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RESEARCH ARTICLE

On the phenology of protists: recurrent patterns reveal seasonal variation of protistan (Rhizaria: Cercozoa and Endomyxa) communities in tree canopies

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One sentence summary: Investigation into the seasonality of protistan communities colonizing different microhabitats across the surface of tree canopies in a temperate floodplain forest.

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ABSTRACT

Tree canopies are colonized by billions of highly specialized microorganisms that are well adapted to the highly variable microclimatic conditions, caused by diurnal fluctuations and seasonal changes. In this study, we investigated seasonality patterns of protists in the tree canopies of a temperate floodplain forest via high-throughput sequencing with group-specific primers for the phyla Cercozoa and Endomyxa. We observed consistent seasonality, and identified divergent

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spring and autumn taxa. Tree crowns were characterized by a dominance of bacterivores and omnivores, while eukaryvores gained a distinctly larger share in litter and soil communities on the ground. In the canopy seasonality was largest among communities detected on the foliar surface: In spring, higher variance within alpha diversity of foliar samples indicated greater heterogeneity during initial colonization. However, communities underwent compositional changes during the aging of leaves in autumn, highly reflecting recurring phenological changes during protistan colonization. Surprisingly, endomyxan root pathogens appeared to be exceptionally abundant across tree canopies during autumn, demonstrating a potential role of the canopy surface as a physical filter for air-dispersed propagules. Overall, about 80% of detected OTUs could not be assigned to known species—representing dozens of microeukaryotic taxa whose canopy inhabitants are waiting to be discovered.

Keywords: unicellular eukaryotes; metabarcoding; plant microbiome; microhabitats; forest ecosystems; plant pathogens

INTRODUCTION

Tree canopies—an ephemeral environment for microbes

The forest canopy is defined as 'the aggregate of all tree crowns in a stand of vegetation, which is the combination of all foliage, twigs, fine branches, epiphytes as well as the air in a forest' (Parker 1995). With an estimated area exceeding 100 million km^2 globally, the foliar surface forms the largest biological surface on Earth (Morris and Kinkel 2002; Peñuelas and Terradas 2014). Nevertheless, knowledge on microorganisms inhabiting the phyllosphere, i.e. the whole aerial region of plants dominated by leaves (Vorholt 2012), is far less advanced than that of belowground counterparts (i.e. rhizosphere, soil, litter layer). The phyllosphere is subject to recurrent microclimatic dynamics due to rapid changes in abiotic stressors, such as UV radiation, temperature, humidity and osmotic pressure, during daily fluctuations that only specially adapted microorganisms can cope with (Baldocchi and Collineau 1994; Vorholt 2012; Manching, Balint-Kurti and Stapleton 2014; Stone, Weingarten and Jackson 2018). Considering that perennial deciduous plants produce and shed their leaves annually, the phyllosphere represents a highly ephemeral environment (Vorholt 2012; Mwajita *et al.* 2013). Thus, it can be presumed that microorganisms dwelling within this habitat opportunistically colonize, multiply and occupy newly formed niches after leaf emergence throughout the year.

On the seasonal variability of microbial plant dwellers

Former studies on foliar microecology observed bacteria to be, by far, the most abundant inhabitants, with on average 10^6–10^7 bacterial cells per cm^2 of foliar surface (Lindow and Brandl 2003; Rastogi, Coaker and Leveau 2013). Investigations into the variation of microbial communities on leaves over multiple temporal and spatial scales provided detailed knowledge on the taxonomy and the ecology of bacterial leaf inhabitants (Thompson *et al.* 1993; Jacques, Kinkel and Morris 1995). Seasonal variability turned out to be a major driver of variation in these prokaryotic communities (Copeland *et al.* 2015; Bao *et al.* 2019; Grady *et al.* 2019). Another, but still neglected important factor shaping foliar bacterial communities are microbial predators, i.e. bacterivorous protists (Mueller and Mueller 1970; Bamforth 1973, 2007, 2010; Flues, Bass and Bonkowski 2017). Protistan predation has a profound influence on the structure and function of bacterial communities (Matz and Kjelleberg 2005; Rosenberg *et al.* 2009; Jousset 2012; Amacker *et al.* 2020). Since these microbial eukaryotes comprise a vast array of functional traits in morphologies, locomotion and nutrition types (Fiore-Donno *et al.* 2019; Dumack *et al.* 2020), we presume that different protistan taxa likely play complementary ecological roles within the highly heterogeneous habitat of forest canopy. In contrast to molecular surveys on seasonal changes in prokaryotic diversity (Rastogi *et al.* 2012; Copeland *et al.* 2015; Agler *et al.* 2016), studies on community shifts of protists over time were commonly conducted in aquatic systems for dominant taxa (Rynearson, Newton and Armbrust 2006; Aguilera *et al.* 2007) or at higher taxonomical level (Tamigneaux *et al.* 1997; Araújo and Godinho 2008). Studies on terrestrial protists often lack a temporal dimension. Consequently, analyses of seasonality in terrestrial protistan communities are still rare and limited to a relatively small range of ecosystem types, dominated by soil habitats (Fiore-Donno *et al.* 2019; De Gruyter *et al.* 2020; Fournier *et al.* 2020). Hence, the effect of a seasonal niche separation as a possible selective force for temporal shifts in protistan communities dwelling on plant surfaces remains largely unexplored.

