From forest soil to the canopy: increased habitat diversity does not increase species richness of Cercozoa and Oomycota in tree canopies

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Abstract

Tree canopies provide habitats for diverse and until now, still poorly characterised communities of microbial eukaryotes. One of the most general patterns in community ecology is the increase in species richness with increasing habitat diversity. Thus, environmental heterogeneity of tree canopies should be an important factor governing community structure and diversity in this subsystem of forest ecosystems. Nevertheless, it is unknown if similar patterns are reflected at the microbial scale within unicellular eukaryotes (protists). In this study, high-throughput sequencing of two prominent protistan taxa, Cercozoa and Oomycota, was performed. For a comprehensive assessment of their diversity across all ecological compartments from forest soils to the canopy, group specific primers were used. When taking OTU abundances into account, our results showed highly dissimilar protistan communities within the investigated microhabitats. We observed no pattern of nestedness, because the majority of OTUs was present in all sampled microhabitats. According to the microbiological tenet 'Everything is everywhere, but, the environment selects', habitat diversity strongly favoured distinct protistan taxa in terms of abundance, but due to their almost ubiquitous distribution the effect of species richness on community composition was negligible.

Running title: Cercozoa and Oomycota in forest ecosystems

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Abstract

Tree canopies provide habitats for diverse and until now, still poorly characterised communities of microbial eukaryotes. One of the most general patterns in community ecology is the increase in species richness with increasing habitat diversity. Thus, environmental heterogeneity of tree canopies should be an important factor governing community structure and diversity in this subsystem of forest ecosystems. Nevertheless, it is unknown if similar patterns are reflected at the microbial scale within unicellular eukaryotes (protists). In this study, high-throughput sequencing of two prominent protistan taxa, Cercozoa and Oomycota, was performed. For a comprehensive assessment of their diversity across all ecological compartments from forest soils to the canopy, group specific primers were used. When taking OTU abundances into account, our results showed highly dissimilar protistan communities within the investigated microhabitats. We observed no pattern of nestedness, because the majority of OTUs was present in all sampled microhabitats. According to the microbiological tenet 'Everything is everywhere, but, the environment selects', habitat diversity strongly favoured distinct protistan taxa in terms of abundance, but due to their almost ubiquitous distribution the effect of species richness on community composition was negligible.

Keywords : protists, canopies, metabarcoding, environmental heterogeneity, forest ecosystems

1 INTRODUCTION

Role of forest ecosystems. Communities inhabiting tree canopies are believed to contribute significantly to the maintenance of the diversity, resiliency, and functioning of forest ecosystems (Thompson *et al.* , 2009). On a global scale, there are more than 3 trillion trees on Earth, of which 43% can be found in tropical and subtropical regions and 22% in temperate biomes (Crowther *et al.* , 2015). Forest ecosystems harbour a large proportion of global biodiversity, contribute extensively to biogeochemical cycles, and provide countless ecosystem services (Bonan, 2008). Moreover, tree canopies form the most important interface between Earth's terrestrial biomass and the atmosphere (Ozanne *et al.* , 2003; Ellwood & Foster, 2004) and contain a multitude of heterogeneous microhabitats conducive to the evolution of epiphytic plants, animals and microorganisms (Nadkarni, 2001).

Environmental heterogeneity and biodiversity in tree canopies. One of the most general patterns in community ecology is the increase in species richness with increasing habitat heterogeneity (MacArthur & MacArthur, 1961; Williams, 1964). Accordingly, the presence of different structurally complex microsites (microhabitats) within tree canopies was shown to favour biodiversity of a variety of organisms, including epiphytes (Lyons, Nadkarni, & North, 2000; Nadkarni, 2001), birds (Goetz *et al.* , 2007), small mammals (Carey & Wilson, 2001) and arthropods (Hijii, Umeda, & Mizutani, 2001; Ishii, Tanabe, & Hiura, 2004).

State of the art (microorganisms). In a similar way, tree-colonizing microorganisms (i.e., Bacteria, Archaea and microfungi) formed highly specific communities across broader microhabitat classes (soil, stems, leaves) (Cregger *et al.* , 2018). In addition, it was shown that different plant species harbour species specific leaf-associated bacterial communities (Lambais *et al.* , 2006; Vorholt, 2012) as well as highly specific bacterial communities inhabit different cryptogamic epiphytes (bryophytes, macrolichens) (Aschenbrenner *et al.* , 2017). Accordingly, it can be assumed that unicellular eukaryotes (protists) show similar diversity patterns. So far, molecular studies reported distinct protistan communities in mosses (Mitchell, Bragazza, & Gerdol, 2004; Mieczan & Tarkowska-Kukuryk, 2014), lichens (Bates *et al.* , 2012; Mazei *et al.* , 2016), phytothelmata (Carrias, Cussac, & Corbara, 2001; Dunthorn *et al.* , 2012) as well as root associated communities (Turner *et al.* , 2013; Dumack *et al.* , 2020). A recent study on protistan diversity in tropical forest soils hypothesised that some soil protists could be a subset of tree canopy communities that have rained down from above (Mahé *et al.* , 2017). Nevertheless, a comprehensive comparative assessment of the protistan communities across different microhabitats from forest soils to the canopy region is still lacking.

