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THE PROTISTS OF TREE CANOPIES

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“Yet another continent of life remains to be discovered, not upon earth, but one to two hundred feet above it… At present we know almost nothing of it. Up to now gravitation and tree-trunks swarming with terrible ants have kept us at bay, and of the tree-top life we have obtained only unconnected facts and specimens.“

- William Beebe, 1918

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Abstract

Eukaryotic microorganisms, i.e. protists, are the base of all multicellular life on Earth, which independently evolved in three of the 26 major eukaryotic lineages. Apart from dominating eukaryotic diversity, protists play fundamental roles in many Earth system processes – representing all trophic levels, including autotrophs, mixotrophs, saprotrophs, eukaryvores, omnivores as well as parasites and their hyperparasites. Recent progress in molecular methods for the first time opens a window into the black box of protistan diversity in terrestrial ecosystems. Among terrestrial habitats, the highly heterogeneous habitat represented by tree canopies is presumed to harbour diverse but, until now, poorly characterized communities of these microbial eukaryotes. A majority of protists that have been identified to strongly interact with plant surfaces can be assigned to the Stramenopiles-Alveolata-Rhizaria (SAR) supergroup, in particular representatives belonging to the monophyletic group of Cercozoa (Rhizaria). Their ability to rapidly excyst, feed and multiply within short generation times, represents an advantageous evolutionary adaptation to the highly variable microclimatic conditions prevailing up in the tree crowns. For this PhD thesis we perfomed a series of metabarcoding analyses with highly group-specific primers to comprehensively analyse communities of Cercozoa in various ecological compartments (microhabitats), from the forest floor to the canopy region, across two biomes (temperate and tropical forest).

The first objective of this thesis was to investigate to which degree the environmental heterogeneity of tree canopies represents a deterministic force governing the structure and diversity of cercozoan communities. Furthermore, we wanted to elucidate the adaptive functional traits that are facilitating the survival of taxa via adaption to the physicochemically different microhabitats across the tree crown. In the first part of Chapter I, we were able to demonstrate how habitat filtering in canopy microhabitats led to specific differences in the composition of cercozoan communities, and ultimately within the entire ecosystem of a temperate floodplain forest. Interestingly, the majority of taxa were present in all microhabitats, and therefore differences in beta diversity were mainly related to taxa performance (i.e. relative abundance of taxa).

In order to obtain a comparison on the same basis, we investigated cercozoan diversity in a palaeotropical rainforest in the northeast of Papua New Guinea in the second part of Chapter I. Tropical forest canopies harbour a vast diversity of multicellular organisms, but it is still little explored if eukaryotic microorganisms reflect similar hyperdiversity patterns compared to temperate zones. Our data indicated a higher taxa richness in the tropical biome compared to data obtained from the temperate forest. In fact, taxa richness increased with the number of sampled microhabitats, which is a pattern hitherto only observed at a macroscopic scale. Overall, our results led to the conclusion that certain cercozoan taxa and lineages were exclusively present in the palaeotropical forest.

Taxonomic assignment of our sequence data from temperate and tropical biomes identified the cercozoan family of Rhogostomidae (Cryomonadida), a group of omnivorous thecate amoebae, to be highly abundant and extremely diverse in the tree canopy. In Chapter II of this thesis we focussed on the putative cryptic diversity of Rhogostomidae by critical re-evaluating Rhogostomidae sequence data obtained from environmental sequencing studies from various terrestrial habitats, inter alia, the forest canopies. This study revealed 23 new clades and extended the untapped diversity of Rhogostomidae by the description of eleven new *Rhogostoma* species.

Besides investigating deterministic factors (e.g. habitat filtering for better adapted taxa) that contributed to cercozoan community assembly in the tree crowns, we also shed light on the hitherto unknown stochastic processes that appeared to be responsible for the ubiquitous distribution of Cercozoa in the canopy region. With the studies represented by Chapter III and IV we identified air-dispersal and phenology to play major roles for cercozoan colonization and community assembly in the forest roof. In this context we uncovered the seasonal variability of Cercozoa in the canopy surrounding air and canopy microhabitats. Moreover we found first evidence for the function of tree canopies as a physical barrier for air dispersed microbial pathogens and identified ecological priority effects to be responsible for the outcome of cercozoan community assembly in the canopy**.** With the findings described herein, we were the first to assess quantitative and qualitative informations on canopy associated cercozoan communities, which, in turn, contribute to the understanding of the processes governing distribution and selection of microbial eukaryotes in terrestrial ecosystems. Moreover, our investigations provide new insights into the ecosystem functioning as well as ecosystem services tree canopies provide.

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GENERAL INTRODUCTION

General Introduction

Forest Ecosystems – A Reservoir for Biodiversity

On a global scale, forests cover nearly one third of the land area and harbour over 80% of terrestrial biodiversity (Aerts and Honnay 2011). This forest biodiversity comprises all life forms colonizing forested areas as well as their ecological roles. As such, the prevailing biodiversity encompasses not just a multitude of plants, but also animals as well as microbial life inhabiting forest areas and their associated genetic diversity. The biodiversity of forest ecosystems is expected to result in high levels of adaptation as a consequence of evolutionary processes, formed over the course of thousands and even of millions of years, which, in themselves, are driven by strong ecological forces such as competition and disturbance (Schamp and Aarssen 2009; Martín, Díaz-Raviña and Carballas 2012; Jensen and Löf 2017). As a result, forest biological diversity is directly linked to services provided by these forest ecosystems, including the prevention of soil erosion, pest control (e.g. control of disease vectors), and thus local ecosystem stability and resilience (Thompson *et al.* 2009). Consequently, the ability of forests to provide ecosystem services is expected to be dependent upon the maintenance of the forest biological diversity (Emmett Duffy 2009; Brockerhoff *et al.* 2017).

In total, more than three trillion trees exist on Earth, of which 22% can be found in temperate and 24% in boreal regions. However, the undisputed champion in forest tree densities is clearly represented by the tropical and subtropical biomes, harbouring 43% of all trees worldwide (Crowther *et al.* 2015). Thus, it can be presumed that the tropical vegetation represents the most fundamental rainforest component by highly contributing to the structure and biomass within this ecosystem (Gentry 1992). Despite the high tree density, the enormous tree diversity of the tropics appears to be even more astounding: 0.5km² of rainforest in Borneo or Amazonia are reported to contain as many tree species as 4.2×10^6 km^2 of the temperate forests that cover Europe, North America and Asia combined (Wright 2002).

