance in the environment, and will not be discussed further.

Among the other groups, ciliate diversity showed a bimodal pattern with peaks in spring and fall (Supplementary Fig. S2). Some clone sequences were typically found in high numbers during winter conditions, such as for instance PR2_3E_66 (Supplementary Fig. S3). This clone appeared associated with many chrysomonad OTUs in the DCA ordination (Fig. 8). Given that members of genus *Uroleptus* are large protist predators (Foissner and Berger 1996) chrysophytes might be their main prey. In contrast, PR3_4E_59, affiliated to the genus *Cyclidium* (Fig. 5), a genus of small bacterivorous ciliates, appeared associated with high bacterial numbers found during the warm months. This clone represented up to 42% of the total number of sequences in May 2008 (Supplementary Fig. S3). Such a peak in abundance of small bacterivorous ciliates during the summer has been observed in a eutrophic freshwater system in Finland, where they were shown to be the most important regulators of bacterial populations (Zingel et al. 2007).

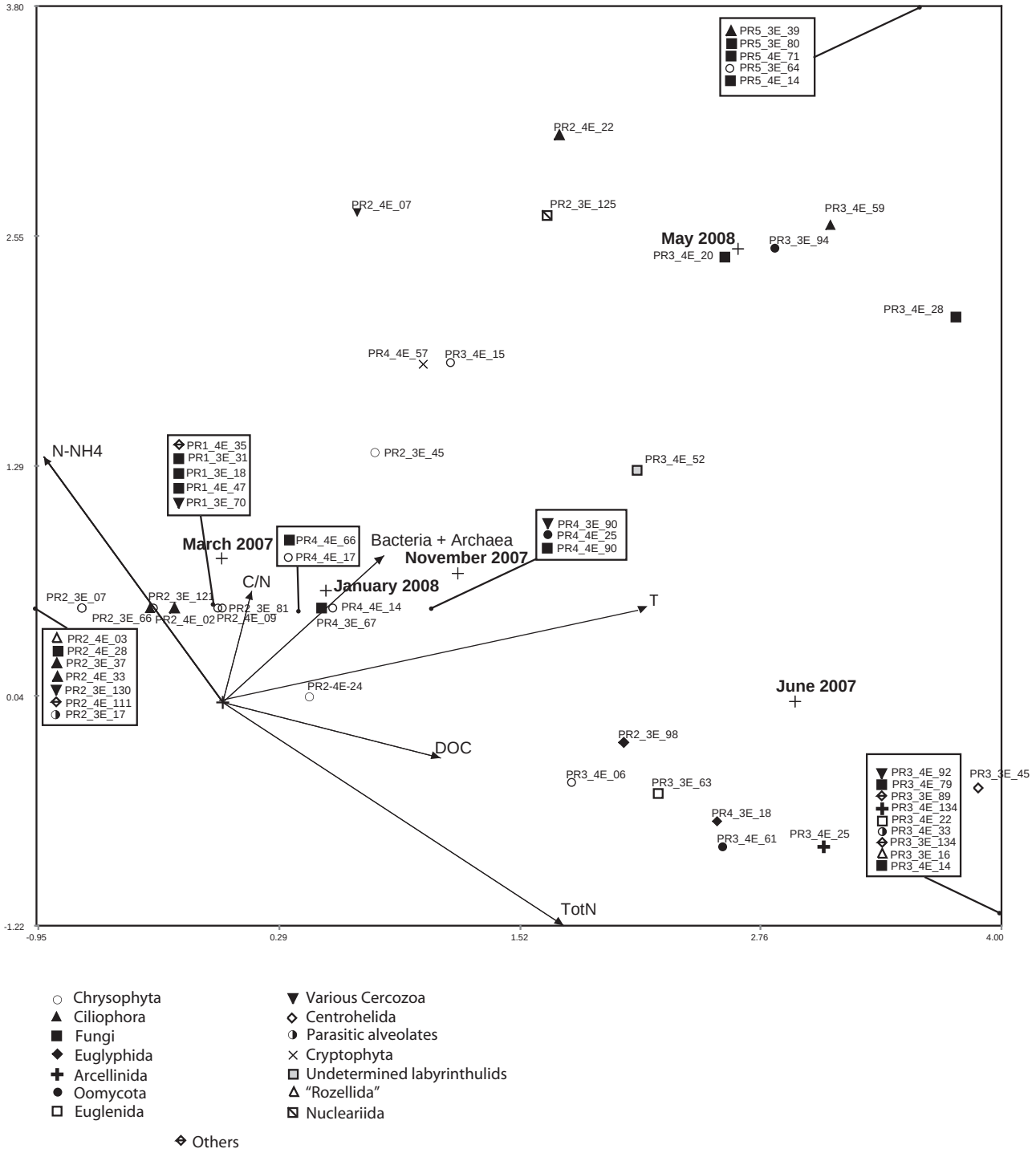

Figure 8. Detrended correspondence analysis (DCA) analysis of Eukaryotic clones obtained from water samples collected at the Praz-Rodet Bog (Swiss Jura). Environmental variables are projected passively in the ordination space. Only clones representing more than 0.97% of the total number of clones of the sample were included in the analysis. Metazoa were excluded. Symbols represent the taxonomic affiliation of the clones. The length of the vectors representing the environmental parameters has been multiplied five times for clarity. Symbols and clone codes figuring in boxes linked by a line to a point share the exact same coordinates in the ordination space.

The total amount of OTUs related to testate amoebae (Arcellinida and Euglyphida) was higher in abundance and diversity in the warmer months (summer and fall), in agreement with direct observations of *Sphagnum* mosses taken from the field (Heal 1964), although such differences are not always clear (Warner et al. 2007). Likewise, sphenomonad euglenids (PR3_3E_63) and centrohelid heliozoa followed the same trend (PR3_3E_45), as well as an unidentified stramenopile related to *Diplophrys* sp. (PR3_4E_52, Supplementary Fig. S3).

