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Contrasted Micro-Eukaryotic Diversity Associated with *Sphagnum* Mosses in Tropical, Subtropical and Temperate Climatic Zones

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Abstract

Sphagnum-dominated ecosystem plays major roles as carbon sinks at the global level. Associated microbial communities, in particular, eukaryotes, play significant roles in nutrient fixation and turnover. In order to understand better the ecological processes driven by these organisms, the first step is to characterise these associated organisms. We characterised the taxonomic diversity, and from this, inferred the functional diversity of microeukaryotes in *Sphagnum* mosses in tropical, subtropical and temperate climatic zones through an environmental DNA diversity metabarcoding survey of the V9 region of the gene coding for the RNA of the small subunit of the ribosomes (SSU rRNA). As microbial processes are strongly driven by temperatures, we hypothesised that saprotrophy would be highest in warm regions, whereas mixotrophy, an optimal strategy in oligotrophic environments, would peak under colder climates. Phylotype richness was higher in tropical and subtropical climatic zones than in the temperate region, mostly due to a higher diversity of animal parasites (i.e. Apicomplexa). Decomposers, and especially opportunistic yeasts and moulds, were more abundant under warmer climates, while mixotrophic organisms were more abundant under temperate climates. The dominance of decomposers, suggesting a higher heterotrophic activity under warmer climates, is coherent with the generally observed faster nutrient cycling at lower latitudes; this phenomenon is likely enhanced by higher inputs of nutrients most probably brought in the system by Metazoa, such as arthropods.

Keywords Sphagnosphere · V9 region of the SSU rRNA gene · Protist · Mould · Yeast · Microbial food webs

Introduction

Documenting the distribution patterns of diversity, its drivers and consequences for ecosystem functioning is a major topic in ecological research. Until recently, however, it was difficult to conduct such research for the multitude of poorly known microscopic eukaryotes (i.e. all eukaryotes except plants and animals) living in the soil. Recent developments of sequencing technology, such as high-throughput sequencing (HTS) and the associated bioinformatics and biostatistics, are

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currently revealing the extent of micro-eukaryotic diversity. Indeed, hundreds of thousands of sequences possibly representing millions of biological species are discovered, including previously overlooked forms such as symbionts, parasites or small heterotrophs [1–3]. HTS is increasingly used to reveal not only the diversity of soil protists but also functional relationships [4], illustrating their central role as a major hub linking microbial and aboveground communities [5]. Indeed, microeukaryotes are increasingly shown to play key roles in nutrient cycling [4, 6], in controlling bacterial populations as well as community composition, thus affecting their functions [7]. Eukaryotic phototrophs were long known to be major primary producers in aquatic systems, and recent studies suggest they also play an important role in soils contributing substantially to labile carbon input [4]. Such data can also be used in a semi-quantitative way, as the number of sequences obtained in environmental DNA surveys was shown to be proportional to the biomass of organisms, be it with mitochondrial [8] or ribosomal markers [9]. It is therefore possible to estimate with reasonable confidence the relative abundance and thus the likely importance of a given functional group of organisms in the environment. In addition, taxonomic assignment of HTS can give insights in the ecological function of the organisms present. Mycologists are already using an automated pipeline to assign fungal phylotypes built on internal transcribed spacer (ITS) sequences to ecological functions [10]. When surveying the whole eukaryotic communities, similar inferences can be made, at least to the level of main lifestyle categories [11]. Altogether, it is possible to categorise environmental eukaryotic communities to a level of accuracy that has never been attained before, and HTS is now the silver bullet method for exploring micro-eukaryotic environmental diversity.

Recent studies on soil microeukaryotes have revealed a huge of unknown diversity [2, 6] as well as new unexpected pathway in nutrient cycling [4]. However, the patterns and functional diversity across biomes are not yet well explored. The choice of the specific environment to assess these patterns are challenging due to the heterogeneity of soils [12].

Sphagnum mosses are very distinctive bryophytes that change considerably their immediate surroundings in terms of hydrology and chemistry [13]. These mosses have a specific requirement to growth [14] and have a cosmopolite distribution across all major climatic zones. *Sphagnum* mosses are ubiquitous bryophytes in habitats where humidity is important year-round, from the tropics to subarctic environments. They are the main components of high latitude peatlands especially in the Northern Hemisphere. These particular wetlands are well-known carbon sinks, sequestering 12% of all anthropogenic emissions of CO_2 [15]. In spite of covering only 3% of the Earth's emerged surface [16], at the global scale, this process is of considerable significance: an estimated 30% of the world's pool of organic carbon is stored in peat bogs [17], and the total amount of stored carbon has been estimated around 500 petagrams of carbon [18]. However, under a global warming scenario, it has been demonstrated that these ecosystems revert into carbon sources [19]. The processes responsible for carbon fluxes in peatlands depend to a large extent on microbial eukaryotes. Indeed, although *Sphagnum* mosses are most likely the main primary producers, the contribution of mixotrophic protists was recently shown to also be significant [20]. Regarding decomposition, and therefore CO_2 production, Fungi play a dominant role in being able to tolerate the acidic environments and degrade highly resistant organic compounds [21].