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Investigation of protistan niche-level composition across trees. Accordingly, we hypothesised (1) to find microhabitat-specific protistan communities in tree canopies, and (2) an increase of species richness with habitat diversity. Following the terminology of Stein and Kreft (2015), we define *environmental heterogeneity* as an "umbrella term for all kinds of spatial heterogeneity, complexity, diversity, structure, or variability in the environment", while we are focusing here in particular on the sub-categorical term *habitat diversity* as a measurement of habitat richness, i.e. the number of distinct (micro-)habitats and habitat types. To investigate our hypotheses, we sampled numerous microhabitat compartments across a vertical gradient, from forest soils to the canopy region of three autochthonous tree species in a temperate floodplain forest. In a metabarcoding approach, we focused on two exemplary prominent taxa of protists which are commonly known to be plant associated (Ploch *et al.*, 2016; Flues, Bass, & Bonkowski, 2017; Sapp *et al.*, 2018), namely the **Cercozoa** (Rhizaria) and the **Oomycota**(Stramenopila). In order to improve coverage of investigated taxa, taxon-specific primers were used to amplify protistan DNA. Considering variation in taxonomic resolution of DNA barcodes, two different markers were targeted in this study: the hypervariable V4 region of the 18S rRNA gene and the Internal Transcribed Spacer 1 (ITS1) for barcoding cercozoan and oomycete communities, respectively.

The aim of this study was to shed light on unicellular eukaryotic diversity and community composition in forest soils and the canopy region. Unveiling these protistan distribution patterns will contribute to the understanding of environmental factors shaping cercozoan and oomycete communities across different ecological compartments represented by the structural complex ecosystem of tree canopies.

2 MATERIAL AND METHODS

2.1 Sampling and DNA extraction

Microhabitat samples were collected in October 2017 in cooperation with the Canopy Crane Facility in the floodplain forest in Leipzig, Germany (51.3657 N, 12.3094 E). We sampled three different specimens of three autochthonous tree species: The small-leaved lime (*Tilia cordata*), the European ash (*Fraxinus excelsior*) and the pedunculate oak (*Quercus robur*). The samples can be classified into two strata: (i) canopy samples and (ii) ground samples. Canopy samples were taken at 20 to 30 m height with replicates at all four cardinal directions of each tree. At each of the four replicate sites on every tree, following seven microbial microhabitat compartments related to tree surface were sampled: Fresh leaves, dead wood, bark, arboreal soil and three cryptogam epiphytes (lichen and two moss genera,*Hypnum* and *Orthotrichum*). In addition, two ground samples (soil and leaf litter with four replicates per tree) at 2 m distance from each trunk were sampled. The soils were collected at the surface layer (~10 cm depth after removal of leaf litter and stones) throughout each station. All 324 samples were stored at -22°C until further processing. For DNA extraction, all canopy and litter samples were decorticated and/or chopped with a sterile razor blade and cut into small, regular pieces. DNA extraction was done according to the manufacturer's instruction with the DNeasy PowerSoil kit (QIAGEN, Hilden, Germany). DNA concentration and quality were checked using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, USA). For following PCR amplification, all four replicates of each microhabitat per tree were pooled.

2.2 PCR amplification, barcoding and sequencing

PCRs with taxon specific primers were conducted in two steps. The hypervariable V4 region of the 18S ribosomal RNA gene (SSU rDNA) was used for cercozoan community profiling with specific primers (Fiore-Donno, Richter-Heitmann, & Bonkowski, 2020). For the first PCR the forward primers S616F_Cerco and S616F_Phyt were mixed in the proportions of 50% and 50%, and used with the reverse primer S963R_Phyt. For a following semi-nested PCR a mixture of the reverse primers S947_Phyt and S947_Vamp in an equal proportion has been used. The thermal program consisted of an initial denaturation step at 95degC for 2 min, 24 cycles at 95degC for 30 s, 52degC for 30 s, 72degC for 30 s; and a final elongation step at 72degC for

5 min. For amplifying the ITS 1 of the oomycete communities we used the specific primer pair ITS_177F and 58SR_Oom (Fiore-Donno & Bonkowski, 2020). Amplicons of the first PCR were again used as template for a semi-nested PCR with the primer pair I1786F_Stra and 58SR_Oom. The thermal program started with a denaturation step at 95degC for 2 min, followed by 24 cycles at 95degC for 30 s, 58degC for 30 s, 72degC for 30 s; and a final extension step at 72degC for 5 min.

We used 1 µl of DNA template for the first PCR amplification and 1 µl of the obtained amplicons as a template for a second semi-nested PCR which was conducted with tagged primers. Tags were designed as described in Fiore-Donno, Richter-Heitmann, & Bonkowski (2020). The used primers and tag combinations are provided in Supplementary Tables 3 and 4.