Protists and their distribution mechanisms

Dispersal of unicellular eukaryotes in terrestrial environments is facilitated by dormant stages, i.e. resting cysts or spores (Foissner 1987, 2006; Verni and Rosati 2011). These can be carried over large distances by wind (Wilkinson 2001), rain and fog (Finlay 2002), or animals and humans (Revill, Stewart and Schlichting 1967; Schlichting and Sides 1969; Perrigo, Romeralo and Baldauf 2012). Recent studies on protistan diversity with taxon-specific primers allow for the first time a thorough recovery of the existing species richness in a habitat and indeed suggest a largely ubiquitous distribution within the same terrestrial ecosystem (Fiore-Donno et al. 2018, 2019; Degrune *et al.* 2019; Jauss *et al.* 2020). Considering the large surface area that trees extend into the atmosphere, the forest canopy may act as huge physical filter for airborne microorganisms and, after litter fall, may be conducive to their further spread into soils (Jauss *et al.* 2021). Accordingly, it may be suggested that colonization is largely driven by random dispersal, but because the canopy is subject to harsh and highly variable environmental conditions where only adapted species will successfully replicate and survive, we expect markedly distinct patterns of beta diversity instead of random community assembly throughout the seasons. Consequently, the composition of these communities will initially reflect the product of passive colonization, with persistence then selected by deterministic processes driven by biotic and abiotic factors (e.g. environmental filters). Moreover, the question arises whether protistan communities undergo further seasonal changes, forced by changing abiotic conditions and subsequent selective pressures, and/or by seasonal invasion of passively dispersed propagules.

In this study, we investigated seasonal changes in protistan communities of structurally different ecological compartments (microhabitats) across the canopy of three autochthonous tree species in a temperate floodplain forest. Therefore, four samplings were conducted in two consecutive spring and autumn

seasons, over a period of 2 years. We applied a MiSeq Illumina sequencing protocol using taxon-specific primers, which target the hypervariable V4 region within the 18S rRNA gene of the protistan phyla Cercozoa and Endomyxa (Rhizaria; Fiore-Donno, Richter-Heitmann and Bonkowski 2020). Cercozoa are a highly diverse phylum, with many taxa encompassing a broad variety of functional traits (Dumack *et al.* 2020). Further, Cercozoa appear to contain well adapted phyllosphere taxa (Ploch *et al.* 2016; Dumack *et al.* 2017; Flues *et al.* 2018), that withstand environmental extremes by quickly responding to fluctuating environmental conditions (Ekelund, Olsson and Johansen 2003; Holtze *et al.* 2003). In particular, their ability to rapidly excyst, feed and multiply within short generation times (Ekelund 1996; Glücksman *et al.* 2010; Flues 2017), is a perfect adaptation to the highly fluctuating environmental conditions up in the tree canopies over the seasons. The phylum Endomyxa, which was only recently separated from Cercozoa (Cavalier-Smith, Chao and Lewis 2018), is of particular interest for comprising diverse plant parasites of economic importance (Neuhauser *et al.* 2014; Bass, Ward and Burki 2019; Dumack *et al.* 2020).

We hypothesized that (I) cercozoan and endomyxan communities differ in their seasonal composition in tree canopies. (II) Functional diversity of communities differs spatially and temporally between different microhabitats. (III) Despite the presumption that tree canopies act as a filter for air-dispersed propagules, we expected highly distinct patterns of beta diversity to dominate over randomness in community assembly throughout all samplings.

MATERIAL AND METHODS

Sampling, DNA extraction and sequencing

Environmental samples were collected during spring and autumn within a period of 2 years: October 2017 and 2018, and May 2018 and 2019. The sampling took place in cooperation with the Leipzig Canopy Crane Facility (LCC) in a temperate deciduous floodplain forest in Leipzig, Germany (51.3657 N, 12.3094 E). All samples were obtained and processed as described in Jauss *et al.* (2020). Briefly, seven different microhabitat compartments were sampled related to the canopy surface at 20–30 m height: fresh leaves, deadwood, bark, arboreal soil and three cryptogamic epiphytes comprising lichen and two moss species, *Hypnum* sp. and *Orthotrichum* sp. For comparison, two microhabitats on the ground (leaf litter layer and mineral soil underneath up at to 10 cm depth) were sampled. All microhabitat samples were taken with replicates at all four cardinal directions from three autochthonous tree species (*Quercus robur, Tilia cordata* and *Fraxinus excelsior*) with biological triplicates each. DNA extraction was done according to the manufacturer's protocol with the DNeasy PowerSoil kit (QIAGEN, Hilden, Germany). DNA concentration and quality were checked using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, United States). For subsequent PCR amplification, all four replicates of each microhabitat per tree were pooled. Semi-nested PCRs with tagged group-specific primers (Fiore-Donno, Richter-Heitmann and Bonkowski 2020) and Illumina sequencing were performed as described in Jauss *et al.* (2020), the used primer and barcode combinations are provided in Tables S1 and S2 (Supporting Information).