Reaching the Forest Roof

The canopy region has always represented a reservoir of difficult-to-access diversity. Historically, forest canopies have been among the most poorly understood regions of all terrestrial ecosystems (Erwin 1983). Many difficulties in forest canopy research arose from obstacles related to access the treetops and the subsequent challenges of sampling – thinking back to early European explorers who were hiring climbers or even trained monkeys to collect specimens that were far out of reach (Trichon 2002). But even many decades later, so called 'low-tech' access methods, like slingshots or ropes, were still in use: in the late 1970s and early 1980s several articles on single-rope climbing techniques had been published (Perry 1978; Perry and Williams 1981; Whitacre 1981; Landsberg and Gillieson 1982) and even a handbook on techniques of access and study in the canopy (Mitchell 1982), which has been out-of-print for several years. However, considering the large surface area that tree canopies extend into the atmosphere, it is conceivable that rope-based access methods were not exactly ideal to reach the leafy perimeters, since these techniques were restricted to positions close to the tree trunk (Lowman 2009). Hitherto, forest canopy science is still an emerging frontier of biodiversity studies, but high-tech methods like the usage of towers and construction cranes allowed, for the first-time, access to any region of the canopy without regard to the tree trunk. In total, 12 forest canopy research cranes are operating in the world today (Basset, Horlyck and Wright 2003), providing the ability to carry a gondola with researchers and instruments from the bottom to the top of trees in even difficult to reach areas. Therefore, canopy cranes became an indispensable tool for investigating biological diversity as well as the ecophysiology in the forest roof.

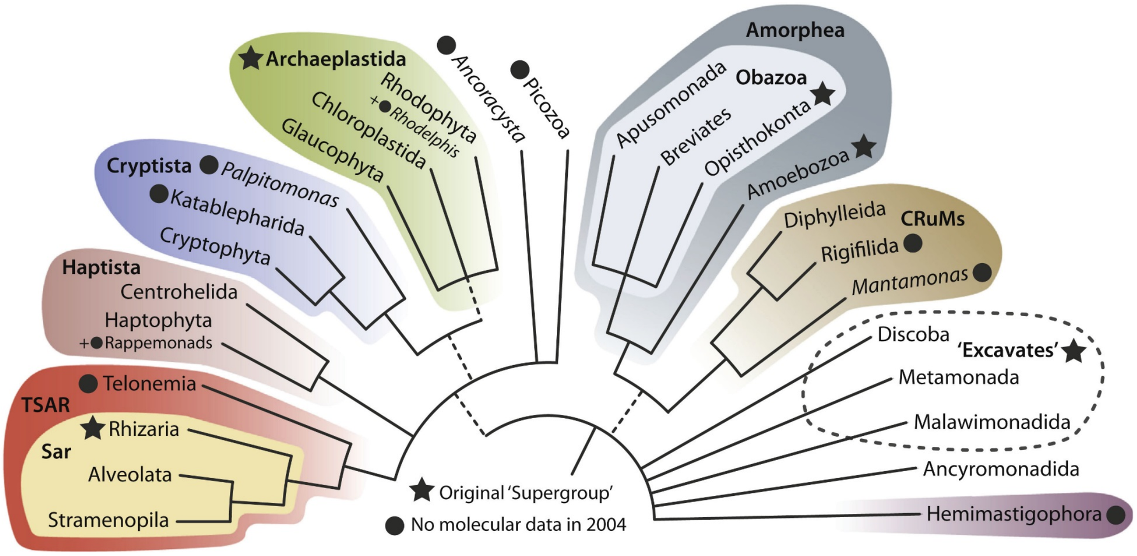
The Nature of Forest Canopies

Vegetation diversity has been reported to have an impact on animal diversity by affecting the quantity and quality of food for consumers, and by enhancing the habitat complexity, thereby affecting niche availability for specialist species (Kissling, Field and Böhning-Gaese 2008; Castagneyrol, Jactel and Cardinale 2012). In particular, the structurally and physicochemically highly heterogeneous habitat of forest canopies is expected to largely affect biodiversity in terms of niche availability (Nadkarni 2001). Forest canopies are the primary interface between the Earth's terrestrial biomass and the atmosphere, controlling gas exchange and evaporation on regional scales (Ozanne *et al.* 2003; Ellwood and Foster 2004; Karl *et al.* 2004). 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, Lowman and Nadkarni 1995), the forest canopy provides numerous of highly diverse ecological compartments (microhabitats) that were conducive to the evolution of epiphytic plants (Lyons, Nadkarni and North 2000; Nadkarni 2001), small mammals and birds (Carey and Wilson 2001; Goetz *et al.* 2007) as well as arthropods (Ishii, Tanabe and Hiura 2004).

A recent study on tree-colonizing microorganisms (i.e. Bacteria, Archaea and microfungi) revealed highly specific communities across broader microhabitat compartments (i.e. soil, stem and leaves) (Cregger *et al.* 2018). Moreover, significant community turnover across canopies of different tree species was reported for bacterial canopy inhabitants (Lambais *et al.* 2006). Investigations on wood-decaying fungi in tree crowns further revealed distinct variation in their community composition, with respect to the tree species, microhabitat and substrate preference (Unterseher, Otto and Morawetz 2005; Unterseher and Tal 2006). In the phyllosphere, i.e. the whole aerial region of plants dominated by leaves (Vorholt 2012), microbial fungal communities were reported to differ markedly between the interior and surface of individual leaves, whereas several fungal taxa showed significant preference between the sampling height and/or at the distance from the trunk (Osono and Mori 2004). These observations were corroborated by a study on the distribution patterns of leaf-inhabiting endophytic fungi by Unterseher et al. (2007), where tree species, different light exposures as well as sampling season were highly deterministic for several endophytic taxa. Thus, the microenvironmental conditions (e.g. UV radiation, temperature and humidity) prevailing across these highly heterogeneous microhabitats in the tree canopy are presumed to induce deterministic processes effectuating habitat filtering. In the light of microbial communities, habitat filtering is therefore expected to select for optimal eco-physiological traits that confer appropriate physiological tolerance to specific environmental conditions, and thus lead to either convergent or divergent distribution in traits of taxa coexisting within a microhabitat (Freschet *et al.* 2011).