We applied the following final concentrations: DreamTaq polymerase (Thermo Fisher Scientific, Dreieich, Germany) 0.01 units, Thermo Scientific DreamTaq Green Buffer, dNTPs 0.2 mM and primers 1 µM. To reduce the artificial dominance of few amplicons by PCR competition, all PCRs were carried out twice. PCR products were pooled, then purified and normalized using SequalPrep Normalization Plate Kit (Invitrogen GmbH, Karlsruhe, Germany). Sequencing was performed with a MiSeq v2 Reagent kit of 500 cycles for the shorter ITS amplicons (c. 250 bp) of Oomycota and a MiSeq v3 Reagent kit of 600 cycles for the amplified V4 Region fragments (c. 350 bp) of Cercozoa. Sequencing was conducted by a MiSeq Desktop Sequencer (Illumina Inc., San Diego, CA, USA) at the Cologne Center for Genomics (Germany).

2.3 Sequence processing

All bioinformatic and statistical methods were applied to both Oomycota and Cercozoa datasets independently if not stated otherwise. Raw reads were merged using vsearch v2.10.3 (Rognes *et al.* , 2016) at default settings. Merged contigs were demultiplexed with cutadapt v1.18 (Martin, 2011) allowing no mismatches in neither primer nor tag sequence. Cutadapt was also used to trim primer and tag sequences after demultiplexing. Sequences were then clustered into operational taxonomic units (OTUs) using swarm v2.2.2 (Mahé *et al.* , 2015) with $d = 1$ and fastidious option on. Chimeras were *de novo* detected using vsearch. OTUs were removed from the final OTU table if they were flagged as chimeric, showed a quality value of less than 0.0002, were shorter than 150bp (Oomycota) or 300bp (Cercozoa), or were represented by less than 0.005% of all reads (i.e. 141 reads for Oomycota or 269 reads for Cercozoa).

For taxonomic assignment, OTUs were first tentatively assigned by using BLAST+ v2.9.0 (Camacho *et al.* , 2009) with default parameters against the non-redundant NCBI Nucleotide database (as of June 2019). OTUs were removed if the best hit in terms of bitscore was a non-oomycete or non-cercozoan sequence, respectively. For a finer taxonomic assignment, two databases were used: The PR2 database (v4.12.0, Guillou *et al.* , 2012) served as a taxonomic reference set for cercozoan V4 sequences, while for the Oomycota all available oomycete sequences were downloaded from NCBI Nucleotide (as of July 2019). Both databases were used as a template for an *in-silico*PCR with cutadapt, with the same primer sequences used in this study. The resulting virtual amplicons served as a database with the same length and genetic origin as our sequenced amplicons, which offers the advantage of penalising terminal gaps during the taxonomic annotation - which was performed with vsearch. The annotation was refined by assigning the species name of the best vsearch hit to the corresponding OTU if the pairwise identity was over 95%. OTUs with lower percentages were assigned higher taxonomic levels.

To account for random effects due to low sequencing depth, the final OTU table was loaded into Qiime2 v2018.11 (Bolyen *et al.* , 2019) to explore the sequencing depth by sample metadata. The minimum sequencing depth was determined depending on how many samples per metadata would be excluded. It was set as high as possible while retaining at least five samples per microhabitat and 15 samples per tree species.

2.4 Statistical analyses

All statistical analyses were conducted in R v3.5.3 (R Core Team, 2019). Rarefaction curves were carried out with the iNEXT package (Chao *et al.* , 2014; Hsieh, Ma, & Chao, 2019) to determine if a higher sequencing depth would have revealed more OTUs. Alpha diversity indices were calculated for each sample using the *diversity* function in the vegan package (Oksanen *et al.* , 2019). Both former methods were applied on the OTU table with absolute abundances. To explore differences in the community composition across the samples, the following beta diversity-based methods were conducted on relative abundances. Non-metric multidimensional scaling was performed on the Bray-Curtis dissimilarity matrix of the log transformed table (functions *vegdist* and *metaMDS* in the vegan package, respectively). The same method was used in a permutational multivariate analysis of variance (permANOVA, function *adonis*), to test if oomycete and cercozoan OTU diversity differed across the strata, habitats and tree species. To analyse the effects of environmental factors to the variance of the community composition, a redundancy analysis was carried out on the Hellinger-transformed table (function *rda* in the vegan package). The function *nestedtemp* was used to test if the community of a microhabitat might be a subset of a larger one. Species accumulation curves were calculated using the *specaccum* function and the number of shared OTUs between different combinations of microhabitats was visualised using the UpSetR package (Lex *et al.* , 2014; Gehlenborg, 2019). All figures were plotted with the ggplot2 package (Wickham, 2016).

3 RESULTS

3.1 Sequencing and bioinformatic pipeline

We obtained 550 OTUs from 1.381.839 sequences (Cercozoa) and 331 OTUs from 1.610.374 sequences (Oomycota). The average number of cercozoan OTUs was 516 ± 15 and 546 ± 3 per microhabitat and tree species, respectively, while the average number of oomycete OTUs was 236 ± 25 and 304 ± 4 per microhabitat and tree species, respectively. Tree canopies contained a substantially unknown diversity of oomycetes, with 57 oomycete OTUs with less than 70% percent identity to any reference sequence, while this was the case for only three cercozoan OTUs (Figure 1). While most of the reads and OTUs showed a similarity of 97-100% to any known reference sequence, oomycete reads revealed two additional peaks at about 76% and 87%, indicating that a small number of highly abundant OTUs in oomycetes is still not taxonomically recorded (see Supplementary Figure 1 and Supplementary Tables 1 and 2 for taxonomic composition and annotation).