Sequence processing

Sequence processing followed the pipeline described in Fiore-Donno, Richter-Heitmann and Bonkowski (2020). Briefly, paired reads were assembled using MOTHUR v.39.5 (Schloss *et al.* 2009) allowing no differences in the primer and the barcode sequences and no ambiguities. Next, assembled sequences smaller than 300 bp and with an overlap less than 200 bp were removed. The obtained sequences were checked for their quality and removal/cutting of low-quality reads were conducted with the default parameters. Afterwards, sequences were clustered into Operational Taxonomic Units (OTUs) using VSEARCH (Rognes *et al.* 2016) with abundance-based greedy clustering (agc) and a similarity threshold of 97%. Clusters represented by ≤0.005% (≤440 sequence reads) of the total number of reads were removed to mitigate sequencing noise due to errors during amplification and sequencing (Fiore-Donno *et al.* 2018). Sequences were taxonomically assigned with the PR^2 database (Guillou *et al.* 2013) using BLAST+ (Camacho *et al.* 2009) with an e-value of $1^{e\text{-}50}$, keeping only the best hit. Cercozoan and endomyxan sequences were aligned with a template provided in Fiore-Donno *et al.* (2018) in MOTHUR. Chimeras were identified using UCHIME (Edgar *et al.* 2011) as implemented in MOTHUR; chimeras and misaligned sequences were removed.

To explore the sequencing depth of each sample per sampling period, the final OTU table was loaded into QIIME2 v2018.11 (Bolyen *et al.* 2019). To discard samples suffering from shallow sequencing, a threshold for a minimum number of sequences per sample was determined for further analyses. The threshold was set as high as possible: at least five samples per microhabitat and 15 samples per tree species within each sampling period (≤7525 reads/sample).

Functional trait assignment

We classified the protistan OTUs according to their nutrition type into bacterivores, eukaryvores and omnivores (i.e. feeding on both, bacteria and eukaryotes) as in Dumack *et al.* (2020). The phytomyxean parasites, due to their peculiar life cycle, were considered separately. We assigned the nutrition types at the genus level (Table S3, Supporting Information).

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 (Hsieh, Ma and Chao 2021) to determine if a higher sequencing depth would have revealed more diversity. Alpha diversity (i.e. Shannon diversity index) was calculated for each microhabitat per sampling season using the *diversity* function in the vegan package (Oksanen *et al.* 2019). Pairwise differences were tested with ANOVA, differences between multiple means by subsequent Tukey's HSD (function *HSD.test*), as implemented in the agricolae package (De Mendiburu and Yaseen 2020). Analysis of season correlated OTU abundances was performed with the DESeq2 package (Love, Huber and Anders 2014) at the 1% significance level.

In order to assess the main environmental factors responsible for differences in beta diversity, separately for canopy and ground (litter and mineral soil) samples, variance partitioning analyses were carried out on the Hellinger-transformed table (function *varpart* in the vegan package); the explanatory variables that significantly explained variation in protistan community composition were determined by forward selection using the *ordistep* function in vegan. Non-metric multidimensional scaling (NMDS) on the Bray–Curtis dissimilarity matrix was performed to analyse beta diversity patterns between protistan communities detected across different microhabitats, tree species and sampling seasons (function *metaMDS* in the vegan

package). A permutational multivariate analysis of variance (PERMANOVA, function *adonis*) was conducted to test if protistan communities differed between the microhabitats, tree species as well as the seasons. The number of shared OTUs between different combinations of sampling periods was visualized using the UpSetR package (Lex *et al*. 2014; Gehlenborg 2019). Figures were plotted with the ggplot2 package (Wickham 2016). Cercozoan and endomyxan diversity was illustrated using the Sankey diagram generator (http://sankeymatic.com/, 21 March 2021, date last accessed).

RESULTS

Sequencing results

We obtained 783 genuine cercozoan and endomyxan OTUs from 324 ground and canopy microhabitat samples representing ca. 1.5 million filtered sequences per sampling period and 6 157 731 high quality sequence reads in total (Table S4, Supporting Information). However, 34 samples (ca. 10%) were removed because the yield was not sufficient (≤7525 reads/sample). The remaining 290 samples yielded on average 20 657 reads/sample (min. 7633, max 57 404 and SD 9521). The average number of OTUs was 780 ± 2781 ± 2 and 774 ± 2 per microhabitat, tree species and sampling period, respectively. In total 22% of the OTUs showed a sequence similarity of 97–100% to any known reference sequence (Fig. 1B). OTU001 occurred with exceptionally high read abundances in the canopy, being 18-fold higher than in the ground stratum (1 183 933 vs 67 009 reads; ANOVA: $F = 68.98$, $P < 0.001$, Fig. 1A). This OTU001 had 86.14% sequence similarity to a sequence of an undetermined glissomonad taxon (Fig. 1A and Table S5, Supporting Information).

Sequencing effort was sufficient for the majority of microhabitats in both autumn samplings, where the total OTU richness was reached after only ca. 200 000 sequences. In spring samples, however, rarefaction curves for several microhabitats did not reach a plateau, especially for the samples of fresh leaves (Figure S1, Supporting Information), suggesting that we underestimated the OTU richness in this habitat. A database with OTU abundances, taxonomic and functional assignment is provided (Table S3, Supporting Information).