An artificial warming of *Sphagnum* biota of 5 °C under controlled conditions has been shown to double microbial respiration and to increase decomposition (and hence increase C release) [22], which is directly related to fungal communities and saprotrophy [23]. On the other hand, experimentally warming of peatlands showed a decrease in mixotrophic protists, a guild that contributes significantly to C input in the system [20]. Microbial eukaryotic communities develop in the constantly wet environment created by *Sphagnum* mosses [13] which can efficiently stock water (ca. 20× their dry mass) in specialised, hollow cells (hyalocysts) of their leaves and stems [24]. *Sphagnum* mosses maintain a high humidity content by taking up water (passively, by capillarity) from soil [25], or air [26], thus compensating the water lost by evaporation [27, 28].

This micro-environment is strongly selective for microbial life, as it has typically low pH and nutrient content [14] and a high concentration of humic acids which are known to have biocidal properties [29]. These conditions strongly shape the composition of microbial communities, leading to highly specialised assemblages, as shown for prokaryotes [14], fungi [30], Peronosporomycetes [31] and micro-Metazoa [32]. Overall eukaryotic communities are not only different from those of other environments, they are also highly diverse [33, 34]. This very particular micro-environment, which is key for assessing the role of *Sphagnum* in the global ecosystem, has been coined "sphagnosphere" [35, 36]. Such a specific habitat presents the advantage of allowing comparative observational studies in contrasted climatic settings while minimising the possible impact of other drivers, as was done in several studies on specific microbial groups [37, 38].

Here, we present the first survey focussed exclusively on eukaryotic diversity associated to *Sphagnum* based on HTS, comparing temperate (Europe), subtropical (Yaku, Japan) and tropical (Costa Rica) regions. We hypothesise that (1) warmer climates should favour a higher overall diversity; (2) decomposition should be faster under warmer climates (due to the temperature-sensitivity of enzymatic activity; [36]). In addition, higher decomposition rates should also be mirrored in higher number of osmotrophic decomposers, especially opportunistic saprotrophic moulds and yeasts which

take up labile compounds; (3) mixotrophy, as a strategy, should be favoured under less productive conditions where N availability is lowest [39], i.e. under cold climates where decomposition is slower.

Materials and Methods

Sampling, PCR and Sequencing

The study was carried out in three contrasted climatic zones: Tropical (Costa Rica, 9° 30′ N, 83° 28′ W), subtropical (Yaku Island, Japan, 30° 20′ N, 130° 30′ E) and temperate (Switzerland–Italy 46° 50′ N 8° 10′ E) (S1). We collected 21 samples (8 tropical, 7 subtropical and 6 temperate) of the top 3 cm of *Sphagnum* mosses under sterile conditions (S2). These samples were stored into LifeGuard® buffer to fix and preserve the DNA prior DNA extraction. DNA was extracted directly from *Sphagnum* stems using a PowerSoil® DNA Isolation Kit (Carlsbad, CA, USA) according to the manufacturer's instructions. The 18S SSU rRNA V9 regions (amplicon size about 180 bp) was amplified using the broad-spectrum eukaryotic primers 1380F/1510R described in [40]. PCR protocols and conditions were done following [33] and amplicon purification was made using a Wizard® SV Gel and PCR Clean-Up System (Madison, WI, USA) according to the manufacturer's instructions. Quantification of the obtained amplicons was performed using the Qubit 4 system (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation and sequencing was performed by the company Fasteris (Geneva, Switzerland) with an Illumina's HiSeq 2500 (paired-end, 2 × 125 bp), using V3 chemistry.

Sequences Processing

Quality check (Phred score filtering, elimination of reads without perfect forward and reverse primers, and chimera removal) of the sequences was performed following the pipeline developed by [1] and applied in [31]. Sequences were grouped in phylotypes using SWARM clustering algorithm [41]. We retained phylotypes that comprised more than 10 sequences in at least 3 samples, as rare sequences tend to blur ecological signal [42]. Taxonomic assignment of the phylotypes was performed by aligning the dominant sequences of each phylotypes to the reference PR^2 database [43] using GGSearch script in the FASTA package [44]. We filtered out sequences belonging to groups that were not assigned to microbial eukaryotes (Metazoa, Embryophyceae, Archaea, Bacteria, Streptophyta), an important step as we needed to remove sequences of *Sphagnum*, which represented up to 50% of the number of reads per sample. To homogenise the number of sequences and to be able to compare samples richness, we normalised the numbers of reads by reducing it to the smallest sample (here, 8070 sequences). Extra sequences were removed randomly from the dataset by using the function "rrarefy" from the R vegan package (V. 2.3-5) [45]. To verify if the total diversity were reached, we computed rarefaction curves with a visual check of the asymptotic behaviour for each sample (S3) and for each climatic zone (S4). We also computed the Chao estimator for each climatic zone.