Protists

Eukaryotic unicellular organisms (i.e. protists) are the base of all multicellular life on Earth, which independently evolved in three of the 26 major eukaryotic lineages (Figure 1). Thus, the polyphyletic group of protists represents a vast majority of the eukaryotic diversity that has thrived for hundreds of millions of years before multicellularity rose to prominence during the Proterozoic–Phanerozoic transition (Javaux, Knoll and Walter 2001; Bengtson *et al.* 2017). For centuries, protists were most often studied by light microscopy, and thus were solely classified on the base of morphological characteristics into four major morphogroups (i.e. flagellates, ciliates, naked and testate amoebae), irrespective of their fundamentally different evolutionary relationships and lifestyles (Hausmann, Hülsmann and Radek 2003). However, previous estimates indicate a worldwide existence of 60.000-300.000 species (Foissner 2008; Mora *et al.* 2011) of which only a small fraction is known to the scientific world (Adl *et al.* 2007).



Trends in Ecology & Evolution

Figure 1: The New Tree of Eukaryotes. The colored groupings correspond to the current 'supergroups'. Unresolved branching orders among lineages are shown as multifurcations. Dashed lines reflect lesser uncertainties about the monophyly of certain groups. Star symbols denote taxa that were considered as supergroups in early versions of the supergroup model; thus, all original supergroups except Archaeplastida have either disappeared or been subsumed into new taxa. The circles show major lineages that had no molecular data when the supergroup model emerged (Extracted from Burki *et al.* 2020).

During the last decades, progress in molecular methodology has finally led to a widely accepted consensus in protist taxonomy. The discovery of DNA barcodes (relatively short standardized gene sequences in well-defined genes) revolutionized the field of microbial taxonomy research. As a consequence, Sanger sequencing of DNA barcodes has become the 'gold standard' for species identification and taxonomic classification (Purty and Chatterjee 2016). The method of choice for protistan barcoding surveys soon consisted in sequencing fragments of the small subunit ribosomal RNA (SSU or 18S) gene, representing the gene with the largest reference database (Pawlowski *et al.* 2012). For the first time, it was possible to investigate species boundaries by grouping unknown taxa based on their barcode sequence to already known or even new species. Moreover, sequencing of DNA barcode markers uncovered a remarkably high cryptic diversity within morphologically defined protistan taxa (Fenchel 2005; Kosakyan *et al.* 2012; Škaloud and Rindi 2013) – the term 'cryptic species' is used for morphologically indistinguishable species, which are often identified only by taking genetic information into account (Hebert *et al.* 2004). Soon, the cultivation-dependent Sanger sequencing technology became inferior to Next-Generation Sequencing (NGS) technologies, which were suddenly capable of producing millions of sequence reads from environmental samples, simultaneously. Finally, application of aforementioned environmental sequencing methods gave first insight into the unimagined diversity among protists, especially in terrestrial environments (Geisen *et al.* 2016; Mahé *et al.* 2017; Venter *et al.* 2017).

To target a wide range of eukaryotic microorganisms usually so-called 'universal primers' were used (Baldwin *et al.* 2013; Bates *et al.* 2013). However, experience has revealed numerous drawbacks of this approach: apart from missing a large proportion of protistan diversity due to selection against some major protistan lineages (Lentendu *et al.* 2014; Geisen *et al.* 2015a), applying universal eukaryotic primers on terrestrial samples ultimately results in a majority of sequence reads derived from multicellular organisms (e.g. animals and fungi) (Baldwin *et al.* 2013; Dupont *et al.* 2016). In order to improve the resolution of protistan diversity from environmental samples, the most promising way is to apply highly group-specific primers for targeting only selected monophyletic groups (Fiore-Donno *et al.* 2018, 2019; Sapp *et al.* 2018; Degrune *et al.* 2019; Fiore-Donno, Richter-Heitmann and Bonkowski 2020; Roshan *et al.* 2021). Whereby, the application of specific primers has a further advantage which should not be disregarded: only by encompassing the whole diversity of a taxon of interest, qualitative conclusions can be drawn on the structuring effects of envriomental factors that shape protistan communities in natural systems (Fiore-Donno *et al.* 2018).

Protist Diversity in Forest Ecosystems

Protists represent all trophic levels, including autotrophs, mixotrophs, saprotrophs, eukaryvores, omnivores as well as parasites of plants and animals and their hyperparasites (Geisen *et al.* 2016; Bonkowski, Dumack and Fiore-Donno 2019). Apart from dominating eukaryotic diversity, protists play fundamental roles in many Earth system processes, particularly by catalysing carbon and nutrient cycling in the food webs of aquatic and terrestrial environments (Wardle *et al.* 2004; Van Der Heijden, Bardgett and Van Straalen 2008; Singer *et al.* 2021). In forest ecosystems, protists occur in high abundances, and their numbers in forest soils generally range between 10^4–10^7 active protistan individuals per gram of dry soil and litter (Adl and Gupta 2006). Despite their substantial impacts on belowground food webs, studies based on microscopical observation of coarse protistan morphotypes (i.e. flagellates, ciliates and amoebae) also highlighted the complexity of taxa-rich and highly dynamic litter communities (Bamforth 1971, 1973, 2010; Krivtsov *et al.* 2003). Moreover, in a metatranscriptomic study, Voss and colleagues (2019) revealed an unsuspected protistan diversity in leaf litter across temperate beech forests, where protists represented nearly a quarter of the total eukaryotic diversity. While leaf litter is an important source of recalcitrant organic matter (Baldrian 2017), these litter protists thus seemingly play a major role in forest nutrient cycling, channelling energy from litter decomposition to higher trophic levels by grazing on bacterial biomass as well as fungi, other protists, and even microfauna (Schröter, Wolters and De Ruiter 2003; Geisen *et al.* 2016; Trap *et al.* 2016; Bonkowski, Dumack and Fiore-Donno 2019).

Beside their function as microbial grazers, protists, in particular highly host-specific parasites within the Apicomplexa, have been suggested to drive the high animal diversity in tropical rainforests by reducing their population size in a density-dependent manner (Mahé *et al.* 2017). Furthermore, representatives of the plant parasitic Oomycota (Stramenopiles) have been reported to represent a major threat for the stability of entire forest ecosystems, by being directly associated with the mortality and decline of various tree populations worldwide (Brasier, Robredo and Ferraz 1993; Brasier, Rose and Gibbs 1995; Jung, Blaschke and Obwald 2008; Hansen 2015). Therefore, protistan diversity has a far greater impact on the forest ecosystem functioning than it was thought to be possible; ranging from major consumers providing nutrients to higher trophic levels, to selective biotic forces that have been, and continue to be, conducive to the evolution as well as the extinction of various host organisms in forest ecosystems.