Seasonal variation and spatial structuring

Investigation into seasonality patterns of OTUs revealed 81 OTUs with a higher frequency ($P < 0.01$) in one of the two different seasons (Fig. 2). These comprised 54 OTUs in autumn, with four OTUs belonging to the phylum Endomyxa and 50 cercozoan OTUs. In spring, 27 distinct cercozoan OTUs were particularly abundant. OTU394, within the genus *Rhogostoma*, appeared to be the most temporarily abundant OTU in autumn, followed by OTU627 assigned to the genus *Thaumatomonas* and three endomyxan OTUs (OTU274, OTU230 and OTU566) of which more than >96% of their reads were solely found in autumn 2017 (Figure S2 and Table S6, Supporting Information). The endomyxan OTUs were assigned to root parasites (*Polymyxa betae*, OTU274; *Spongospora nasturtii*, OTU230) in the order Plasmodiophorida and a vampyrellid (OTU566). Interestingly, these endomyxan OTUs were equally distributed across all canopy microhabitats and the ground. In spring, *Bodomorpha* sp. (OTU429), was highly abundant together with OTUs assigned to the genus *Thaumatomonas* (OTU472), two different *Euglypha* OTUs (OTU670, OTU675) and one *Paracercomonas* sp. (OTU735).

Alpha diversity of microhabitats showed similar patterns for both seasons (Figure S3A, Supporting Information): fresh leaves and deadwood showed lower OTU richness, Shannon diversity and evenness as compared to bark and mosses (*Orthotrichum* sp., *Hypnum* sp.). On the ground, leaf litter contained lower OTU richness, Shannon diversity and evenness than the mineral soil in both seasons. Shannon diversity and evenness of canopy microhabitats did not change between seasons, with the exception of deadwood, which harbored a higher alpha diversity in autumn (Figure S3B, Supporting Information). However, OTU richness of fresh leaves showed high variation in spring, and was significantly lower as compared to autumn.

Variance partitioning showed that most variation in protistan communities was explained by microhabitat identity, with 31% and 18% variation explained in the ground and canopy stratum, respectively (Fig. 3). Yet, a small, but significant proportion of variation accounted for differences between the two seasons (ground: 5% and canopy: 2%). Further, differences between tree species explained 2% of community variation in the ground stratum and 5% of community variation in tree canopies, respectively. However, communities of fresh leaves did not differ between tree species (PERMANOVA: R^2 0.097, $P = 0.087$, Supplementary Table S7), while the seasonal effect in the litter layer was dependent on tree identity (PERMANOVA: R^2 0.087, $P = 0.041$; Table S7, Supporting Information).

Differentiation of foliar communities

Interestingly, cercozoan and endomyxan leaf litter communities were more similar to canopy communities than to the communities from the mineral soil directly underneath, especially in autumn 2017 and spring 2018 (NMDS; Figure S4, Supporting Information). Beta diversity of protistan communities on fresh leaves changed markedly between spring and autumn: In spring, communities scaled closer to other canopy microhabitats, but they became completely distinct in autumn. Arboreal soil contained highly variable communities in all four sampling periods, ranging from communities very similar to those of bark and epiphytes to communities closely resembling the mineral soil communities below the litter layer. Highly significant seasonal differences in beta diversity could be detected across all microhabitats (Table S7, Supporting Information), however, almost 98% of OTUs were shared between all sampling periods (Figure S5, Supporting Information). Accordingly, differences in community composition were almost entirely based on temporal and spatial changes in the relative abundance of OTUs. A separate NMDS analysis of fresh leaves communities only showed highly distinct spring and autumn communities (Fig. 4, PERMANOVA: R^2 0.149 and $P = 0.001$). Foliar communities turned out to be highly variable in spring and, and furthermore differed between the two sampling periods (PERMANOVA: R^2 0.175, $P = 0.025$). In autumn communities the variation was much lower, but communities of both autumn samplings still showed significant differences (PERMANOVA: R^2 0.216, $P = 0.001$), suggesting a variable outcome after the recurrent colonization of fresh leaves over the seasons.

Functional diversity

More than three-quarters of the cercozoan and endomyxan reads within the canopy were bacterivores (77 ± 9%), followed by omnivores (18 ± 7%), sequences of unknown nutrition type (4 ± 2%) and only very few eukaryvores (2 ± 1%; Fig. 5). Communities of ground microhabitats showed a relatively smaller