The clone sequence PR4_4E_57, affiliated to cryptomonads, was found in great numbers in the November sample, where it represented 19% of all sequences. This percentage fell in January and in May (respectively 4% and less than 1%). Such a peak of abundance may suggest a bloom, which is typical for *Cryptomonas* species population dynamics in peat bogs, although these events generally occur in summer (Kreutz and Foissner 2006). Another clone which might have followed a "bloom-like" dynamic was PR2_3E_17 (Supplementary Fig. S3), a member of the parasitic alveolate clade Perkinsea which represented up to 14% of all clones encountered in the March sample, and which did not appear elsewhere. Since Perkinsea are parasites of a variety of animal and protozoan hosts, it is likely that their population dynamics followed the variation of their host's abundance.

This study demonstrates that *Sphagnum* bogs are home to a broad diversity of eukaryotic microorganisms. Testate amoebae are one of the best known groups of eukaryotic microorganisms living in peatlands. With a maximum identified diversity of eight phylotypes it is likely, though, that only a fraction of the total diversity of testate amoebae was recorded. Indeed, an average number of 22 species has been reported in bog pool ecosystems with *Sphagnum cuspidatum* in the Jura Mountains (Mitchell et al. 1999). In the case of the arcellinids (three clone types), DNA extraction and PCR biases might explain their low level of detection, as is often the case with amoebozoans in general (Berney et al. 2004). Similar conclusions can be drawn for other groups as well which present long and/or divergent SSU rRNA genes, such as the other amoebozoans, the euglenids, the centrohelids or members of the putative new clade presented in this work. Group-specific amplification protocols would be required to assess the true diversity of individual taxa along temporal and spatial scales, which would improve considerably our understanding of peat bog microbial ecology.

Methods

Sampling: Water samples were taken at the Praz-Rodet peat bog, located in Switzerland (46°33'N; 06°10'E; altitude 1041m) at five different times: 08.03.2007 (ice melting, early spring), 25.06.2007 (summer conditions), 01.11.2007 (autumn conditions), 24.01.2008 (winter, under ca. 5 cm ice) and 08.05.2008 (late spring). Water (1000 ml) was sampled at the same location, in a pool where vegetation was dominated by half submerged *Sphagnum cuspidatum*, with presence of *Drosera rotundifolia, Scheuchzeria palustris* and *Carex limosa*. 500 ml of water were filtered through a 50 μm-pore mesh and then through a 0.2 μm-pore GTTP filter (Millipore). The 0.2 μm filter was used for DNA extraction and the 0.2 μm filtrate for water chemistry analysis. Filtered water was conserved at -20 °C for further chemical analyses. The remaining 500 ml of water was fixed with glutaraldehyde (4% final concentration) and stored at 4 °C for prokaryotic cell counts. All sequences derived from these water samples are coded PR1 to PR5. Additionally peat sediments were collected. Sequences derived from these peat sediment samples are coded PRS.

Water chemistry analyses: Temperature (T), total nitrogen (tot-N), total carbon (tot-C), dissolved organic carbon (DOC), and ammonium (NH_4) were measured on the water samples. Tot-N was measured using a Shimadzu TOC-V TNM1 analyser by thermal decomposition (chemiluminescence method). Tot-C and DOC were measured by the company CAR (Illkirch, France) by chemical oxidation of carbon and subsequent measuring of the released CO_2. NH_4 was determined by Colorimetric-Autoanalyzer Method by Dr. Luca Bragazza at the University of Ferrara (Italy). The C/N ratio was calculated from the tot-C and tot-N data.

Prokaryotic cell counts: Prokaryotic cells were stained with DAPI (4,6 diamino 2 phenylindol), filtered on 0.2 μm black membrane filters, and examined by epifluoresence microscopy. The image was recorded using a digital camera. Twenty to 30 random fields were counted for each sample, with an average of 9-14 cells per field. Counts were expressed as number of prokaryotic cells per millilitre of water.

DNA extraction, PCR amplification, cloning and sequencing: DNA was extracted from the filters as described elsewhere (Lara et al. 2009). In addition, peat was also collected for DNA extraction, which was performed using a MoBio Power Soil™ DNA extraction kit (Carlsbad, CA USA) following the manufacturer's instructions. In order to limit potential PCR biases, two forward eukaryotic domain-specific primers and one reverse were used, resulting in two sets of primers. These primers were, respectively, EK 1F (CTGGTTGATCCTGCCAG), EK 82F (GAAACTGCGAATGGCTC) and EK 1498R (CACCTACGGAAACCTTGTTA). Consequently, two clone libraries per sample were built. PCR reactions were carried out in 25 μl of reaction buffer containing 1 μl DNA template (~1-5 ng), 1.5 mM $MgCl_2$, dNTPs (10 nmol each), 20 pmol of each primer, and 1 U Taq DNA polymerase (Promega). PCR reactions were performed under the following conditions: 35 cycles (denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, extension at 72 °C for 2 min) preceded by 2 min denaturation at 94 °C, and followed by 8 min extension at 72 °C. Amplicons were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and transformed into *E. coli* TOP10' One Shot cells (Invitrogen) according to the manufacturer's instructions. Clone inserts were amplified with vector T7 and M13R primers, and inserts of the expected size were sequenced directly using either specific or vector primers by Cogenics (Meylan, France).

Sequence and phylogenetic analyses: Initially, we obtained partial SSU rRNA gene sequences, which were