3.2 Alpha diversity

The used taxon-specific primers recovered the majority of OTU richness from canopy and ground samples (soil and litter) as indicated by rarefaction curves (Supplementary Figure 2). The extrapolation revealed that doubling the sequencing depth would have yielded no more cercozoan and oomycete OTUs, respectively, for all samples combined. All sampled microhabitats showed high alpha diversity (Figure 2), except for oomycetes in the ground leaf litter (ANOVA F value = 10.79, p <0.001; Figure 2B).

3.3 Beta diversity

Most variation in protistan beta diversity was explained by microhabitat differences (permANOVA, Cercozoa: R^2 0.45, p = 0.001; Oomycota: R^2 0.30, p = 0.001). Another significant proportion of beta diversity was explained by the differences between the two strata ground and canopy (permANOVA; Cercozoa: R^2 0.13, p = 0.001; Oomycota: R^2 0.14, p = 0.001). Only the beta diversity of the oomycete communities, but not that of the cercozoan, differed between tree species, yet with only a small amount of explained variance (permANOVA; Cercozoa: R^2 0.04, p = 0.1; Oomycota: R^2 0.06, p = 0.01) (Supplementary Table 5). Non-metric multidimensional scaling of protistan community profiles revealed a strong separation of canopy and

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ground strata (Figure 3). In Cercozoa, communities inhabiting fresh leaves in the canopy were most distinct to those in mineral soil on the ground (Figure 3A). Leaf litter communities on the ground scaled closer to fresh canopy leaves than to the underlying soil communities. A clear transition in beta diversity occurred from fresh canopy leaves to deadwood, bark and lichen, and finally the moss communities (*Hypnum* sp. and *Orthotrichum* sp.). Communities of arboreal soil were highly variable, ranging from samples with high similarity to mosses to samples closely resembling the mineral soil communities underneath the litter layer. Also in oomycetes (Figure 3B), canopy and ground communities were most distinct. Again, the two ground samples, mineral soil and leaf litter, showed no overlap, with leaf litter having a low alpha diversity. In contrast to Cercozoa, oomycete communities of canopy microhabitats showed a high overlap, and communities of fresh canopy leaves were clearly distinct from litter on the ground. The only common pattern between cercozoan and oomycete communities in the canopy was their high variability in the arboreal soil.

A db-RDA showed the same pattern, but revealed a bit clearer the similarity of communities of fresh canopy leaves to deadwood in both protistan taxa (Figure 4). For the Cercozoa (Figure 4A), both axes explained approximately the same amount of variance. Bark and epiphyte communities were again more similar to each other; thus these factors explained the same kind of variance in the community compositions. The soil communities were clearly different, while leaf litter clustered together with fresh leaves and deadwood communities. Little variance was found between tree species and arboreal soil. In the Oomycota (Figure 4B) a similar pattern could be observed. Bark and epiphytes communities were similar, and soil - together with leaf litter communities - appeared as separated from the other microhabitats. Tree species communities appeared further away along the second RDA axe, which however only explained approx. 5% of the variance.

3.4 Shared OTUs

Despite high differences in beta diversity, the majority of OTUs were shared between all microhabitats irrespective of the protistan phylum (Figure 5). Only few combinations yielded more than ten unique OTUs shared between distinct microhabitats, which is negligible given the high OTU richness per sampled microhabitat, which varied between 498 (deadwood) and 537 OTUs (fresh leaves) for the Cercozoa (Figure 5A) and between 189 (leaf litter) and 270 (*Orthotrichum* moss) for the Oomycota, respectively (Figure 5B). Because almost all OTUs were shared between all microhabitats with the species accumulation curve showing only a flat increase, communities revealed no patterns of nestedness (Supplementary Figure 3, Supplementary Figure 4).

4 DISCUSSION

Why did the protists not obey the rule of increased species richness with increasing habitat diversity? Our findings show how discrete microhabitat niches lead to compositional heterogeneity of microbial communities within tree canopies, and ultimately within whole ecosystems (Peay, Kennedy, & Talbot, 2016; Cregger *et al.* , 2018). Application of taxon-specific primers ensured an exhaustive coverage of the investigated protistan taxa, a crucial precondition to minimise the number of taxa absent due to undersampling (Supplementary Figure 2). Furthermore, a study comparing the outputs of general and group specific primers has shown that structuring effects of the environment on protistan communities could only be seen in the data sets generated with specific primers (Lentendu *et al* ., 2014). With data obtained this way, we could report a further increase in the diversity of investigated protistan taxa, as it was already demonstrated by Fiore-Donno et al. (2018). Thus, the majority of detected OTUs account for a large amount of undescribed diversity (Figure 1) and may represent so far uncharacterised lineages, especially in the taxon of oomycetes, as only 34% of the OTUs were 97%–100% similar to any known sequence.