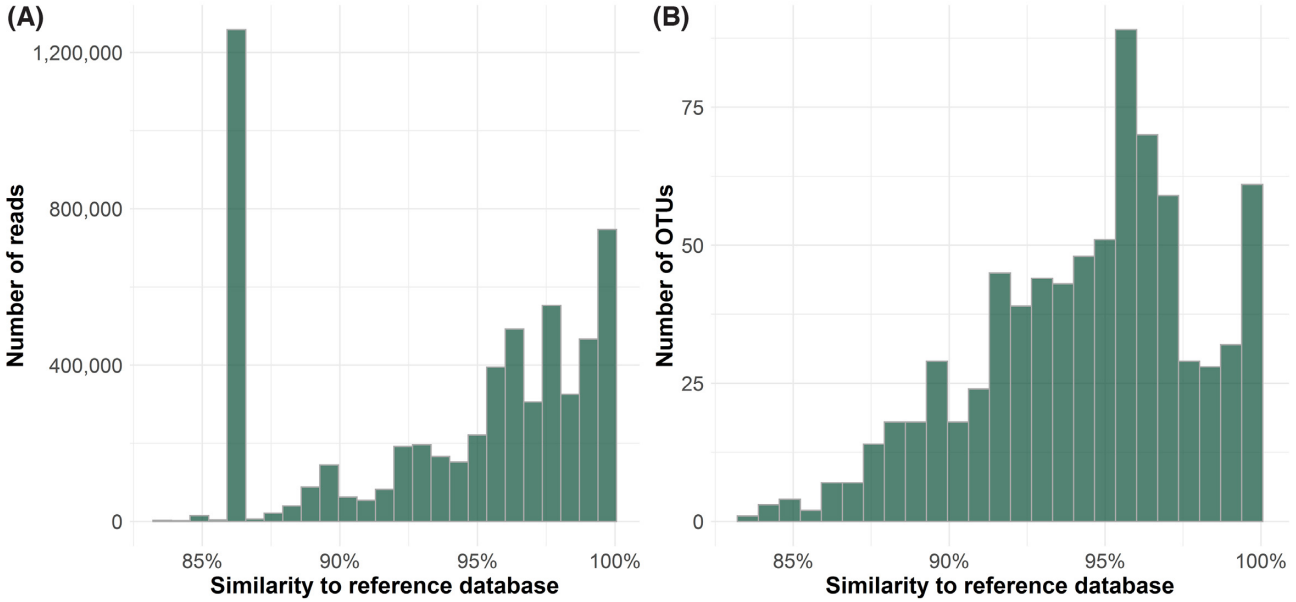

Figure 1. Similarity of protistan reads and OTUs to the reference database. Only 37% of all reads **(A)** and 22% of all OTUs **(B)** were ≥97% similar to sequences within the PR^2 database. Read numbers of OTU001 (long bar in Fig. 1A) exceed more than one million reads in tree canopies.

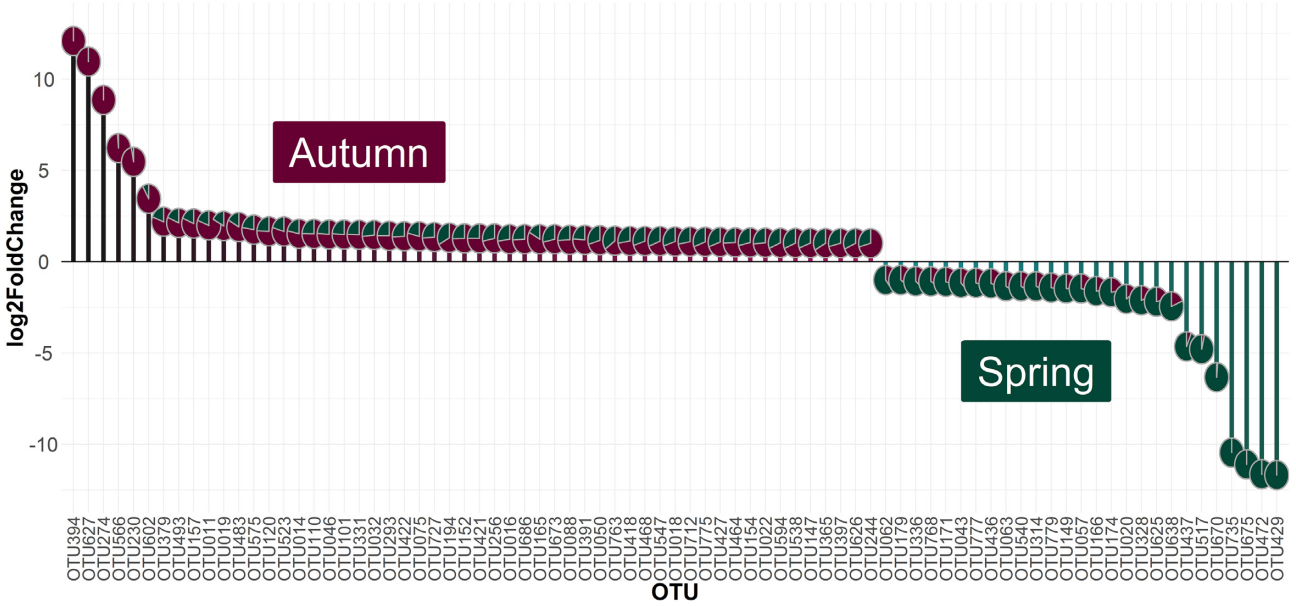

Figure 2. Analysis of season correlated OTUs. Investigation of autumn and spring communities revealed 54 and 27 OTUs with predominance in autumn and spring samplings, respectively ($P < 0.01$). Pie charts on the top of the bars represent the relative proportion of each OTU either in the autumn (purple) or spring (green) season.

proportion of bacterivores (55 ± 12%; ANOVA: $F = 31.09$ and $P < 0.001$) and more omnivores (26 ± 7%; ANOVA: $F = 8.14$ and $P < 0.01$), as well as a greater share of eukaryvores (5 ± 2%; ANOVA: $F = 49.87$ and $P < 0.001$) as compared to the canopy microhabitats. Plant parasites and parasites of other host organisms were only marginally present, on average <1%, except in autumn 2017, where soil communities contained 2.4% of reads derived from parasitic taxa. Thus, most variation in protistan functional diversity was explained by differences between the microhabitats (PERMANOVA: R^2 0.721, $P = 0.001$; Table S8, Supporting Information). However, differences between the two sampling seasons were not detected (PERMANOVA: R^2 0.002 and $P = 0.802$).