trimmed for ambiguities and aligned using the software MUSCLE v. 3.6 (Edgar 2004). Groups of similar sequences (greater than 97% identity), which will be referred in this work as OTUs (Operational Taxonomic Units), were identified by building distance (neighbour joining) trees using the software Clustal X v.1.82 (Thompson et al. 1997). Representative sequences of the different OTUs were then selected and fully sequenced to obtain almost complete SSU rDNAs. Full-length sequences were checked for the presence of chimeras: the affiliation of each half of the suspicious sequences was determined using BLAST (Altschul et al. 1997). These sequences were also analysed with KeyDNAtools (Zimmerman et al., unpublished). Final phylogenetic trees were reconstructed using the maximum likelihood (ML) method with the software TREEFINDER (Jobb et al. 2004), under the GTR + Γ+ I model with four substitution rate categories. Nonparametric bootstrap analyses were performed with TREEFINDER using 1000 replicates. In parallel, the same datasets were analysed using RAxML (Stamatakis et al. 2008) to test if the general topology of the trees remained congruent, which they did, when the RAxML algorithm was applied. Ambiguous positions in the alignment were discarded from phylogenetic analyses. When the placement of a sequence in a tree required confirmation, we performed a Bayesian analysis on the same dataset, using the software Mr Bayes v. 3.1.2 (Ronquist and Huelsenbeck 2003) with the GTR + Γ+ I model of sequence evolution. They were repeatedly run from a random starting tree and run well beyond convergence. We used four hidden Markov chains, and up to 5 000 000 generations. The number of sequences and that of unambiguously-aligned positions used in the construction of the different trees were, respectively: (1) 139 and 1195 for the Opisthokonta, the root being placed between Fungi + Nucleariida and Mesomycetozoa + Choanomonadea + Metazoa; (2) 74 and 939 for the Amoebozoa, rooted with some Opisthokonta; (3) 130 and 1233 for the Alveolata, rooted with the Heterokonta; (4) 146 and 1161 for the Heterokonta plus Rhizaria, the root being placed between these two groups; (5) 86 and 1261 for the Chrysophyta, rooted with some closely related heterokonts; and (6) 108 and 1090 for the general eukaryote alignment, with the root placed between unikonts and bikonts. In this last tree, the analysis was performed with and without long-branch forming sequence PR3_3E_134, to ensure that the topology of the tree was not affected by long branch attraction. The eukaryotic taxa that formed too long branches were analysed separately; these were the Gregarinasina (14 sequences, 798 positions, rooted with the Coccidiniasina) and the Euglenida (15 sequences, 1188 positions, rooted with the Kinetoplastida + Diplonemida). In addition, Arcellinida were treated separately due to the fact that most sequences available in GenBank are significantly shorter than the whole SSU rRNA gene sequence (19 sequences, 668 positions, rooted with some Tubulinea). Alignments are available from the authors upon request. Sequences reported in this paper have been deposited in the GenBank database under accession numbers GQ330569-GQ330643 and FJ976648-FJ976650.

Statistical analyses: Rarefaction calculations as a measure of coverage of the clone libraries were performed using the software DOTUR (Schloss and Handelsman 2005). In addition, the composition of clone libraries from the same sample and different primer combinations were compared between themselves using the software LIBSHUFF v 0.96 (Singleton et al. 2001). We assessed the seasonal patterns of eukaryotic microbial communities and associated environmental variables by studying a reduced data set that included 59 phylotypes, which appeared with a minimum percentage of 0.97% of all clones, thus removing 32 rare phylotypes. We performed a detrended correspondence analysis (DCA) on this community data (phylotype absolute frequency data) in which the water chemistry variables and the prokaryotic cell counts were passively projected in the ordination space defined by the clone's data. The DCA was done using the software Canoco version 3.1 (Ter Braak 1988-1992).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2010.05.003.

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Supplementary Tables

Highly diverse and seasonally dynamic protist community in a pristine peat bog

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Table S1. Total numbers of metazoan clones per phylotype (defined with a 3% threshold) encountered in the libraries derived from peat bog water in relation with closest BLAST hit. Numbers at the bottom of the table represent total clone numbers.

Metazoa taxa	Number of sequences per date					First BLAST hit (% sequence similarity)
	3.2007	6.2007	11.2007	1.2008	5.2008	
Gastrotrich 1	0	105	10	0	21	Uncultured Chaetonotus-like gastrotrich clone CH1_S1_38 AY821982 (97%)
Gastrotrich 2	1	30	0	4	3	Uncultured Chaetonotus-like gastrotrich clone CH1_S1_38 AY821982 (97%)
Gastrotrich 3	0	1	0	0	0	Chaetonotus neptuni AM231774 (93%)
Turbellaria 1	0	7	5	0	0	Stenostomum bryophilum isolate K04_50 FJ384807 (99%)
Turbellaria 2	0	0	6	0	2	Eukaryote clone Elev_18S_5021 EF025005 (94%)
Nematod 1	36	3	0	0	0	Prismatolaimus cf. dolichurus JH-2004 isolate PriMDol1Z AY284727 (99%)
Nematod 2	0	0	1	0	0	Malaconothrus gracilis EF091424 (99%)
Nematod 3	0	0	0	0	1	Uncultured nematode clone SType 5 AJ875122 (98%)
Copepod 1 Harpacticoida	0	0	0	6	0	Attheyella crassa EU380307 (98%)
Copepod 2 Cyclopoida	0	1	0	0	0	Cyclops sp. UK-RJH-2004 AY626998 (98%)
Acari: Oribatid	0	0	1	1	0	Hydrozetes lacustris EU433987 (99%)
Rotifera: Bdelloid	0	5	0	0	0	Rotaria rotatoria 18S DQ089736 (99%)
Rotifera: Monogonont 1	0	11	0	0	0	Polyarthra remata DQ297716 (99%)
Rotifera: Monogonont 2	0	2	0	0	1	Uncultured metazoan clone Orly20 FJ577821 (97%)
Rotifera: Monogonont 3	0	9	3	2	6	Lepadella patella 18S AY218117 (99%)
total clones (unicell + metazoan)	280	265	202	155	130	

Table S2. Total numbers of microbial eukaryote clones per phylotype (defined with a 3% threshold) encountered in the libraries derived from peat bog water. Clones that were considered for DCA analysis are indicated, as well as the primer set which successfully amplified them. In addition, taxonomic identification, sequence length and percentage of similarity with closest BLAST hit are indicated.