A world-wide survey on forest litter to investigate patterns of diversity in a group of testate amoebae observed that this common group of terrestrial protists behave like macroscopic organisms. Documented community structures were strongly correlated with climatic and physicochemical parameters but also with geographical

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barriers (Lara*et al.*, 2015). We observed Canopy communities to be strikingly different in terms of beta diversity compared to litter and soil communities on the ground, and as expected, different microhabitats within tree canopies were further colonized by distinct protistan communities, especially by Cercozoa (Figure 3A). However, the hypothesised rule of increased species richness with increasing habitat diversity could not be confirmed due to the almost ubiquitous distribution of protistan taxa (Figure 5). Our data exemplify that microorganisms do not always obey ecological rules assigned to multicellular organisms. Moreover, our findings are in contrast to patterns observed for bacteria (Lundberg *et al.*, 2012; Ottesen*et al.*, 2013; Wagner *et al.*, 2016), epifoliar fungi (Gilbert, Reynolds, & Bethancourt, 2007) or lichens (Boch *et al.*, 2013; Marmor *et al.*, 2013). Most of these groups are directly dependent on specific environmental conditions or the availability of resources offered by the habitat (Vandenkoornhuyse*et al.*, 2015; Sasse, Martinoia, & Northen, 2018). However, high turnover rates and the ability of protists to form cysts as resting stages imposes less constraints on their distribution over large distances due to a reduced likelihood of isolation and high diversity of source pools for local community assembly (Fenchel & Finlay, 2004; Bahram *et al.*, 2016). Accordingly, habitat diversity strongly favoured certain protistan taxa in terms of relative abundance, but the effect of OTU richness on community composition was negligible due to their almost ubiquitous distribution.

Mahe *et al.* (2017) hypothesised that protists in soils may be a subset from the canopy that have rained down from above; a pattern confirmed for leaf endophytic fungi in one-year old beech litter of temperate forests (Guerreiro *et al.*, 2018). On the other hand, there is growing evidence of cercozoan species particularly adapted to life in the phyllosphere (Dumack *et al.*, 2017; Flues, Bass, & Bonkowski, 2017, Sapp *et al.*, 2018). Cercozoan phyllosphere communities, isolated from fresh canopy leaves, were overall indeed surprisingly similar to leaf litter communities on the ground (Figure 3A). However, this cannot be confirmed for protists in general, as differences in oomycete communities between phyllosphere and ground litter showed strikingly different patterns. While fresh canopy leaves and ground litter had highest OTU richness of Cercozoa (Figure 5A), ground litter contained a significantly depleted diversity of oomycetes (Figure 2B). Interesting were the resembling patterns of beta diversity between phyllosphere and deadwood Cercozoa and oomycetes which deserve further attention in future studies. The small, but significant differences of oomycete communities between tree species (Figure 4B, Supplementary Table 5) might be explained by differences in host specificity, since oomycetes are well known to contain specific pathogens infecting leaves, stems and roots of forest trees (e.g. Rizzo & Garbelotto, 2003; Lehtijarvi *et al.*, 2017). Apparently, tree species do not shape their associated protistan communities to the same degree as bacteria, where even different genotypes of the same tree species can show distinct spatial patterns in their colonizing bacterial communities (Redford *et al.*, 2010; Leff *et al.*, 2015; Cregger *et al.*, 2018). Heterotrophic (or organotrophic) bacteria experience a direct selection pressure by differences in nutritional resource composition between plant microhabitats (Thapa *et al.*, 2017), while the proportion of bacterivorous Cercozoa at the next trophic level lack this direct dependency on the host-tree species (Figure 4A).

Compared to the highly specific bacterial communities of tree bark, mosses and lichens (Aschenbrenner *et al.*, 2017), canopy protists appear to rather depend on microhabitat characteristics. This is best exemplified within the cryptogamic epiphytes. Lichen and the two moss taxa harboured quite similar protistan communities (Figure 3). These epiphytes are characterised by rapidly changing conditions with rapid swelling and storage of moisture from morning dew and after rainfall to severe dryness at sunshine (e.g. Jonsson *et al.*, 2014; Benitez*et al.*, 2018) and to a certain degree may act as environmental filters selecting specific protistan communities.

Protistan communities of arboreal soil samples showed high variability, spanning from moss-like communities to soil-like communities (Figure 3). Importantly, this indicates that protistan communities resembling those of mineral soil are not restricted to the forest floor. Community variability in arboreal soil might be due to the varying degree of decay of the sampled material and its distinct physico-chemical properties (Nadkarni *et al.*, 2002), and further strengthens our hypothesis that increasing habitat richness may result in increasing compositional heterogeneity of protistan communities.