Protists Associated with Tree Microhabitats

Apart from catalysing carbon in forest ground habitats, protists significantly affect plant physiological processes via production of phytohormones and modifications of the rhizosphere microbiome (Jentschke *et al.* 1995). Moreover, comprehensive growth experiments revealed that protists and mycorrhizal fungi synergistically affect plant performance in the rhizosphere of spruce trees (Bonkowski, Jentschke and Scheu 2001). Although knowledge on protistan ecological functions in the canopy is scarce, several observations point towards their enormous taxonomical diversity associated with trees and their canopy microhabitats. A former study on microbes associated with tree-borne foliose lichens, provided evidence that the presence of lichen patches might be conducive for a robust community of amoeboid protists in forest canopies (Anderson 2014). An investigation on ciliate communities associated with waterfilled tree-holes (dendrotelmae) identified several environmental factors (i.e. tree species, volume and age of dendrotelmae, presence of predatory metazoans) to influence community composition and species richness within this group of protists (Tirjaková and Vdacný 2005). By providing habitat for an array of saproxylic (wood-dwelling) species, deadwood might also play an important role for the protistan diversity in the forest canopy. Microscopical studies on deadwood and bark communities reported a highly specific ciliate fauna, which was characterized by many exclusively autochthonous taxa that appeared to be affected by the prevalent environmental conditions (i.e. moisture, nutrition, pH and temperature) (Bartošová and Tirjaková 2005, 2008). Similar observations have been reported for plasmodial and protostelid slime moulds: namely, the myxomycetes and protostelids. In this context, it may be noted that the life cycle of plasmodial slime moulds is comparable to the one of protostelids, which comprises a motile stage that turns into static fruiting bodies which produce spores for subsequent dispersal across the forest ecosystems (de Haan 2011). Myxomycetes have been observed to exhibit a vast taxonomical diversity, from decaying wood on the forest floor to the living branches up in the canopy region (Clissmann *et al.* 2015; Taylor, Feest and Stephenson 2015). After investigating myxomycete and protostelid communities colonizing the aerial bark from dead and living trees, aerial litter as well as the litter and bark on the ground, habitat filtering was recognized as one of the major drivers for species abundance and distribution within these phyla (Aguilar, Lado and Spiegel 2007; Ndiritu, Spiegel and Stephenson 2009; Shadwick, Stephenson and Spiegel 2009; de Haan 2011; Zahn, Stephenson and Spiegel 2014). Nevertheless, aforementioned studies on protistan diversity are mainly entirely based on microscopical observations and thus only provide a first insight into the diversity of selected groups of protists associated with broader microhabitat classes of trees. But since protists are known to provide a vast array of taxonomical and functional diversity, it can be presumed that the list of hitherto unknown protistan taxa and their ecological functions will most likely increase as other microhabitat types as well as phyla are examined, especially once environmental sequencing will be applied.

Dispersal Strategies of Protists

The fundamental microbiological tenet 'Everything is everywhere: but the environment selects' was first manifested by the Dutch microbiologist Martinus Wilhelm Beijerinck early in the twentieth century and reinforced in 1934 by Lourens G. M. Baas-Becking. Generally speaking, the aphorism asserts that due to their high abundances and small size, microbial taxa are able to occupy and proliferate in any habitat on Earth, as long as the environmental conditions are appropriate. More specifically, it proposes that the high dispersal potential of microbes would lead to distributions generally shaped by environmental factors rather than spatial distance. In this regard, it is important to note that this hypothesis does not rule out the possibility of strong biogeographic patterns, but rather suggests that spatial distance per se does not drive the distribution of microbial taxa (Fondi *et al.* 2016). Apart from neutral processes, the long standing niche theory is considered to underpin microbial biogeography (Dumbrell *et al.* 2010; Dini-Andreote *et al.* 2015). According to the niche theory (Chase and Leibold 2003), microbial dispersal and thus biodiversity is influenced by environmental heterogeneity (i.e. physicochemical properties) and biotic factors via taxa interaction. Further, there is growing evidence that spatial turnover of microbial communities is characterized by a distance-decay relationship and/or taxa-area relationship describing the increasing community dissimilarity with increasing spatial distance as well as the increasing taxa richness with larger area size, respectively (Liu *et al.* 2019).

The dispersal of free-living protists in terrestrial environments is facilitated by passive dissemination of dormant stages of minimal, if any, metabolic activity, i.e. resting cysts or spores (Foissner 1987, 2006; Verni and Rosati 2011). Especially in response to suboptimal environmental conditions (e.g. periods of drought, starvation, or microbial antibiotics) cysts are formed to ensure survival via dormancy (Petz and Foissner 1988; Adl and Gupta 2006; Jousset *et al.* 2006). These protistan propagules can then be dispersed over large distances by wind-blown soil particles in the air (Goudie and Middleton 2001; Pitta *et al.* 2017), bio-aerosolization and precipitation (Finlay *et al.* 2001; Kamono *et al.* 2009; Payne *et al.* 2012), or migrating animals and humans (Revill, Stewart and Schlichting 1967; Schlichting and Sides 1969; Perrigo, Romeralo and Baldauf 2012). Former investigations have reported that viable cysts can be retrieved from temperate and arid environments even after decades of encystment (Moon-Van Der Staay *et al.* 2006; Kageyama and Asano 2009). The excystment is then again usually activated by the return of favourable environmental conditions and is followed by a subsequent reproductive phase in a suitable environment (Khan, Iqbal and Siddiqui 2015).

Applied to forest ecosystems, protistan colonization of highly heterogeneous microhabitats, especially within the forest canopy, might thus be directly affected by formerly described stochastic processes (i.e. the import of propagules via passive dissemination). However, since environmental conditions have to be suitable to induce reproduction of these microbes, the deterministic process of habitat filtering can be presumed to represent one major shaping agent of protistan communities dwelling within these physicochemically different environments.