Phylotype	Taxonomic identification	Number of sequences					Retained for DCA analysis	Amplified with primers 1F/1498R	Amplified with primers 82F/1498R	Sequence length	First BLAST hit (%sequence similarity)
		3.2007	6.2007	11.2007	1.2008	5.2008					
PR2_4E_24	Heterokonta; Chrysophyceae	8	1	40	30	0	yes	yes	yes	1446	Soil flagellate AND30 AY965871 (98%)
PR2_3E_81	Heterokonta; Chrysophyceae	4	0	4	14	0	yes	yes	yes	1445	Chrysophyceae sp. CCMP2296 (96%) EU247834
PR2_3E_07	Heterokonta; Chrysophyceae	40	0	0	3	0	yes	yes	yes	1441	Oikomonas sp. SA-2.2 AY520451 (98%)
PR2_3E_45	Heterokonta; Chrysophyceae	11	1	13	22	4	yes	yes	yes	1444	Chrysophyceae sp. CCCM41 EF165134 (94%)
PR5_3E_64	Heterokonta; Chrysophyceae	0	0	0	0	3	yes	yes	yes	1440	Ochromonas tuberculata AF123293 (99%)
PR2_4E_09	Heterokonta; Chrysophyceae	2	0	2	0	0	yes	no	yes	1445	Uncultured eukaryote clone: CYSGM-8 AB275091 (97%)
PR2_3E_121	Heterokonta; Chrysophyceae	3	0	0	7	0	yes	yes	yes	1466	Chromophyton cf. rosanoffii strain CCMP2751 EF165106 (92%)
PR3_4E_06	Heterokonta; Chrysophyceae	0	2	5	1	0	yes	yes	yes	1436	Chrysamoeba tenera strain UTCC273 (96%) EF165102
PR3_4E_15	Heterokonta; Chrysophyceae	20	2	0	1	6	yes	yes	yes	1436	Spumella-like flagellate JBAF35 AY651071 (96%)
PR4_4E_17	Heterokonta; Chrysophyceae	0	0	10	9	0	yes	yes	yes	1448	Spumella-like flagellate 194f DQ388551 (99%)
PR4_4E_14	Heterokonta; Chrysophyceae	0	0	3	1	0	yes	no	yes	1497	Uncultured marine eukaryote clone SA2_1F7 EF527128 (90%)
PR4_4E_05	Heterokonta; Bacillariophyceae; Eunotia	0	0	1	1	0	no	no	yes	1445	Eunotia sp. AT-73Gel02 AM501963 (98%)
PR3_4E_61	Heterokonta; Oomycetes, Unidentified Oomycetes	1	20	0	2	0	yes	no	yes	1466	Uncultured stramenopile clone BOLA320 AF372762 (91%)
PR4_4E_25	Heterokonta; Oomycetes, Saprolegniales	0	0	2	0	0	yes	no	yes	1461	Aphanomyces invadans DQ403202 (97%)
PR3_3E_94	Heterokonta; Oomycetes, Saprolegniales	0	1	0	1	6	yes	yes	yes	1453	Pythiopsis cymosa AJ238657 (98%)
PR4_4E_12	Heterokonta; bicosoecides; pseudodendromonadales	0	0	1	0	0	no	no	yes	525	Adriamonas peritocrescens AF243501 (95%)
PR2_4E_64	Heterokonta; bicosoecides; pseudodendromonadales	1	0	0	0	0	no	no	yes	207	Uncultured freshwater eukaryote clone LG33-04 AY919797 (94%)
PR2_3E_73	Heterokonta; bicosoecides; pseudodendromonadales	1	0	0	0	0	no	yes	no	1444	Uncultured stramenopile clone PSA11SP2005 EU162646 (94%)
PR2_3E_77	Heterokonta; labyrinthulida (environm. Group RT5iin25)	1	0	0	1	0	no	yes	no	1488	Uncultured labyrinthulid clone CV1_B1_3 AY821979 (86%)
PR3_4E_52	Heterokonta; labyrinthulida; undetermined labyrithulids	4	9	3	3	14	yes	yes	yes	1574	Uncultured eukaryote clone 528-O7 EF586082 (92%)
PR2_3E_66	Alveolata; Ciliophora; Intramacronucleata; Spirotrichea	44	0	4	8	0	yes	yes	yes	1435	Uroleptus pisces AF164131 (99%)
PR2_4E_02	Alveolata; Ciliophora; Intramacronucleata; Spirotrichea	3	0	1	0	0	yes	yes	yes	1438	Environmental sample clone Elev_18S_1438 EF024903 (99%)
PR2_3E_37	Alveolata; Ciliophora; Intramacronucleata; Spirotrichea	6	0	0	0	0	yes	yes	yes	1438	Halteria grandinella AF194410 (98%)
PR2_4E_33	Alveolata; Ciliophora; Intramacronucleata; Spirotrichea	4	0	0	0	0	yes	yes	yes	1437	Hemiurosoma terricola AY498651 (98%)

Table S2. Total numbers of microbial eukaryote clones per phylotype (defined with a 3% threshold) encountered in the libraries derived from peat bog water. Clones that were considered for DCA analysis are indicated, as well as the primer set which successfully amplified them. In addition, taxonomic identification, sequence length and percentage of similarity with closest BLAST hit are indicated.