Conclusions

Beta diversity of protists was solely driven by differences in the relative abundance of OTUs, because almost all taxa were ubiquitously distributed among tree crowns and soil of the floodplain forest. Accordingly, species richness did not increase with habitat diversity as hypothesized, although strong differences in beta diversity between protistan communities of the forest floor and tree crowns, and among microhabitats within tree crowns, demonstrate strong differences in relative abundance. Different tree species had a surprisingly low influence on protistan community assembly; even the mostly plant-parasitic oomycetes did not show a high degree of host-specificity. However, a high number of OTUs from canopy communities could not be assigned to any known sequence, giving evidence that protistan communities of tree canopies are largely understudied. Both strata show unique protistan communities, indicating no top-down relationship of investigated protistan taxa in trees. Thus, our findings illustrate that the diversity of soil protists is solely shaped by the habitat itself, from which no conclusions regarding the total diversity of the canopy can be drawn. The occurrence of only a few specialist OTUs does not imply functional homogenization at the community level across microhabitats, but rather indicates increasing functional diversity to a greater extent than increasing OTU richness with increasing habitat diversity (Ofek-Lalzar *et al.* , 2014). Future studies will be needed to further address this hypothesis, supplementing functional traits to the taxonomical assignment of the investigated protistan OTUs.

Acknowledgements

Conflict of Interest

None declared.

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Data Accessibility

Raw sequence data have been submitted to the European Nucleotide Archive (ENA) database under the Bioproject number PRJEB37525, with accession numbers ERS4399743 (Cercozoa) and ERS4399744 (Oomycota) respectively.

All figures, codes and detailed bioinformatic/statistical methods used in this study are available at *https://github.com/RJauss/*
OTU abundance tables will be made publicly available via the GFBio platform.

Author contributions

MB and MS designed the study. RW and StS conceived and conducted the sampling and DNA extraction.
AMF-D contributed the primers, KD helped with the laboratory work. SW and R-TJ conducted the PCRs,
performed the bioinformatic and statistical analyses and drafted the manuscript. All authors contributed to
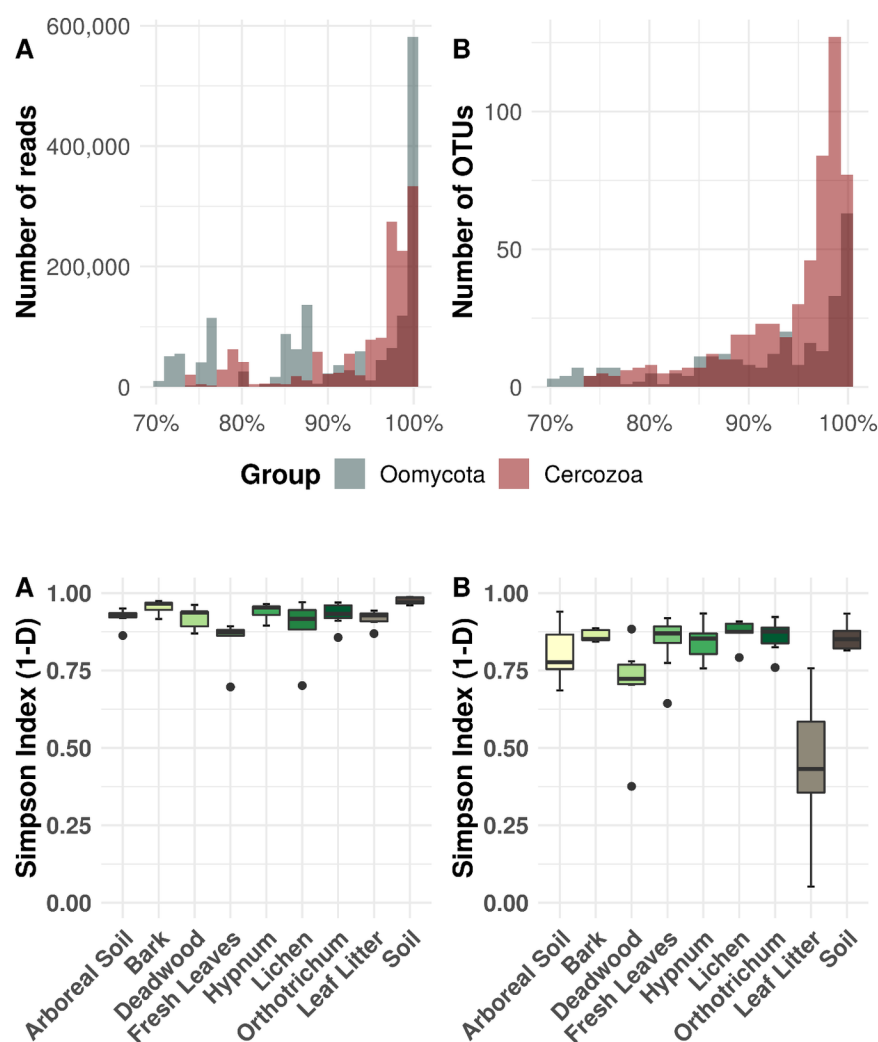and approved the final version.