Cercozoa

The monophyletic protistan phylum Cercozoa within the supergroup Rhizaria (Cavalier-Smith 1998) has been reported to constitute one major lineage in terrestrial habitats (Geisen *et al.* 2015a; Grossmann *et al.* 2016; de Araujo *et al.* 2018). In a metatranscriptomic analysis of protistan soil communities, Geisen et al. (2015b) revealed that 40-60% of all identified protistan small subunit ribosomal RNAs from grassland and forest soils could be taxonomically assigned to Cercozoa. With ca. 600 described species (Pawlowski *et al.* 2012), the phylum Cercozoa consists predominantly of naked and testate amoebae, flagellates as well as amoeboflagellates (Bass *et al.* 2009a, 2009b; Hess, Sausen and Melkonian 2012; Hess and Melkonian 2013), encompassing a broad spectrum of functional traits in morphologies, locomotive modes as well as feeding strategies

(Burki and Keeling 2014; Fiore-Donno *et al.* 2019; Dumack *et al.* 2020). Hence, their ecological roles proved to be multiple: ranging from autotrophic algae and free-living heterotrophic predators to parasites of plants and animals (Burki and Keeling 2014; Neuhauser *et al.* 2014; Bass, Ward and Burki 2019).

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). Especially the order Plasmodiophorida within the class Phytomyxea is widely known for containing plant parasitic taxa that cause major diseases in crops (e.g. brassicas, potatoes, and grain crops) (Neuhauser *et al.* 2014). The most famous species is *Plasmodiophora brassicae*, which causes the clubroot disease in cruciferous plants. In the course of European history there had been several reports of what appears to be clubroot disease as far back as the 13th century and quite possibly much earlier to Roman times (Dixon 2009). Even hundreds of years later, a study by Crête (1981) on the frequency of severity of infestation of *Brassica* crops identified *P. brassicae* to be responsible for up to 10% in-field losses of the global cultivation. Interestingly, a recent environmental sequencing study by Fiore-Donno, Richter-Heitmann and Bonkowski (2020) revealed high abundances of a variety of phytomyxean plant parasites in grassland soils, but none in soils of nearby forests across Germany. They concluded that the establishment of these parasitic lineages in forest soils might depend on the prevailing density of their host organisms. Further, they presumed that the forest soil microbial community might induce an additional suppressing effect on phytomyxean plant parasites by outcompeting these usually highly host-specific protists due to better adaptations to the prevailing environmental conditions within forest sites.

Cercozoa as Phyllosphere Specialists

In nature, coevolution of multicellular plants and diverse microbiota, such as archaea, bacteria, microfungi and protists, led to a functional entity called the holobiont (host plus symbionts) with its hologenome (host genome plus microbiome) (Rosenberg and Zilber-Rosenberg 2016). As a result, plant health, growth and productivity is directly linked to its microbial consortia (Lindow and Brandl 2003; Buée *et al.* 2009; Vorholt 2012). But since interactions between the host plant and its inhabitants are not unidirectional, microbial associates also profit to a high degree from these symbiotic relationships. By providing novel metabolic capabilities to its microbiota, virtually all tissues of a plant are reported to host niche-specialized inhabitants (Hassani, Durán and Hacquard 2018). Microbes dwelling in these niches can either have beneficial (mutualistic), neutral (commensalistic), or even detrimental (pathogenic) impact on their host plant's fitness (Turner, James and Poole 2013).

Most of the protists that have been identified to interact with plants can be assigned to the Stramenopiles-Alveolata-Rhizaria (SAR) supergroup, in particular representatives belonging to Oomycota (Stramenopiles) and Cercozoa (Rhizaria) (Hassani, Durán and Hacquard 2018). Especially in the phyllosphere, cercozoan taxa appear to be the dominant taxa (Ploch *et al.* 2016; Flues, Bass and Bonkowski 2017; Sapp *et al.* 2018). With an estimated area exceeding 100 million km² globally, plant leaves form the largest biological surface on Earth (Morris and Kinkel 2002; Peñuelas and Terradas 2014). Termed as the 'interkingdom crossroads' by Shepherd and Wagner (2007), the phylloplane, or leaf surface, has particularly been noted for its bacterial species diversity (Lambais *et al.* 2006). However, microbial communities dwelling in the phylloplane are taxonomically more diverse, and thus comprise besides bacteria also filamentous fungi, yeasts, algae as well as protists (Lindow and Brandl 2003).

Wherever there is prey, there will be predators. In particular, Cercozoa have been reported to shape bacterial communities on leaves by exerting substantial predation pressure (Flues, Bass and Bonkowski 2017). Bacteria, in turn, respond to protistan predation by induced alteration in secreted secondary metabolites and cell shape as well as micro-evolution (Jousset *et al.* 2006; Young 2006; Friman, Jousset and Buckling 2014), which would adversely affect the surface characteristics of the phylloplane, especially since epiphytic bacteria have been reported to regulate the prevailing physicochemical properties of the phyllosphere (Knoll and Schreiber 1998; Lindow and Brandl 2003; Schreiber *et al.* 2005).

In comparison to their belowground counterparts (microbial communities of rhizosphere and rhizoplane), epiphytic microbial communities are expected to experience a tremendous influence of environmental fluctuations (Lindow and Brandl 2003). As a result, phyllosphere protists are characterized by short diurnal life cycles, usually with metabolic active periods at nighttime when dew is accumulating on leaves (Mueller and Mueller 1970). Especially Cercozoa appear to contain numerous well adapted phyllosphere taxa (Ploch *et al.* 2016; Dumack *et al.* 2017; Flues *et al.* 2018). By quickly responding to fluctuating environmental conditions, taxa within this phylum are highly capable to withstand environmental extremes (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, Bass and Bonkowski 2017), represents a perfect evolutionary adaptation to the highly variable microclimatic conditions up in the tree crowns.

Aims & Hypotheses

The research performed for this thesis was supported by the Priority Program (SPP 1991): '**TaxonOmics – New Approaches for Discovering and Naming Biodiversity**' of the German Research Foundation (DFG). The survey was conducted as a joint collaboration between the universities of Cologne and Leipzig, focussing on the two prominent protistan taxa Cercozoa (Rhizaria) and Oomycota (Stramenopiles), respectively. The main objective of this PhD thesis was to characterize the cercozoan diversity and community composition in forest soils and the canopy region in a metabarcoding approach, using highly group-specific primers. Consequently, this PhD thesis had five major goals:

1.) Comparative analysis of the taxonomic and functional diversity of cercozoan communities along a vertical gradient, from forest soil to the canopy, of different autochthonous tree species.

2.) Comparative analysis of the cercozoan diversity in the forest canopy across two biomes (temperate versus tropical forest).

3.) Shed light on a cercozoan cryptic-species-complex by using data obtained from the forest canopy communities together with data from other environmental sequencing studies.