Phylotype	Taxonomic identification	Number of sequences					Retained for DCA analysis	Amplified with primers 1F/1498R	Amplified with primers 82F/1498R	Sequence length	First BLAST hit (%sequence similarity)
		3.2007	6.2007	11.2007	1.2008	5.2008					
PR5_4E_23	Alveolata; Ciliophora; Intramacronucleata; Spirotrichea	0	0	0	0	1	no	no	yes	1434	Oxytricha sp. Misty AF508764 (97%)
PR3_4E_59	Alveolata;Ciliophora; Oligohymenophorea; Scuticociliatia	0	6	10	0	54	yes	yes	yes	1440	Cyclidium glaucoma EU032356 (91%)
PR2_3E_53	Alveolata;Ciliophora; Oligohymenophorea; Scuticociliatia	1	0	0	0	0	no	yes	no	1424	Uncultured marine eukaryote clone NA2_2E2 EF526761 (92%)
PR4_4E_42	Alveolata;Ciliophora; Oligohymenophorea; Scuticociliatia	0	0	0	1	0	no	no	yes	1447	Cyclidium glaucoma EU032356 (91%)
PR2_4E_22	Alveolata; Ciliophora; Oligohymenophorida; Peniculida	1	0	0	0	2	yes	no	yes	1396	Uncultured marine eukaryote clone M2_18E08 DQ103844 (88%)
PR2_4E_94	Alveolata; Ciliophora; Colpodida; Cyrtolophosida	1	0	1	0	0	no	no	yes	1417	Environmental sample clone Amb_18S_1429 EF023962 (94%)
PR5_3E_39	Alveolata; Ciliophora; Prostomatea	0	0	0	0	8	yes	yes	yes	1434	"Uncultured Amoebophrya" clone F AY829526 (97%)
PR1_4E_51	Alveolata; Apicomplexa; Gregarinida	0	0	0	1	0	no	no	yes	1417	Uncultured eukaryote clone LEMD012 AF372799 (97%)
PR3_4E_33	Alveolata; Apicomplexa;	0	1	0	0	0	no	no	yes	1435	Uncultured alveolate clone BOLA566AF372780 (96%)
PR2_3E_1 16	Alveolata; Apicomplexa;	1	0	0	0	0	no	yes	no	1453	Uncultured marine alveolate clone PAF8AU2004 DQ244031 (89%)
PR2_3E_17	Alveolata; Perkinsea	28	0	0	0	0	yes	yes	yes	1447	Uncultured alveolate clone AT4-98AF530536 (92%)
PR2_3E_74	Alveolata Incertae sedis	1	0	0	0	0	no	yes	no	1434	Ichthyophonida sp. LKM51 AJ130859 (84%)
PR2_3E_98	Rhizaria; Cercozoa; Filosa; Euglyphida; Euglyphidae	2	4	4	0	1	yes	yes	no	1471	Euglypha cf .ciliata EF456754 (98%)
PR4_3E_18	Rhizaria; Cercozoa; Filosa; Euglyphida; Euglyphidae	0	22	14	1	0	yes	yes	no	1469	Euglypha compressa EF456755 (99%)
PR1_3E_44	Rhizaria; Cercozoa; Filosa; Euglyphida; Assulinidae	0	0	0	1	1	no	yes	no	1469	Assulina seminulum EF456749 (99%)
PR1_3E_70	Rhizaria; Cercozoa; Filosa; Proteomyxidae	0	0	0	4	0	yes	yes	no	1913	Uncultured cercozoan isolate DB-2703-11 EU567236 (96%)
PR1_4E_55	Rhizaria; Cercozoa; Filosa; Tectofilosida	0	0	0	1	0	no	no	yes	1454	Pseudodifflugia cf. gracilis AJ418794 (97%)
PR3_4E_92	Rhizaria; Cercozoa; Filosa; Tectofilosida	0	1	0	0	0	no	no	yes	1472	Protaspis obliqua isolate 2 FJ824122 (92%)
PR2_3E_130	Rhizaria; Cercozoa; Filosa; Glissomonadina	2	0	0	0	0	yes	yes	no	1467	Cercozoa clone Amb_18S_1122 EF023523.1 (97%)
PR4_3E_90	Rhizaria; Cercozoa; Endomyxa; Phytomyxea;Vampyrellidae	0	0	2	0	0	no	yes	no	1457	Uncultured eukaryote clone LEMD004 (87%)
PR1_3E_26	Rhizaria; Cercozoa; Endomyxa; Phytomyxea; Plasmodiophorida	0	0	0	1	0	no	yes	no	1480	Uncultured plasmodiophorid clone D20 EU910610 (96%)
PR4_4E_73	Opisthokonta; Ichthyophonida; Eccrinales	0	0	1	0	0	no	yes	no	1465	Eccrinidus flexilis isolate SP A11C45 AY336700 (90%)
PR2_4E_07	Opisthokonta; Ichthyophonida	3	0	0	0	1	no	yes	yes	1450	Ichthyophonida sp. LKM51 AJ130859 (97%)
PR5_4E_81	Opisthokonta; Choanoflagellida; Codonosigidae	0	0	0	0	1	no	no	yes	1435	Sphaeroeca volvox Z34900 (97%)

Table S2. Total numbers of microbial eukaryote clones per phylotype (defined with a 3% threshold) encountered in the libraries derived from peat bog water. Clones that were considered for DCA analysis are indicated, as well as the primer set which successfully amplified them. In addition, taxonomic identification, sequence length and percentage of similarity with closest BLAST hit are indicated.