Functional Assignment of Phylotypes

We assigned all well-represented phylotypes to major functional groups, based on their trophic mode: osmotrophic (Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Mucoromycotina), phototrophic (Archaeplastida, Bacillariophyta), parasitic (Apicomplexa, Entomophtoromycota, Ichthyosporea, Peronosporomycota = Oomycota), phagotrophic (Ciliophora, Rhizaria, Amoebozoa) and mixotrophic (Cryptophyta, Dinophyta, Haptophyta) (S5). Although many Peronosporomycota are facultative parasites that can live in pure cultures, these organisms live and develop generally in association with a host; it is actually believed that the ancestral condition was parasitic [46]. Mixotrophic organisms are defined here as those organisms that use both phagotrophy and photosynthesis as carbon sources [47]. We considered as mixotrophic all cryptophytes with the exception of the Goniomonadales and related early branching taxa [48]. Although Dinophyta include many heterotrophic species in marine environments, these are anecdotic in freshwater systems, representing about 12% of all known species [49]; therefore, we counted them as mixotrophic. Phylogenetic analyses of Chrysophyceae (sensu [50]) show several cases of shifts in nutrient acquisition strategies from mixotrophic to heterotrophic [51], as well as several groups containing organisms using a single strategy [52]. For these organisms (as well as Euglenida, that can be either phototrophic or phagotrophic), we verified manually the precise phylogenetic affiliation of all phylotypes to infer their lifestyle, i.e. phylotypes included within a strictly mixotrophic group, we considered as being mixotrophic (S6); all taxa that could not be assigned to a functional category were labelled "unknown". After verification, all Euglenida belonged to the phototrophic group (S5). We combined all phylotypes within the different mentioned functional categories (S7). We also highlighted indicator fungal phylotypes corresponding to saprotrophic moulds and yeasts, a clade that degrades labile compounds; the assignment of phylotype was based mostly on Clemmensen et al. [53].

Climatic Data

We used the coordinates for each sample to extract the biologically relevant bioclimatic variables from the finest resolution grids (30 arc-second) of the WorldClim database [54]. These

19 variables were extrapolated from data on monthly temperatures and rainfall. They have been used for species distribution modelling in two soil protist groups, protosteloid amoebae [55] and euglyphid testate amoebae [56]. To avoid excessive correlation among the explanatory variables, we performed a stepwise selection based on the variance inflation factors (VIF) with the recommended (and most stringent) threshold of five. Stepwise selection was performed using the customs script (https://beckmw.wordpress.com/2013/02/05/collinearity-and-stepwise-vif-selection/) on R (V.3.1.2) [57]. The following variables were selected for further analysis: Mean Temperature of the Wettest Quarter (MTWeQ), Mean Diurnal Range (MDR), Mean Temperature of the Driest Quarter (MTDQ), Mean Temperature of the Warmest Quarter (MTWaQ) and Precipitations of the Coldest Quarter (PCQ) (S8).

Data Analyses

We assessed the relationships between the phylotypes found in the 21 samples and environmental variables using a partial redundancy analysis (RDA) on Hellinger transformed data [58]. The significance of the variables and ordination axes (first and second) was assessed using a one-way analysis of variance (ANOVA; 1000 permutations, p value threshold = 0.05) [59]. These analyses were performed with R V.3.0.1 [57] and the R vegan package (V. 2.3-5) [45].

We investigated if phylotype richness and functional types were differently represented in the respective biomes using a Pairwise Test for Multiple Comparisons of Mean Rank Sums (Nemenyi tests) with the R package PMCMR V4.1. [60]. To identify characteristic organisms for each biome, we performed an indicator species analysis. In this purpose, we used a multi-level pattern analysis with the function "multipatt" from the R package indicspecies V1.7.5 [61] to find the significant indicator phylotypes in our dataset. Then a heat map was computed using the "heatmap.2" function of the gplots package [62]. We considered only phylotypes that occurred at least 100 times in the dataset in order to avoid noise and bias due to rare species for the indicator species analysis [42].