4.) Investigate potential dispersal of cercozoan taxa by air in natural forest ecosystems.

5.) Examine seasonal variability of cercozoan communities colonizing the forest canopy over a period of two years.

Therefore, we addressed the following hypotheses:

H1 Using highly group-specific primers will yield a comprehensive survey of hitherto unknown taxonomic diversity and community composition of Cercozoa across all major ecological compartments in forest canopies across two biomes (temperate versus tropical biome).

H2 Microhabitats in the tree canopies harbour microhabitat-specific cercozoan communities, and foliar communities represent a subset of the diversity in litter communities on the forest floor.

H3 Tropical forest canopies harbour a specific, mainly undescribed diversity of Cercozoa compared to temperate forests.

H4 Microhabitat identity affects functional diversity; thus habitat filtering induces selection for specific cercozoan taxa.

H5 Stochastic processes contribute to the distribution of Cercozoa in the tree canopy.

Chapter Summary

CHAPTER I

Opening the Microbial Back Box of Tree Canopies

Part 1.1: From Forest Soil to the Canopy: Increased Habitat Diversity Does Not Increase Species Richness of Cercozoa and Oomycota in Tree Canopies.[2,6]

Knowledge on protistan diversity up in the tree crowns is scarce and former studies on eukaryotic microorganisms dwelling in this heterogeneous habitat were almost entirely based on morphological examinations by a threatened species: the taxonomist. Fortunately, progress in sequencing methods and growing databases enabled to elucidate protistan diversity on a molecular base by the development and improvement of environmental sequencing methods. This study aimed to characterize for the first time the diversity and community composition of protists (Rhizaria: Cercozoa and Stramenopiles: Oomycota) dwelling in various structurally different microhabitats within the canopy region in a temperate floodplain forest by the use of high-throughput sequencing with group-specific primers. To get a first overview of the protistan plant-dwellers, the following main questions were addressed: who is there? How many are there? To which extent do they differ from the forest soil to the canopy?

Part 1.2: Exploring the "Last Biotic Frontier" – Unraveling the Diversity of Cercozoa and Oomycota in Palaeotropical Tree Canopies.[2,6]

Tropical forest canopies harbour a vast diversity of multicellular organisms, but it is still little explored if eukaryotic microorganisms (i.e. protists) reflect similar hyperdiversity patterns compared to their counterparts in temperate biomes. The second part of this chapter aimed to investigate protistan diversity (Rhizaria: Cercozoa and Stramenopiles: Oomycota) in the canopy region of three autochthonous tree species in a palaeotropical rainforest in Papua New Guinea. Further, we proposed a tentative approach of assigning ecological niches to metabarcoding data lacking from missing reference sequences by assigning possible ecological niches on the base of significant correlations with well-studied taxa.

CHAPTER II

Forest Canopies – A Reservoir for Cryptic Species

What Drives the Diversity of the Most Abundant Terrestrial Cercozoan Family (Rhogostomidae, Cercozoa, Rhizaria)?[4,5]

Recent Metabarcoding studies on environmental samples from terrestrial habitats revealed the cercozoan family of Rhogostomidae (Cryomonadida), a group of omnivorous thecate amoebae, to be highly abundant and extremely diverse. However, only very few species within this family had been described so far. Thus, the aim of this chapter was to phylogenetically investigate the putative cryptic diversity by re-evaluating Rhogostomidae sequence data obtained from environmental sequencing studies from terrestrial habitats, inter alia, the forest canopies. Furthermore, this study aimed to identify the major environmental drivers that shape Rhogostomidae communities in terrestrial habitats.

CHAPTER III

On the Aerobiology of Microbial Eukaryotes

To the Canopy and Beyond: Air Dispersal as a Mechanism of Ubiquitous Protistan Pathogen Assembly in Tree Canopies.[3]

The air has been reported as an effective means of long-distance propagation for a wide range of microbial organisms. In this chapter the collaborators from the University of Leipzig aimed to reveal a potential distribution mechanism by air of formerly analyzed protistan groups in the forest canopy. Therefore, samples of canopy and ground surrounding air were taken during two different sampling time points to investigate if colonization of canopy habitats takes place through inoculation of these microorganisms via aerial transport. Moreover, the major aim was to show the role of protistan air dispersal as a potential prerequisite to understand the mechanisms of protistan community assembly in the canopy region.

CHAPTER IV

How Seasonality Affects Protistan Communities and their Functional Diversity in Tree Canopies

Part 4.1: On the Phenology of Protists: Recurrent Patterns Reveal Seasonal Variation of Protistan (Rhizaria: Cercozoa, Endomyxa) Communities in Tree Canopies.[1,6]

Tree canopies represent a highly ephemeral environment, characterized by diurnal fluctuations and recurrent seasonal changes. Thus, this study aimed to examine seasonality within the investigated alpha and beta diversity patterns of protistan communities (Rhizaria: Cercozoa, Endomyxa) in tree canopies over a period of two years. Further, potential habitat and season correlations of detected genera were investigated by a comparative analysis of assignable feeding strategies (i.e. feeding modes: bacterivory, eukarivory, autotrophy, parasitism) prevailing in the distinct cercozoan communities detected across the microhabitats throughout the seasons.

Part 4.2: A Parasite's Paradise: Biotrophic Species Prevail Oomycete Community Composition in Tree Canopies.[4,6]

Oomycota (Stramenopiles) are among the most severe plant pathogens with high economic and ecologic impact on forest ecosystems. Thus, in the second part of this chapter the collaborators from the University of Leipzig aimed to investigate if this group of protists would show similar patterns of seasonal variability within tree crowns as reflected by the group of Cercozoa. Moreover, they aimed to investigate if their functional diversity would differ between canopy and ground habitats, as it was already reported for the cercozoan communities.

(1) This study was designed and planned together with Prof. Dr. Michael Bonkowski and Prof. Dr. Martin Schlegel. Sampling, laboratory work, bioinformatics, taxonomic and statistical analyses were carried out mainly by the author, if not stated otherwise.
The article was mainly written by the author under guidance of Prof. Dr. Michael Bonkowski and Prof. Dr. Martin Schlegel.

(2) Both first authors performed the sampling, laboratory work, bioinformatic analyses, if not stated otherwise, and their complementary contributions merit equal credit. This study was designed and planned together with Prof. Dr. Michael Bonkowski and Prof. Dr. Martin Schlegel. The article was mainly written by both first authors under guidance of Prof. Dr. Michael Bonkowski and Prof. Dr. Martin Schlegel.