Phylotype	Taxonomic identification	Number of sequences					Retained for DCA analysis	Amplified with primers 1F/1498R	Amplified with primers 82F/1498R	Sequence length	First BLAST hit (%sequence similarity)
		3.2007	6.2007	11.2007	1.2008	5.2008					
PR2_3E_125	Opisthokonta; Nucleariidae	1	0	4	0	4	yes	yes	yes	1538	Nuclearia thermophila AB433328 (97%)
PR2_4E_03	Opisthokonta; "Rozellida"	4	0	0	0	0	yes	yes	yes	1415	Uncultured eukaryote clone Ivry06 FJ577810 (87%)
PR5_4E_71	Opisthokonta; "Rozellida"	0	0	0	0	2	no	no	yes	1450	Rhizophlyctis rosea isolate AFTOL-ID 43 AY635829 (90%)
PR3_3E_16	Opisthokonta; Fungi; Basal fungal lineages	0	1	0	0	0	yes	yes	no	593	Lepidostroma akagerae voucher Ertz 7673 FJ171730 (85%)
PR1_3E_18	Opisthokonta; Fungi; Zoopagomycotina	0	0	0	4	0	yes	yes	no	300	Uncultured fungus clone T6_II_2b_09 EF629013 (91%)
PR5_4E_14	Opisthokonta; Fungi; Mucoromycotina; Mucorales 2	0	0	0	0	5	yes	no	yes	1448	Mortierella parvispora AY129549 (99%)
PR3_4E_28	Opisthokonta; Fungi; Mucoromycotina; Mucorales 2	0	2	0	0	4	yes	no	yes	1478	Basidiobolus ranarum isolate AFTOL-ID 301 AY635841 (87%)
PR1_4E_32	Opisthokonta; Fungi; Chytridiomycota; Chytridiales	0	0	0	1	0	no	no	yes	1447	Chytriomyces angularis isolate AFTOL-ID 630 AF164253 (99%)
PR1_4E_45	Opisthokonta; Fungi; Chytridiomycota; Rhizophydiales	0	0	0	1	0	no	no	yes	1450	Rhizophydium sp. JEL223 isolate AFTOL-ID 2007 DQ536492 (96%)
PR3_4E_79	Opisthokonta; Fungi; Chytridiomycota	0	1	0	0	0	yes	no	yes	1450	Blyttiomyces helicus isolate AFTOL-ID 2006 DQ536491 (99%)
PR5_4E_15	Opisthokonta; Fungi; Ascomycota; pezizomycotina; Helotiales	0	0	0	0	1	yes	no	yes	1452	Botryotinia fuckeliana strain DSM877 (=Botropsis cinerea) EF1 10887 (100%)
PR3_4E_20	Opisthokonta; Fungi; Ascomycota; pezizomycotina; Helotiales	0	1	1	1	7	yes	no	yes	1452	Hyphozyma variabilis var. odora AJ496240 (99%)
PR2_4E_93	Opisthokonta; Fungi; Ascomycota; pezizomycotina; Eurotiales	1	0	0	0	0	no	no	yes	1461	Penicillium purpurogenum strain HS-A82 DQ365947 (99%)
PR4_3E_67	Opisthokonta; Fungi; Ascomycota; pezizomycotina; dothideales	0	0	2	1	0	yes	yes	yes	1452	Phoma exigua var. exigua clone 26-7 EU342890 (96%)
PR1_4E_47	Opisthokonta; Fungi; Ascomycota; pezizomycotina; leotideomycetes; dothideales	0	0	0	4	0	yes	yes	yes	1452	Symbiotaphrina kochii isolate AFTOL-ID 1902 FJ176833 (98%)
PR2_4E_28	Opisthokonta; Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Lipomycetales	2	0	0	0	0	yes	no	yes	1460	Dipodascopsis (=Babjevia) anomala strain NRRL Y-7931 DQ519001 (99%)
PR5_3E_80	Opisthokonta; Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Lipomycetales	0	0	0	0	2	yes	yes	yes	1451	Candida sp. BG02-7-15-009-2-1 AY520194 (95%)
PR1_3E_31	Opisthokonta; Fungi; Basidiomycota; Pucciniomycotina; Microbotryomycetes; Leucosporidiales	0	0	0	2	0	yes	yes	yes	1458	Leucosporidium antarcticum isolate AFTOL-ID 1550 DQ785788 (99%)
PR1_4E_31	Opisthokonta; Fungi; Basidiomycota; Agaricomycotina;Tremellomycetes; Cystofilobasidiales	0	0	0	1	0	no	no	yes	1450	Mrakia frigida AFTOL-ID 1818 DQ831017 (99%)
PR4_4E_66	Opisthokonta; Fungi; Basidiomycota; Agaricomycotina;Tremellomycetes; Tremellales	0	0	2	2	0	yes	no	yes	1452	Cryptococcus skinneri AB032646 (99%)
PR4_4E_90	Opisthokonta; Fungi; Basidiomycota; Agaricomycotina;Tremellomycetes; Tremellales	0	0	2	0	0	yes	yes	yes	1450	Bullera taiwanensis AB072234 (98%)
PR1_4E_64	Opisthokonta; Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricales; Lycoperdaceae	0	0	0	1	0	no	no	yes	1455	Lopharia mirabilis AY293141 (99%)
PR3_4E_14	Opisthokonta; Fungi; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricales; Strophariaceae	0	1	0	0	0	yes	no	yes	1456	Psilocybe montana isolate AFTOL-ID 820 DQ465342 (99%)
PR2_3E_38	Opisthokonta; Fungi; Basidiomycota;Agaricomycotina; Agaricomycetes; Agaricales; Armillaria group	1	0	0	0	0	no	yes	no	1878	Oudemansiella radicata AY654884 (93%)

Table S2. Total numbers of microbial eukaryote clones per phylotype (defined with a 3% threshold) encountered in the libraries derived from peat bog water. Clones that were considered for DCA analysis are indicated, as well as the primer set which successfully amplified them. In addition, taxonomic identification, sequence length and percentage of similarity with closest BLAST hit are indicated.