Results and Discussion

Broad Scale Patterns of Diversity Among the Three Climatic Zones

After removing metazoan and embryophyte phylotypes, as well as low quality and chimeric sequences, we obtained a total of 169,470 sequences and a total of 3035 phylotypes (tropical = 2236, subtropical = 2105, temperate = 1441), representing all major eukaryotic clades (Fig. 1a, b). The rarefaction curves computed for each biome and for each sample

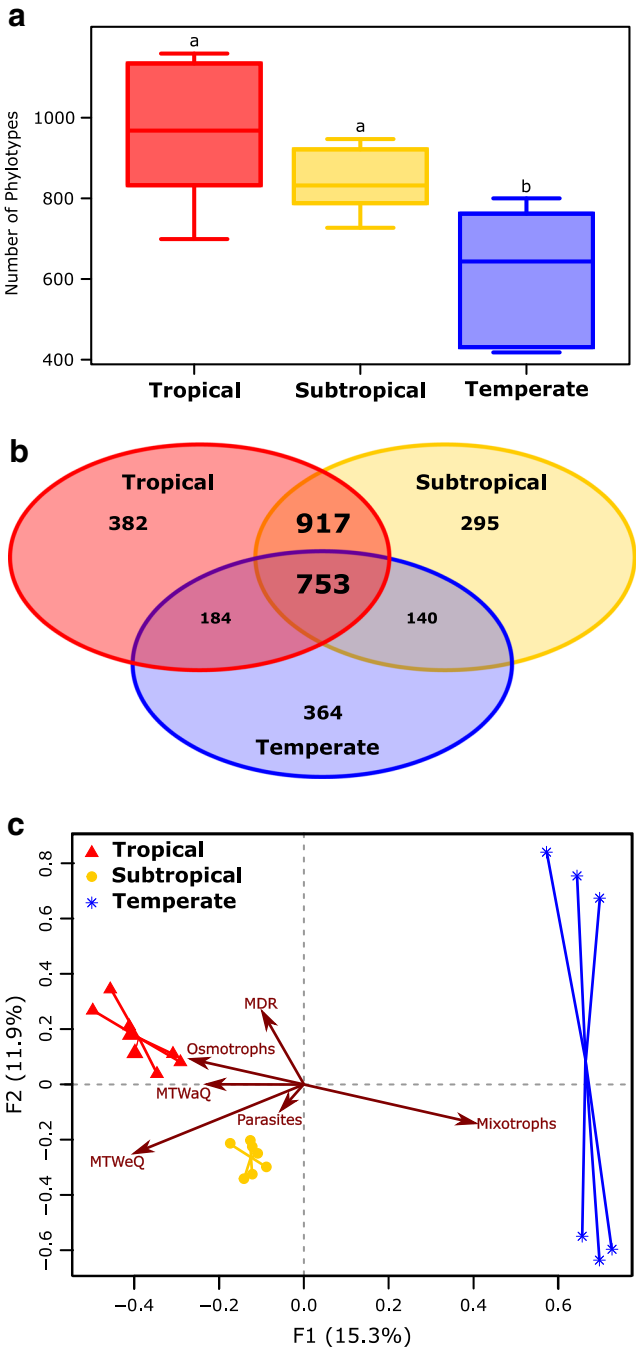

Fig. 1 Diversity and community structure of *Sphagnum*-inhabiting microeukaryotes in three climatic zones (red, tropical; yellow, subtropical; blue, temperate) based on Illumina sequencing of the V9 18S SSU rRNA gene. **a** Phylotype richness per climatic zones. Box and whisker diagram showing the median, inter-quartile and extreme values; letters above the boxplots represent groups of environments expressing significant different diversity distribution according to a Nemenyi test ($P < 0.05$). **b** Phylotype richness specific to each climatic zone and shared between pairs or among all three zones. **c** Redundancy analysis (RDA) build with the 6 significant variables ($p < 0.05$): Mean Diurnal Range (MDR), Mean Temperature of Wettest Quarter (MTWeQ), Mean Temperature of Warmest Quarter (MTWaQ), mixotrophs, parasites and osmotrophs.

showed that the major part of diversity had been covered and Chao estimates for the whole dataset showed saturation was reached (S3-S4). The data analysed in this study are available in the European Nucleotide Archive (ENA) under the reference number PRJEB29714.

High rank phylotype composition in all three climatic zones was overall similar, and no less than 24.8% of all phylotypes (i.e. 753 phylotypes) were present in all three climatic zones. The diversity of the micro-eukaryotic sphagnosphere was dominated by opisthokonta, which represented half of all reads. Most Opisthokonta were Fungi, which were dominated by Dikarya (Asco- and Basidiomycota), and a high diversity of Mucoromycotina (ex-Zygomycetes, *pro parte*) in tropical samples. Mucoromycotina are almost exclusively opportunist saprotrophic moulds that take up labile nutrients [63]. Fungi are key actors in the nutrient-depleted sphagnosphere, both as decomposers of organic matter and root-associated mediators of C transport and respiration [53].