Figures

Figure 2: Alpha diversity of microhabitats for cercozoan (A) and oomycete (B) communities. Boxplots
describe the Simpson Index of the samples grouped by microhabitat, outliers are given by dots. Simpson

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Index revealed high alpha diversity irrespective of the investigated protistan group, with the exception of lower alpha diversity of the leaf litter samples within the Oomycota.
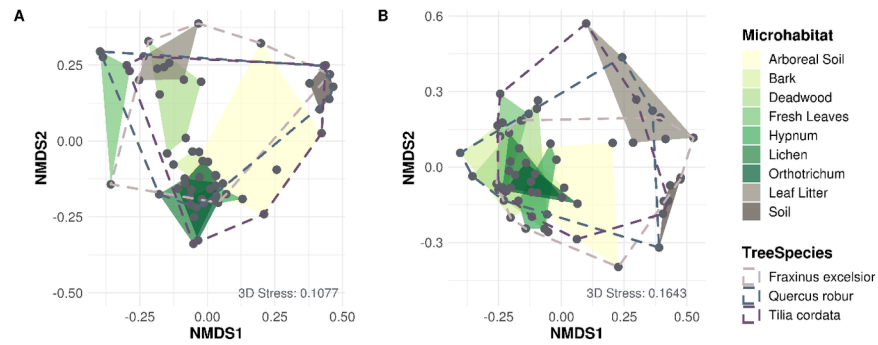