Phylotype	Taxonomic identification	Number of sequences					Retained for DCA analysis	Amplified with primers 1F/1498R	Amplified with primers 82F/1498R	Sequence length	First BLAST hit (%sequence similarity)
		3.2007	6.2007	11.2007	1.2008	5.2008					
PR3_3E_45	Centrohelida; Acanthocystidae	0	5	0	0	1	yes	yes	yes	2076	Acanthocystis spinifera AY749630 (95%)
PR1_4E_1 1	Centrohelida; Acanthocystidae	0	0	0	1	0	no	no	yes	2069	Acanthocystis sp. Oxford8 AY749626 (87%)
PR3_4E_25	Amoebozoa; Tubulina; Arcellinida; unidentified Arcellinid	0	3	1	0	0	yes	no	yes	1541	Argynnia dentistoma EU392158 (91%)
PR3_4E_134	Amoebozoa; Tubulina; Arcellinida; core Nebelas; *Hyalosphenia*	0	1	0	0	0	yes	no	yes	1958	Hyalosphenia papilio from Sweden EU392153 (99%)
PR4_4E_85	Amoebozoa; Tubulina; Arcellinida; core Nebelas; *Nebela*	0	0	1	0	0	no	no	yes	1989	Nebela carinata from Sweden EU392143 (99%)
PR5_3E_71	Amoebozoa; Tubulina; Euamoebida; Hartmanellidae	0	0	0	0	1	no	yes	no	1577	Hartmannella cantabrigiensis AY294147 (81%)
PR2-3E-03	Amoebozoa; *Phalansterium* clade	1	0	0	0	0	no	yes	no	1442	Environmental clone Elev_18S_1278 AF386643 EF024776 (94%)
PR1_3E_80	Amoebozoa; LKM74 environmental clade	0	0	0	1	0	no	yes	no	1581	Environmental clone Elev_18S_1517 EF024971 (87%)
PR3_3E_63	Excavata; Euglenozoa; Euglenida; Sphenomonadales	0	2	7	0	0	yes	yes	no	1716	Uncultured sphenomonad euglenozoan clone CH1_S2_19 AY821958 (96%)
PR3_4E_22	Excavata; Euglenozoa; Euglenida; Aphagea	0	1	0	0	0	no	no	yes	1814	Distigma elegans SAG 224.80 (79%)
PR1_4E_35	Archaeplastida; Viridiplantae; Chlorophyta; Trebouxiophyceae; Microthamniales	0	0	0	2	0	yes	no	yes	513	Trebouxia asymmetrica Z21553 (99%)
PR2_4E_1 11	Archaeplastida; Viridiplantae; Chlorophyta; Trebouxiophyceae; Microthamniales	2	0	0	0	0	yes	no	yes	513	Chloromonas sp. 047-99 AF514406 (99%)
PR1_3E_30	Archaeplastida; Viridiplantae; Chlorophyta; Chlorophyceae; Chlamydomonadales	0	0	0	1	0	no	yes	no	1448	Chloromonas reticulata strain SAG 29.83 AJ410448 (97%)
PR4_4E_57	Cryptophyta; Cryptomonadina	0	0	34	5	1	yes	yes	yes	1432	Cryptomonas sp. M1634 AM901361 (99%)
PR4_4E_41	EK Incertae sedis	0	0	1	0	0	no	no	yes	1485	Cyanidium caldarium strain:55B AB091232 (76%)
PR3_3E_134	EK Incertae sedis	0	2	0	0	0	yes	yes	no	1310	Uncultured eukaryote clone R T5iin3 AY082996 (80%)
PR2_3E_18	EK Incertae sedis	1	0	0	0	0	no	yes	no	1386	Uncultured eukaryote clone R T5iin3 AY082996 (79%)
PR3_3E_89	EK Incertae sedis	0	1	0	0	0	no	yes	no	1371	Environmental sample clone Elev_18S_792 EF024493 (80%)
total Unicell		206	91	176	142	130					

Table S3. Values obtained for the different environmental parameters measured in peat bog water at the different sampling times. TN= total nitrogen, N-NH4= total ammonium, DOC =dissolved organic carbon, TOC =total organic carbon, Temp= water temperature, and Bact/ml = bacterial cells per ml water.

Date	TN (mg/l)	N-NH4 (mg/l)	DOC (mg/l)	TOC (mg/l)	Temp °C	Bact / ml
March 2007	0.903	0.232	24.9	25.1	2.0	9.08E+04
June 2007	1.780	0.084	10.1	10.3	16.5	1.36E+05
November 2007	1.674	0.059	34.3	34.9	6.5	1.27E+05
January 2008	0.737	0.092	55.3	57.2	1.0	1.21E+05
May 2008	1.102	0.178	31.9	32.5	20.0	1.39E+05

Supplementary Figures

Highly diverse and seasonally dynamic protist community in a pristine peat bog

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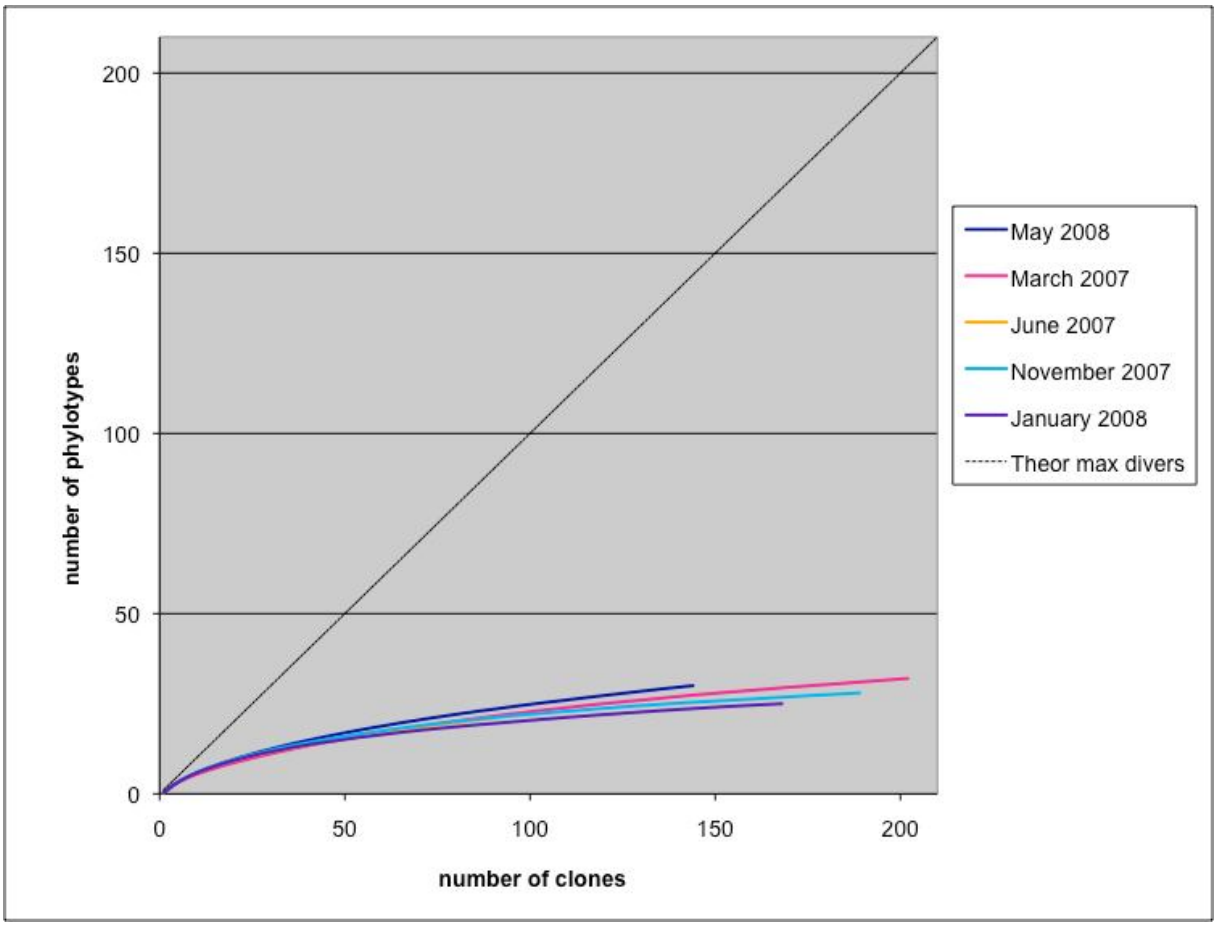