(3) This study was designed and planned together with Prof. Dr. Martin Schlegel. The article was written and adjusted by Robin-Tobias Jauss, by usage of the developed thesis of a supervised student, under the guidance of Dr. Martin Schlegel. The author assisted the sampling and contributed contentual to the adjusted version.

(4) The author contributed in adjusting and refining the final version of the manuscript.

(5) The author contributed in sampling, laboratory work and bioinformatic analysis for this study.

(6) Sampling, DNA extraction and PCRs were mainly performed by the author and the co-author Robin-Tobias Jauss by assistance of Stefan Schaffer, Ronny Wolf and Kai Feng.

CHAPTER I

Opening the Microbial Back Box of Tree Canopies

Part 1.1: From Forest Soil to the Canopy: Increased Habitat Diversity Does Not Increase Species Richness of Cercozoa and Oomycota in Tree Canopies

Reference:

Jauss R-T & **Walden S** *et al.* From Forest Soil to the Canopy: Increased Habitat Diversity Does Not Increase Species Richness of Cercozoa and Oomycota in Tree Canopies. *Frontiers in Microbiology* 2020, DOI: 10.3389/fmicb.2020.592189.

CHAPTER I

Opening the Microbial Back Box of Tree Canopies

Part 1.2: Exploring the "Last Biotic Frontier" – Unraveling the Diversity of Cercozoa and Oomycota in Palaeotropical Tree Canopies

Exploring the “Last Biotic Frontier” – Unraveling the Diversity of Cercozoa and Oomycota in Palaeotropical Tree Canopies

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Keywords: protists, forest canopies, metabarcoding, tropical rainforest, hidden diversity

Abstract

Tropical rainforests are the biodiversity hotspots among the world's ecosystems, containing far higher numbers of species on a per-area basis relative to any other terrestrial ecosystem. Especially, tropical forest canopies harbour a vast diversity of multicellular organisms, but it is still little explored if microorganisms, i.e. protists, reflect similar diversity patterns compared to temperate zones. In this study we investigated protistan diversity of autochthonous tree species in a tropical rainforest in the northeast of Papua New Guinea. We applied a barcoding approach using group specific primers for an extensive assessment of the diversity of Cercozoa (Rhizaria) and Oomycota (Stramenopiles) across forest soils and the canopy region. Together our results indicate taxonomically distinct communities representing different functional traits to inhabit the investigated canopy and ground strata. Palaeotropical trees harboured 652 and 283 OTUs for Cercozoa and Oomycota, respectively. In Cercozoa most taxa detected in this study corresponded to taxa known from temperate zones, only 7% of OTUs could not be assigned to any known order. However, in Oomycetes, approximately 50% of all OTUs showed a sequence similarity of less than 70% to any reference sequence. Co-occurrence analyses of these undetermined OTUs tentatively assigned possible ecological niches on the base of significant correlations with well-studied taxa. Overall, our results show that palaeotropical forest canopies indeed still contain a substantial unknown diversity of microbial eukaryotes.

INTRODUCTION

Tropical tree canopies – Introducing a blackbox of environmental sequencing

On a global scale, more than 3 trillion trees exist on earth with 43% in tropical and subtropical regions and 22% in temperate biomes (Crowther *et al.* 2015). Tropical rainforests contain far higher numbers of animal and plant species on a per-area basis relative to any ecosystem known from subtropical, temperate, and boreal biomes. Defined as the "the aggregate of all crowns in a stand of vegetation, which is the combination of all foliage, twigs, fine branches, epiphytes as well as the interstices in a forest" (Nadkarni 1994), forest canopies are characterized by an exceptional habitat heterogeneity, which was most probably conducive to the evolution of a multitude multicellular organisms, especially in tropical biomes, due to the increased number of aboveground microhabitats.

Termed as “the last biotic frontier” by Erwin (1983), tropical forest canopies still represent a blackbox within molecular diversity studies of microorganisms, particularly given the rapid technological progress in advanced environmental sequencing tools. Hence, it is still little explored if eukaryotic microorganisms, i.e. protists, reflect similar diversity patterns in tropical tree canopies compared to macroorganisms, and their microorganismic counterparts in temperate zones (Jauss *et al.* 2020, 2021; Walden *et al.* 2021).

Microbial diversity in tropical tree canopies – A morphological approach

Former studies on tropical forest canopies recognized epiphytes to contribute to >25% of plant species richness (Küper *et al.* 2004), accumulating with up to 44 t/ha large amounts of humus (i.e. arboreal soil) (Hofstede and Wolf 1993). It is therefore not surprising that the majority of nematode taxa in a rainforest of Costa Rica were found on trees and not on the forest floor (Powers *et al.* 2009). In a morphology-based comparative study of heterotrophic protists from arboreal soils, ground soils and litter sampled within a neotropical rainforest, 115 out of a total of 127 amoebae morphospecies (Amoebozoa and Cercozoa) were detected in the aboveground soils (Bamforth 2007). Moreover, 50 out of 80 ciliate morphospecies were identified in the aboveground soils. In addition, some detailed taxonomic informations on epiphyte-dwelling protists have been documented at the morphospecies level for ciliate and flagellate communities (Foissner 2003; Duarte *et al.* 2013; Buosi *et al.* 2014; Durán-Ramírez *et al.* 2015). However, molecular studies on protistan diversity uncovered a remarkably high cryptic diversity within morphologically defined taxa (Fenchel 2005; Kosakyan *et al.* 2012; Škaloud and Rindi 2013). The term 'cryptic species' is used for morphologically indistinguishable taxa, which are often identified only by DNA barcoding (Hebert *et al.* 2004). These complexes of cryptic species may have diverged only recently and not yet have become morphologically distinct, or they are already distantly related but converged in morphological traits (Fišer, Robinson and Malard 2018; Struck *et al.* 2018). But since the species concept is the cornerstone of diversity studies, comprehensive molecular studies unravelling protistan hidden diversity are essential in order to acquire a complete picture regarding their diversity patterns and associated processes.