Alveolata constitute the second most diverse group; among free-living organisms, the most common are ciliates (mostly phagotrophic) and dinoflagellates (mostly mixotrophic). Stramenopiles are the third most diverse group, and in our

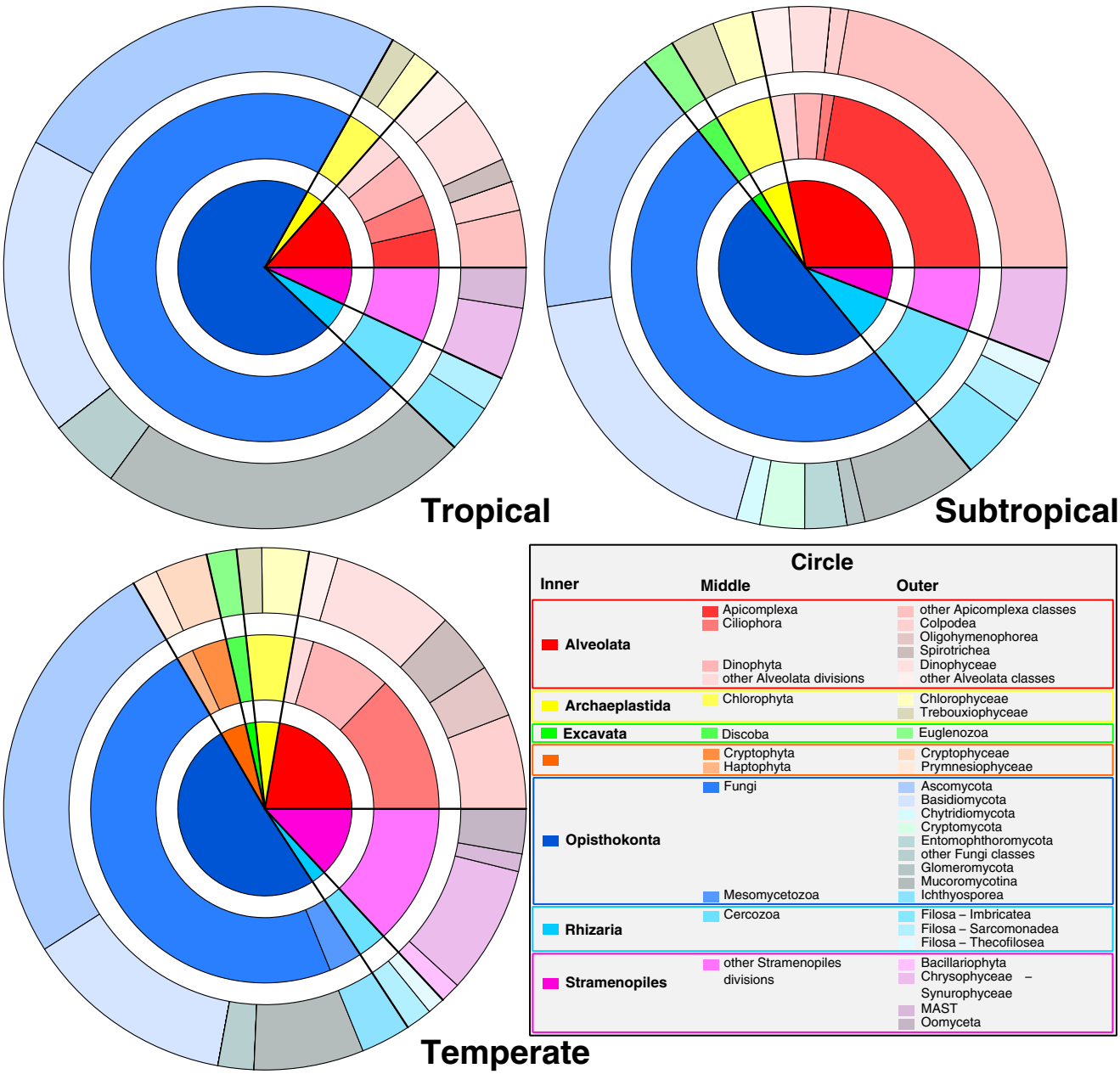

Fig. 2 Pie chart representing the community composition based on the number of reads of microeukaryotes living in *Sphagnum* sp. in three different climatic zones (tropical, subtropical and temperate). Taxa representing less than 1% of the total of a given level/taxa are represented in the "other" sections

samples include chiefly chrysophytes, a group of phagotrophic and mixotrophic flagellates abundant in peatlands and other oligotrophic freshwater systems [11, 33, 42]. Altogether, the composition of the micro-eukaryotic sphagnosphere resembles freshwater systems with a high diversity of dinoflagellates and ciliates [64], and especially the most oligotrophic with typically high diversity of chrysophytes. A likely explanation for this resemblance with freshwater systems is the abundance of free water between *Sphagnum* leaves and within the specialised water-holding cells (hyalocysts). The community composition differs, however, by the higher diversity of Fungi (especially Mucoromycotina; S5), which is more in line with terrestrial habitats. Similarly, phototrophic non-phagotrophic taxa like diatoms (Bacillariophyta) and Chlorophyta were less abundant than in true freshwater environments [64]. Taken together, the composition of the micro-eukaryotic sphagnospheres from different climatic zones is relatively constant at least at broad taxonomic levels, in line with our prediction.