Figure S1: Rarefaction analysis performed on the clone libraries derived from peat bog water

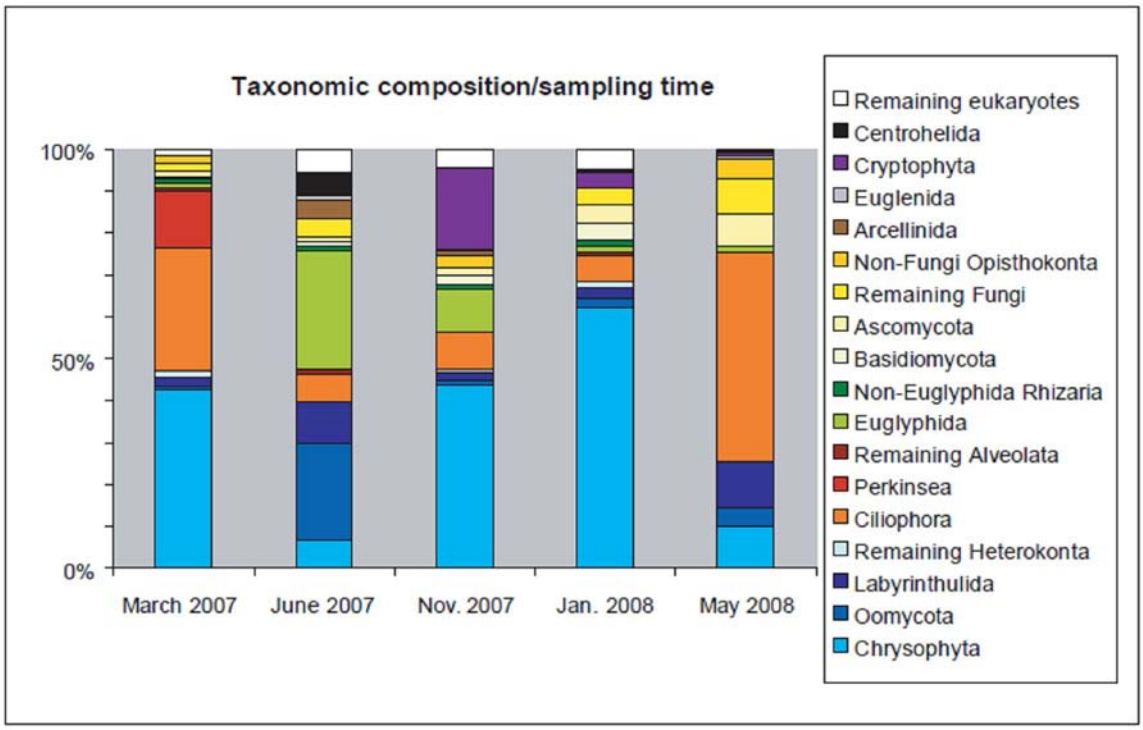

Figure S2: Taxonomic composition of the clone libraries of all peat bog water samples as percentages of total clone numbers.

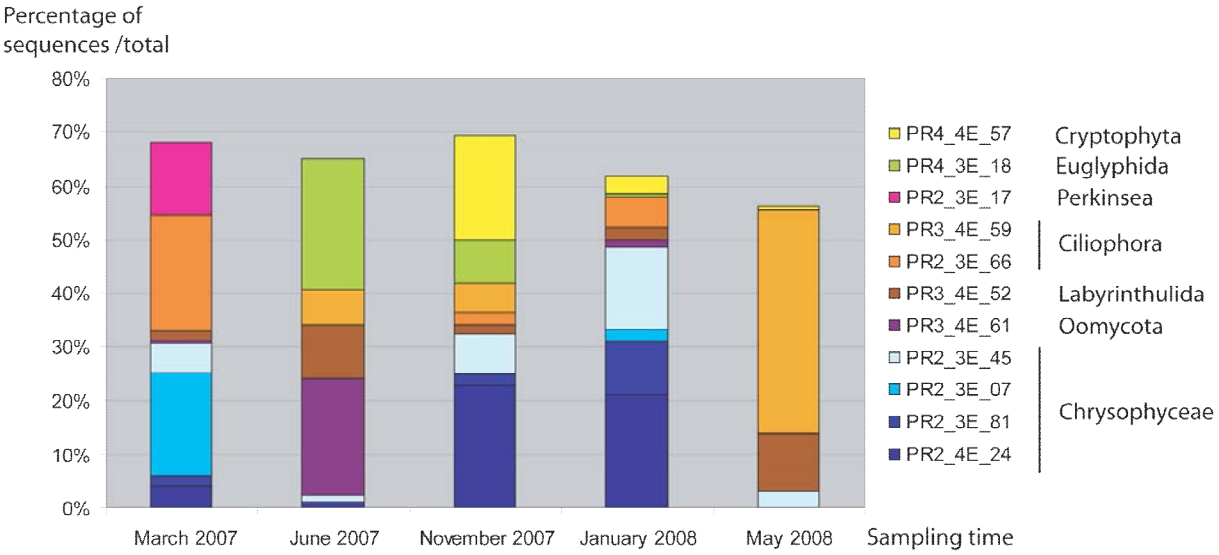

Figure S3: Histograms representing the repartition of the most representative phylotypes in all peat bog water samples as percentage of total clone numbers. The phylotypes presented here either represented more than 5% of all clones, either represented more than 10% of all clones derived from a single library.