DISCUSSION

This study aimed to identify seasonal changes in the community composition of Cercozoa and Endomyxa in tree canopies, over two consecutive years. A total number of 783 OTUs were detected in the temperate floodplain forest, which is 43% of the cercozoan OTU richness that Fiore-Donno, Richter-Heitmann and Bonkowski (2020) retrieved with the same method from mineral soil of 150 different forest sites across Germany. The thorough recovery of the diversity, including even rare taxa due to the taxon-specific primers (Fiore-Donno *et al.* 2018), enabled a direct comparison between protistan communities dwelling

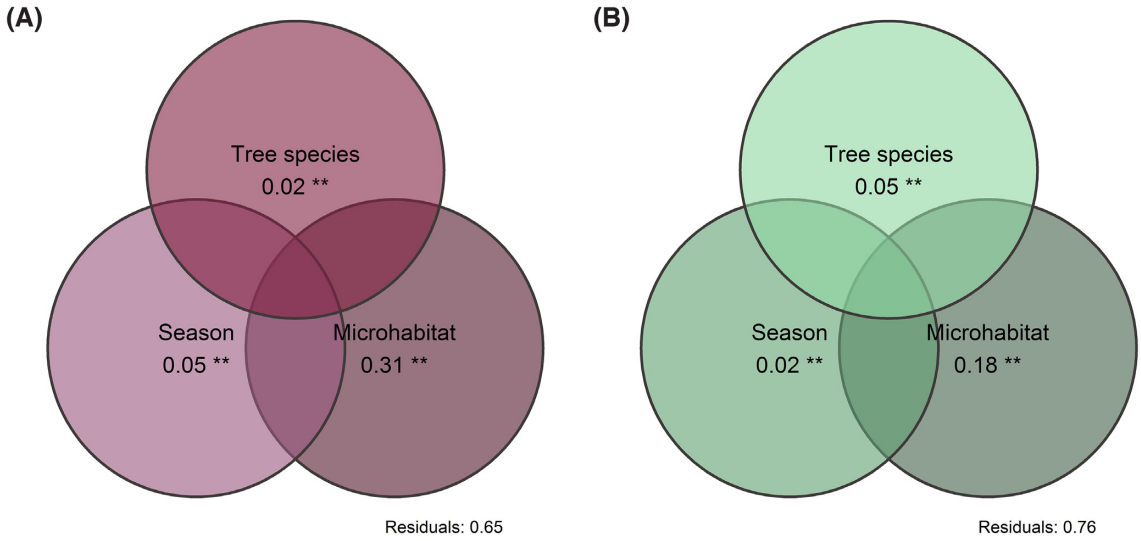

Figure 3. Variance partitioning of cercozoan and endomyxan communities between season, microhabitat and tree species, separately for ground **(A)** and canopy **(B)**. Microhabitat identity always explained most variation, followed by differences between tree species and sampling season. The significance of particular effects was tested by forward selection and is indicated by asterisks (** = $P < 0.01$).

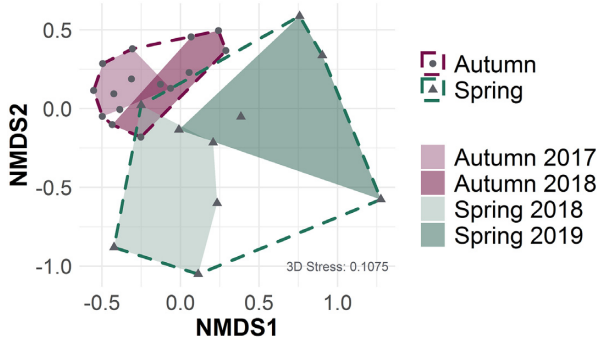

Figure 4. Non-metric multidimensional scaling (NMDS) of Bray–Curtis dissimilarities of foliar communities among sampling periods. Cercozoan and endomyxan communities of fresh leaves where highly distinct between all four sampling periods, especially between the two seasons. The Stress value is shown in the lower right of the graph.

in different microhabitats within the forest canopies throughout both seasons. We showed that, in principle, all detected OTUs could be found across all microhabitats in every sampling period, but habitat diversity strongly favored distinct protistan taxa in terms of abundance, a pattern which was already described by Jauss *et al.* (2020). However, patterns of cercozoan and endomyxan beta diversity in tree canopies were strikingly divergent from communities detected on the ground, showing that distinct species dominated the different communities throughout all samplings. This was in particular true for a highly abundant glissomonad OTU (OTU001), with exceptionally higher relative abundance in the canopy compared to the ground stratum.

Seasonal variability of protists in tree canopies

Seasonality between spring and autumn explained 5% and 2% of the variation in beta diversity of ground and canopy communities, respectively (Fig. 3). About 10% of protistan OTUs were specifically associated with either spring or autumn season (Fig. 2). For example, a *Rhogostoma* sp. (OTU394), belonging to omnivorous thecate amoebae in the Cryomonadida, was temporally the most abundant taxon in autumn, while a bacterivorous *Bodomorpha* sp., from the order Glissomonadida, dominated in spring. Differences between spring and autumn communities were particularly evident on canopy leaves (Fig. 4). In spring, beta diversity of fresh leaves still showed some overlap with other canopy microhabitats (Figure S4, Supporting Information). However, rarefaction curves of fresh leaves did not reach a plateau, and OTU richness appeared to be lower and showed higher variation (Figures S1 and S3B, Supporting Information), indicating high heterogeneity during initial colonization shortly after leaf emergence in spring, while the distinct separation of these communities in autumn indicates that specific foliar communities had established (Figure S4, Supporting Information). Furthermore, beta diversity of fresh leaves communities showed only a slight overlap between both autumn samplings, indicating variable outcomes of community assembly, likely driven by the prevailing seasonal factors; October 2017 was an exceptionally warm and wet month, while October 2018 and the prior spring season were rather dry (DWD 2021). However, in autumn 2017 and spring 2018, leaf litter communities appeared more similar to foliar communities in the canopy than to the underlying mineral soil communities (Figure S4, Supporting Information), suggesting that leaf litter still carries a signature of the preceding foliar community (Jauss *et al.* 2020).