Differences in community composition between climatic zones can be observed mostly at fine taxonomical resolution, especially at the phylotype level. Tropical and subtropical biomes shared 62.5% of their phylotypes while samples of the subtropical and tropical biomes had respectively 33.6 and 34.2% of their phylotypes in common with samples from the temperate biome (Figs. 1b and 2).

The indicator analysis identified 89 phylotypes significantly associated to either one biome or two biomes (tropical = 15, subtropical and tropical = 47, subtropical = 12, temperate = 12, tropical and temperate = 1, and tropical and temperate = 1) (Fig. 3). Many Fungi were sorted as characteristic for subtropical and tropical climatic zones, including 11 yeasts and mould phylotypes out of 30 total fungal phylotypes (and 4 phytopathogens, 2 parasites). Temperate climatic zones were characterised by the presence of four mixotrophic phylotypes, one dinoflagellate, one cryptophyte and two chrysophytes (Fig. 3).

Phylotype richness was significantly higher in the tropical and subtropical zones as compared to the temperate zone ($p < 0.01$) (Fig. 1a). Similar observations of a higher diversity of eukaryotic microbial communities under warmer climates have been made for freshwater

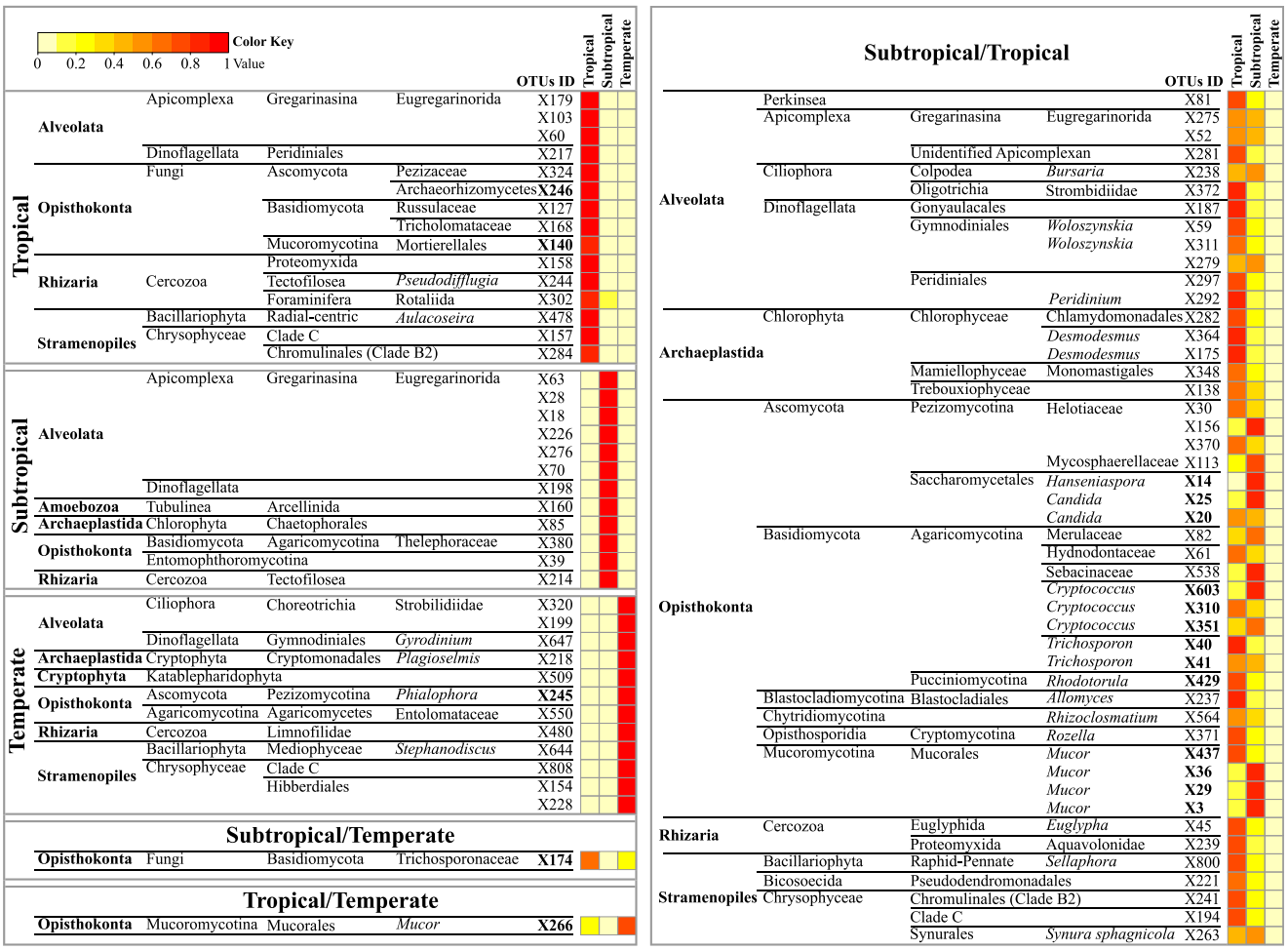

Fig. 3 Heat map representing the abundance of the bioindicator phylotypes occurring at least 100 sequences. All taxonomic affiliations were manually verified against the NCBI database using the BLAST algorithm

phytoplankton [65, 66], but not for soils [67] or marine sediments [68]. Here, the difference in sample richness is due chiefly to a higher diversity of Apicomplexa in the warm climate samples (S5). Apicomplexa is a group of protists parasitizing exclusively Metazoa [69]. This high abundance in warm climates is consistent with findings in tropical soils and