Figure 3: Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities of cercozoan (A) and oomycete (B) communities among microhabitats. Cercozoan communities showed a finer separation between canopy microhabitats compared to oomycetes, while the latter showed a clearer separation of communities between canopy (green and yellow) and ground (brown). Stress values of NMDS are shown in the lower right of each graph. Microhabitats were more influential for protistan community composition than tree species (Supplementary Table 5).
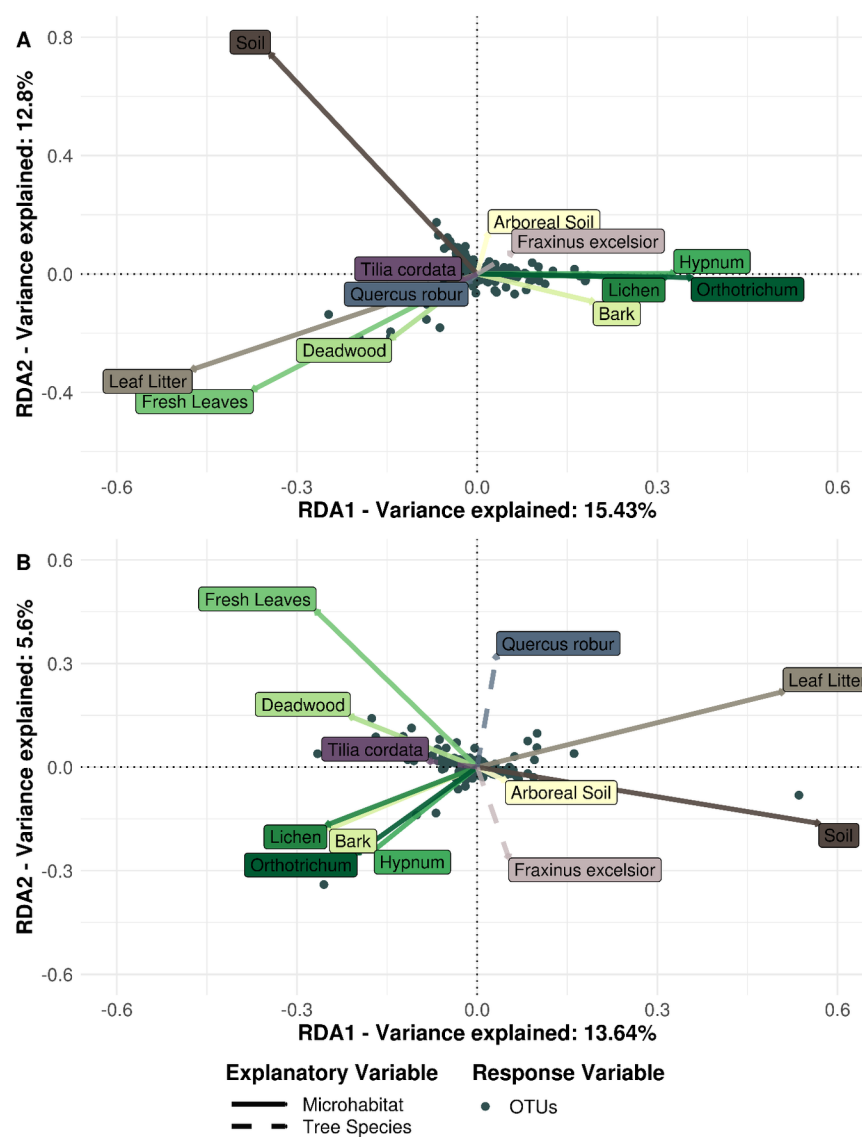
Figure 4: Redundancy analysis (db-RDA) of cercozoan (A) and oomycete (B) OTUs, microhabitats and tree species. Environmental factors of microhabitat identity and tree species were included in the analysis. Dots represent OTUs. The percentages of variability explained by each axis (RDA1 and RDA2) are given in the labels. RDA ordination resulted in three clusters explaining a comparable amount of variance respectively.
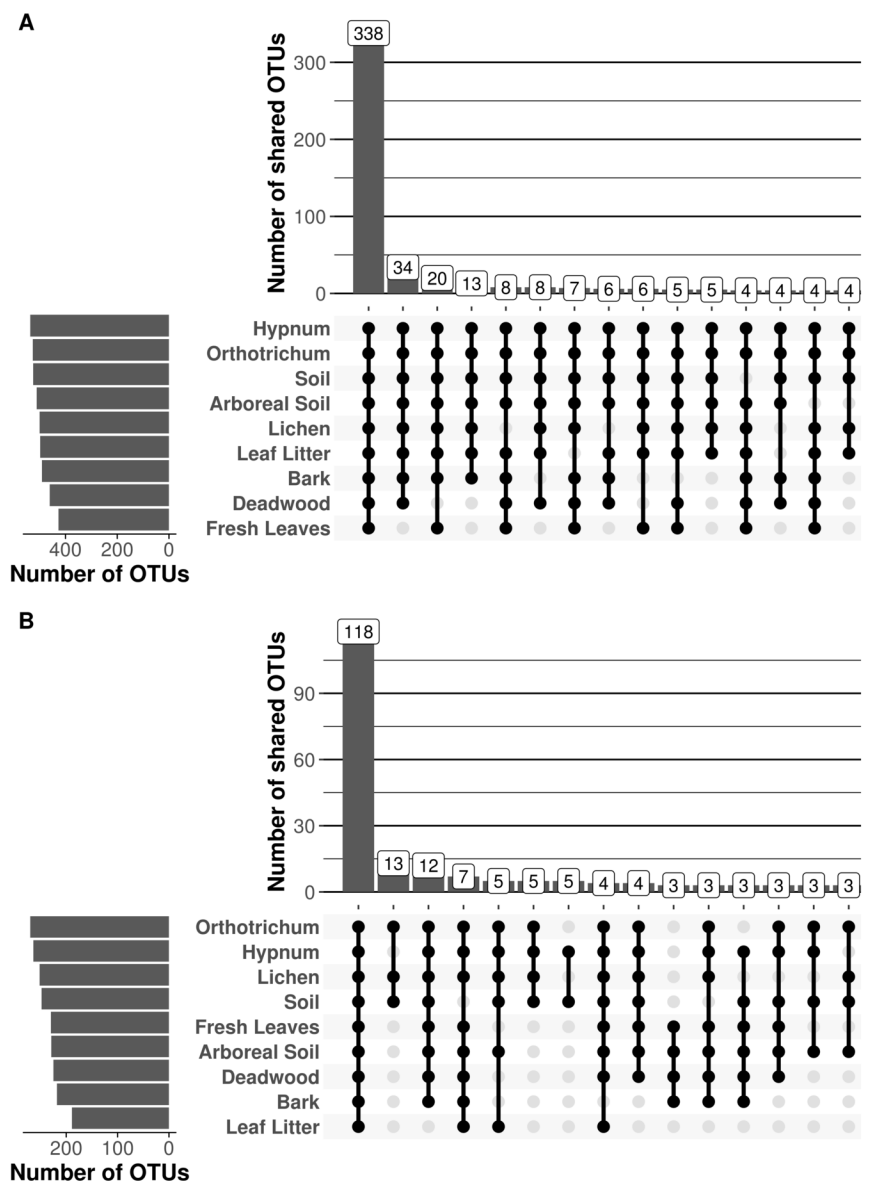
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Figure 5: Shared OTUs of cercozoans (A) and oomycetes (B) between microhabitats. Top bar chart represents the sum of the number of shared OTUs resulting from the combination of microhabitats in the matrix below. Only the 15 combinations with the highest numbers of shared OTUs are shown. The majority of OTUs were shared between all microhabitats, irrespective of the investigated protistan phylum.

Figure captions

Figure 1: Similarity of protistan sequences to the taxonomic reference database. Oomycete sequences and OTUs are given in grey bars, cercozoan in red. Dark colour represents the overlap between the bars. The majority of all reads (A) and OTUs (B) were ≥ 97% similar to the respective database. 17.2% of all oomycete OTUs had <70% similarity to known reference sequences, whereas only 0.5% of the cercozoan reads had a similarity of <70% (not shown).

Figure 2: Alpha diversity of microhabitats for cercozoan (A) and oomycete (B) communities. Boxplots describe the Simpson Index of the samples grouped by microhabitat, outliers are given by dots. Simpson Index revealed high alpha diversity irrespective of the investigated protistan group, with the exception of lower alpha diversity of the leaf litter samples within the Oomycota.

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Figure 4: Redundancy analysis (db-RDA) of cercozoan (A) and oomycete (B) OTUs, microhabitats and tree species. Environmental factors of microhabitat identity and tree species were included in the analysis. Dots represent OTUs. The percentages of variability explained by each axis (RDA1 and RDA2) are given in the labels. RDA ordination resulted in three clusters explaining a comparable amount of variance respectively.

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