Our environmental sequencing method, based on ribosomal DNA, did not allow to distinguish between active protists and their resting or dispersal stages, but instead must be considered as an integrative long-term measure of taxa that replicated well and formed resting stages in respective microhabitats. The consistent differences in beta diversity between microhabitats indicate that well-adapted taxa accumulated and dominated over those that arrived as resting stages by passive dispersal throughout both seasons. This leads to functional differences between communities of spatially separated microhabitats (Fig. 5). As almost 80% of the OTUs showed a similarity of less than 97% to any known sequence in the reference database, nutrition types can only be inferred from related taxa (Dumack *et al.* 2020). Our data confirm the existence of a substantial undescribed taxonomic diversity of Cercozoa, a dominant phylum of microbial eukaryotes in terrestrial ecosystems (Singer *et al.* 2021).

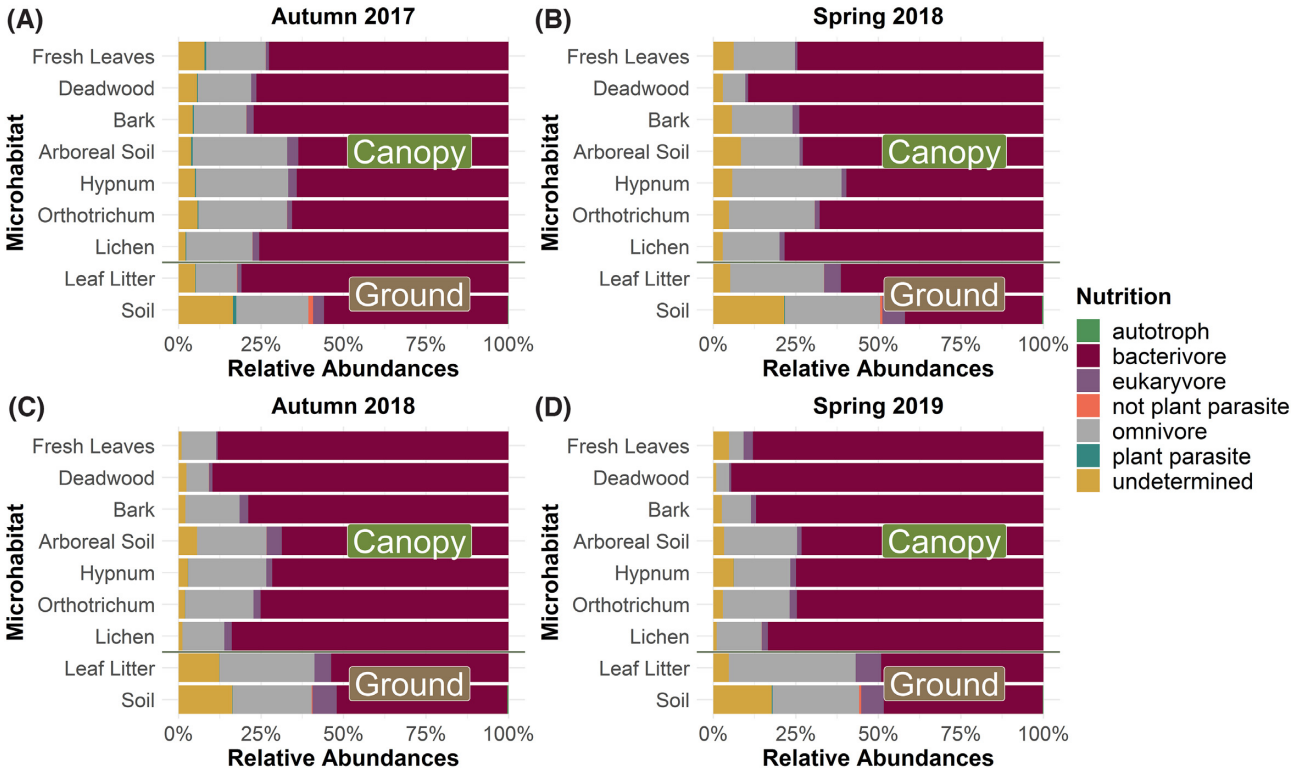

Figure 5. Relative read abundances of functional groups per sampled microhabitat and sampling period. Functional diversity of autumn (**A** and **C**) and spring samples (**B** and **D**) did not differ between seasons. Whereas, differences between the microhabitats were significant throughout all sampling periods: Bacterivores dominated, especially in tree canopies, whereas a higher proportion of omnivores and eukaryvores occurred on the ground.