has been associated to higher metazoan diversity in tropical forest litter [2]. Apicomplexa are rare in temperate biomes, where in contrast, sequences related to Mesomycetozoa (absent in other climatic zones) can be found. Mesomycetozoa also include many Metazoa parasites [70] and could, possibly, replace ecologically Apicomplexa in colder environments.

Within Climatic Zone Community Patterns Between Habitat Type

The six samples from the temperate zone were collected in two types of ecosystems: *Sphagnum* peatlands and poor fens with no or only shallow peat, with three samples in each type. The former can be considered as ombrotrophic (rain-water fed) while the latter are minerotrophic, i.e. influenced by groundwater [71]. This fundamental ecological difference corresponds to clear contrasts in vegetation, water chemistry and microbial community composition [72–75]. These differences are clearly reflected by the position of these samples on the ordination (Fig. 1c, S9), the samples from the high elevation poor fens having a higher score on axis 2 while the samples from lower elevation (lowland to ca. 1000 m in the Jura Mountains) having a lower score. Interestingly, although the micro-eukaryotic communities of these two habitats differ, they lie close together on axis 1 of the ordination that separates samples according to the three climatic zones. Thus, although ecosystem type clearly influences the makeup of microbial communities, the influence of the overall climatic context is stronger.

Higher Abundance of Opportunistic Decomposers Under Warm Climates

As predicted, reads related to osmotrophs (here, Fungi) were more abundant in warmer climate samples (Fig. 4) (Nemenyi test ($P < 0.05$)). While Dikarya display a very diverse array of functional types, Mucoromycotina are, with very few exceptions [76], saprotrophic moulds that require glucose, proteins