Taxonomical and functional diversity

The majority of the 783 OTUs could be assigned to the phylum Cercozoa (97%), the remaining to Endomyxa (3%) and to the *incertae sedis* Novel clade 10 (Tremulida <1%; Figure S6, Supporting Information). With 753 OTUs cercozoan diversity was in line with previous studies, which recognized Sarcomonadea (Glissomonadida and Cercomonadida) as the dominant class in terrestrial habitats (Geisen *et al.* 2015; Bugge Harder *et al.* 2016; Fiore-Donno *et al.* 2018; Fiore-Donno, Richter-Heitmann and Bonkowski 2020). Especially the small and bacterivorous flagellates in the order Glissomonadida dominated in all canopy microhabitats throughout all four sampling periods (Figs 1 and 5; Figure S7, Supporting Information). The Sarcomonadea were followed by the mainly omnivorous testate amoebae within the orders Euglyphida and Cryomonadida. These omnivores feed on both, bacteria and small eukaryotes, such as yeasts, algae and other protists (Dumack *et al.* 2020). While bacteria appeared as an essential food source in tree canopies, cercozoan communities of litter and mineral soil were characterized by a distinctively higher proportion of eukaryvores, which was mostly related to higher relative read numbers of vampyrellid amoebae that feed on a wide range of soil eukaryotes, including fungal mycelia and spores, algae, as well as nematodes (Anderson and Patrick 1980; Pakzad and Schlösser 1998; Hess, Sausen and Melkonian 2012). Our findings reflect the results of Fiore-Donno, Richter-Heitmann and Bonkowski (2020), who found a high proportion of vampyrellids, but almost no other Endomyxa in mineral soil samples of diverse forests in different regions across Germany. In addition, reads derived from taxa of so far undetermined nutrition type were enriched in litter and soil compared to canopy samples (Fig. 5), reflecting a larger proportion of unknown diversity within microeukaryote food webs on the ground than in the physically harsh environment of the tree crown.

Most variation in cercozoan and endomyxan functional diversity was explained by microhabitat identity, whereas seasonal shifts were not detected. However, seasonal differences can be presumed when taking taxonomically assigned relative read abundances into account (Figure S7, Supporting Information). One explanation for this observation could be that the abundance of less dominant orders was more variable between the microhabitats and seasons. But since some functional traits, especially food preferences are still understudied, a measurable proportion of reads could not be assigned to any nutrition type (ground: 12 ± 7%, canopy: 4 ± 2%).

Forest canopies as filters of potential plant pathogens

Distribution of endomyxan plant parasites appears spatially restricted and is likely related to their host plants. Fiore-Donno, Richter-Heitmann and Bonkowski (2020) found diverse and abundant endomyxan plant parasites in grassland soils, but not at all in soils of nearby forests. We, however, found two temporally abundant OTUs among autumn communities which could be assigned to the root pathogens *S. nasturtii* and *Polymyxa betae* (Phytomyxea: Plasmodiophorida, Fig. 2). *Spongospora nasturtii* is an obligate biotrophic root pathogen of watercress (*Nasturtium officinale*), a common herb of river banks in floodplain forests (Down, Grenville and Clarkson 2002); whereas, *P. betae* is an obligate root parasite in beet roots (Tamada and Asher 2016). Although its potential host range also includes Chenopodiaceae, Caryophyllaceae and Papaveraceae, (Barr and Asher 1992; Neuhauser *et al.* 2014), none of these host plants occurred

in the sampled floodplain forest. The ubiquitous distribution of these two endomyxan root pathogens among protists detected in tree crowns, litter and soil in autumn samples (Figure S2, Supporting Information) reflects the complex life cycle of these taxa with distribution via sporangia in autumn (Barr and Asher 1996). The high potential of air dispersal of protistan propagules, was recently emphasized by Jauss *et al.* (2021) and together with our results it indeed appears that tree canopies may play an important role as physical filters of plant pathogenic microbial propagules that may partly prevent their further spread to the environment.

CONCLUSION

Investigating two important protistan lineages, Cercozoa and Endomyxa, over a period of 2 years revealed strong differences in community composition across canopy and soil microhabitats, and a small, but significant fraction of recurrent seasonal variability of these communities. We observed lower beta diversity of canopy communities in spring compared to autumn. Especially foliar communities changed during the aging of leaves, emphasizing the effect of phenology during community assembly. One particular glissomonadid OTU was identified as a clear canopy specialist, while high read numbers of root parasitic phytomyxean OTUs in tree canopies during autumn indicate an important role of the canopy surface as a physical barrier for air-dispersed protistan pant pathogens. In two consecutive seasons, leaf litter communities showed more similarity to foliar canopy communities than to those of the soil directly underneath. This indicates that communities colonizing the foliar surface leave a legacy in the litter layer on the forest floor. The litter, however, becomes strongly enriched in omnivores and eukaryvores relative to bacterivores dominating in the canopy. Overall, the described diversity of Cercozoa and Endomyxa in this study is just one striking example among dozens of microbial eukaryote phyla whose canopy inhabitants still await discovery.

DATA ACCESSIBILITY

Raw sequence data have been submitted to the European Nucleotide Archive (ENA) database under the Bioproject number PRJEB37525, with run accession numbers ERR3994029, ERR4913261 and ERR4911998.

Tables, figures, codes and detailed bioinformatic/statistical methods used in this study are available at: https://github.com/SusanneWalden/OnthePhenologyofProtists.

AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflicts of interest. None declared.

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