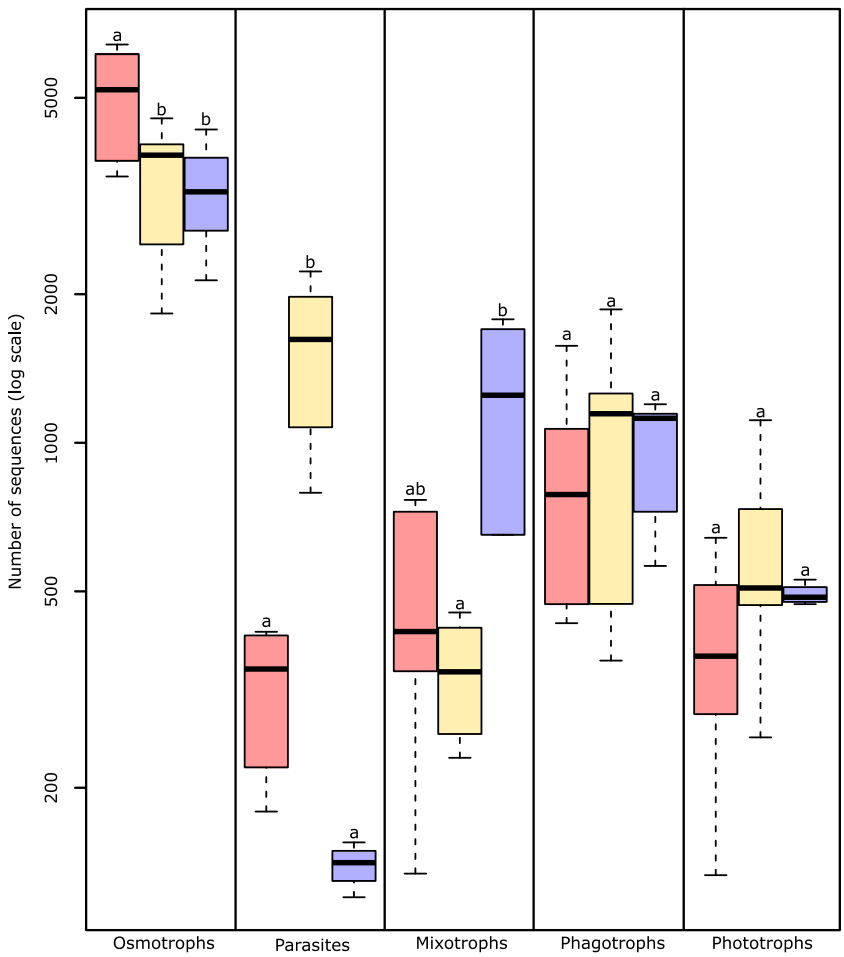

Fig. 4 Abundance of sequences of *Sphagnum*-inhabiting microeukaryotes assigned to each functional group (parasites, osmotrophs, phagotrophs, phototrophs and mixotrophs) in the three climatic zones (first, red: tropical; second, yellow: subtropical; third, blue: temperate). Letters above the boxplots represent groups of environments expressing significantly different diversity distribution according to a Nemenyi test ($P < 0.05$)

and a range of organic and inorganic substances to grow [77]. The important presence of these opportunistic organisms in warm climate samples (Fig. 2) suggests the existence of frequent nutrient pulses like those produced by animal cadavers such as arthropods (most Mucoromycotina are capable of degrading chitin; [77]). Paradoxically, in a world survey of all fungal diversity, Mucoromycotina have been found to be most frequent in tundra biotopes [78] which seems logical if one considers the nutrient pulses caused by the frequent freeze-and-thaw cycles [79].

The typical fungal indicators for tropical/subtropical samples include three phylotypes from genus *Mucor* (Fig. 3), a group of opportunistic moulds that have also the ability of degrading chitin. This could be linked to the presumably higher numbers of chitin-containing animals like arthropods in low latitude systems, a hypothesis which is indirectly corroborated with the higher number of Apicomplexans. Accordingly, warm climate samples included indicators from the saccharomycetous yeasts *Candida* and *Hanseniaspora*, the basidiomycete yeasts *Rhodotorula* and *Cryptococcus* and the equally opportunistic *Trichosporon* (Fig. 3). There are, nevertheless, also Fungi degrading the recalcitrant material produced by *Sphagnum* like, for instance, the warm climate indicator *Allomyces* (Fig. 3), a tropical Blastocladiomycota typically found degrading resistant material in ponds, rice fields and slow-moving rivers [80]. However, the presence of many yeasts and moulds in warm climate samples suggests that the functioning of decomposition processes in tropical *Sphagnum* moss carpets is different from temperate and cold climates, depending more on extraneous imports of materials.

Temperate Samples Include More Mixotrophs

Temperate samples are characterised by the highest diversity of sequences corresponding to mixotrophic organisms, i.e. organisms able to use both CO_2 and organic carbon (through phagocytosis) for carbon acquisition. This concerns members of the Cryptophyta, Haptophyta, Dinophyta and some Chrysophyceae. It has been demonstrated that mixotrophic species tend to use more of their phagocytosis ability under high temperatures [81]. Mixotrophy can be seen as a strategy attempting at obtaining either energy or nutrients where the other component is limiting ("the grand écart hypothesis"; [39]); in nutrient-depleted temperate peatlands, nitrogen is limiting and complementing photosynthesis by phagotrophy is a successful strategy [15]. In warmer *Sphagnum* environments, the high amounts of labile compounds inferred from the higher diversity/abundance of moulds, and yeasts suggest that mixotrophs lose their competitive advantage over heterotrophs, as mixotrophy has an intrinsic metabolic cost [39].

The carbon sequestration of peatlands results from the balance between decomposition and net primary production. Our results show that under warmer climates, the abundance of decomposers is higher and that of mixotrophs lower. It has been suggested that the abundance of mixotrophic protists is a factor determining C fixation in Northern Hemisphere peatlands [20]. Based on this, our result suggests that *Sphagnum*-dominated ecosystems would fix more carbon under cold climates. As opposed to the large extensions found in high latitude peatlands, *Sphagnum* develops under warm climates as patches of moderate extension in favourable microhabitats, usually in forests. There, the input of external material from the surrounding environment (such as, for instance, dead insects) can be expected to be higher, thus increasing decomposition processes by opportunistic and chitinolytic Fungi. In addition, warm climates would rather favour decomposition (and hence, respiration), and therefore, one could expect a different carbon balance shifted towards C release. Direct measurements of CO_2 fluxes on tropical and temperate *Sphagnum*-dominated ecosystems, as well as experimental studies under different climates including tropical regions should be undertaken to further test this. These results would also help better model how peatlands will respond to global warming, which is especially crucial considering that boreal *Sphagnum*-dominated peatlands contain one third of the global soil carbon pool [18, 82].

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