Microbial hyperdiversity in neotropical soils

A recent metabarcoding study with general eukaryotic primers by Mahé *et al.* (2017) on the protistan diversity in soils of neotropical rainforests (Costa Rica, Panama and Ecuador) detected hyperdiversity patterns and highly heterogeneous protistan communities within the same forest. Further, parasitic taxa of the phylum Apicomplexa, which infect arthropods and other animals, were dominating across all soil samples. These dominating parasites were suggested to potentially contribute to the high animal diversity in the forests by reducing population growth in a density-dependent manner. By contrast, for the prominent plant parasitic Oomycota comparatively few OTUs were detected in the soils – an observation, which was contrary to the presumption that Oomycota have been thought to be one major driver of hyperdiversity in tree species in the tropics (Freckleton and Lewis 2006). Instead, the monophyletic, highly diverse phylum of Cercozoa appeared to be the second most diverse protistan taxon in neotropical forest soils. Finally, they hypothesized that some detected Operational Taxonomic Units (OTUs) in the soil may not all derive from soil-dwelling taxa but rather may have originated from canopy communities that had rained down from above. Leading to the presumption that several taxa detected in the soil might be just a shadow of highly diverse communities dwelling in the canopy region, a pattern which could be observed for temperate biomes (Jauss *et al.* 2020).

In this study, we investigated protistan diversity from three autochthonous tree species in a palaeotropical rainforest in the northeast of Papua New Guinea. We applied high-throughput sequencing using group specific primers for a molecular characterization of Cercozoa (Rhizaria) and Oomycota (Stramenopiles) across various microhabitats, from forest soils to the canopy region. Accordingly, our study tackles two hypotheses: (I) Tropical forest canopies harbour a specific, mainly undescribed diversity of Cercozoa and Oomycota and, (II) the diversity of investigated phyla in the tropical zone will be even higher in the tree crowns than on soil, due to increased numbers of aboveground microhabitats, like epiphytes and arboreal soil, while the rapid mineralization of litter layers on the soil surface will reduce habitat complexity compared to relatively thick organic layers in temperate forests.

MATERIAL AND METHODS

Sampling, DNA extraction and sequencing

Microhabitat samples were collected in September 2019 in cooperation with the New Guinea Binatang Research Centre in Madang, Papua New Guinea at the Kakoba Canopy Crane Baitabag, Madang Province (5°08'19.5"S 145°46'23.2"E). Samples were obtained and processed as described in Jauss *et al.* (2020). Briefly, nine microbial microhabitat compartments related to tree surface were sampled in the canopy at 20-30m height: Fresh leaves, flowers, fruits, deadwood, bark, bark covered by a thin layer of crustose lichen, arboreal soil and two distinct cryptogamic epiphytes (foliose lichen and moss). In addition, two ground habitats (soil and leaf litter) were sampled. All microhabitat samples were taken with four replicates, from three autochthonous tree species (*Pometia pinnata*, *Pterocymbium beccarii* and *Pterocarpus indicus*) with three replicates each. DNA extraction was performed with the DNeasy PowerSoil kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. PCR amplification with tagged group specific primers (Fiore-Donno *et al.* 2019; Fiore-Donno and Bonkowski 2021) and sequencing were performed as described in Jauss *et al.* (2020), the used primer tag combinations are provided in Supplementary Table 1.

Sequence processing

Sequence processing and bioinformatic analyses followed the pipeline described in Jauss *et al.* (2020). Briefly, raw reads were merged using VSEARCH v2.10.3 (Rognes *et al.* 2016) at default settings. Primer and tag sequences were trimmed and clustered into operational taxonomic units (OTUs) using Swarm v2.2.2 (Mahé *et al.* 2015). 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 (Nelson *et al.* 2014; Sapp *et al.* 2018) (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) and removed if the best hit in terms of bitscore was a non-oomycete sequence or non-cercozoan sequence, respectively. For a finer taxonomic assignment, two databases were used: The PR^2 database (v4.12.0, Guillou *et al.* 2013) 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). The taxonomic 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. Functional annotation was performed at genus level based on the functional databases published by Fiore-Donno and Bonkowski (2021) for oomycetes and (Dumack *et al.* 2020) for Cercozoa. Samples with low sequencing depth were removed by loading the final OTU table into QIIME 2 v2018.11 (Bolyen *et al.* 2019). The minimum sequencing depth was determined depending on how many samples per metadata would be excluded. It was set as high as possible and resulted in a minimum sequencing depth of 578 sequences for oomycete samples and 1548 sequences for cercozoan samples.

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 and Chao 2019) to determine if a higher sequencing depth would have revealed more OTUs. Alpha diversity indices were calculated for strata and phyla 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 (NMDS) was performed on the Bray-Curtis dissimilarity matrix (functions *vegdist* and *metaMDS* in the vegan package, respectively). To test if oomycete and cercozoan OTU diversity differed across the strata and tree species a Goodness of Fit statistic (function *envit* in the vegan package) was conducted. The number of shared OTUs between different combinations of microhabitats was visualized using the UpSetR package (Lex *et al.* 2014; Gehlenborg 2019). An indicative species analysis (Dufrêne and Legendre 1997) was performed with the indicspecies package using the *multipatt* function (De Cáceres and Legendre 2009) to identify indicator taxa in the different strata. All figures were plotted with the ggplot2 package (Wickham 2016). Phylogenetic trees for both groups were reconstructed with RAxML v8.2.12 (Stamatakis 2014) at default settings with 200 bootstrap replicates and the GTR+G+I substitution model, based on nucleotide alignments calculated with Mafft v7.271 (Katoh and Standley 2013) with the L-INS-I algorithm, a gap open penalty of 0.5, a gap extension penalty of -1.5 and otherwise default settings. Note that the phylogenetic analyses are not meant to provide a curated phylogeny or taxonomy of the protistan lineages, but should rather represent distinct clades of OTUs based on the V4 and ITS1 amplicons, respectively. Distinct phylogenetic clusters of oomycete representative sequences were determined with RAMI v1.2.1 (Pommier *et al.* 2009) with a 0.6 distance threshold. Cercozoan and oomycete phylogenetic diversity was illustrated using the Interactive Tree Of Life tool (http://itol.embl.de/, last accessed January 12, 2020). Co-occurrence correlations between the novel undetermined oomycete clusters (comprising OTUs with a sequence similarity of less than 70% to any known reference sequence) and the other phylogenetic clusters in the strata, ground and canopy, were calculated with the wTO package (Gysi *et al.* 2018).

RESULTS

OTU clustering of amplicons and taxonomic annotation

We obtained 652 genuine OTUs from 1,297,140 sequences (Cercozoa) and 283 OTUs from 446,516 sequences (Oomycota). The average number of cercozoan OTUs was 413 ± 122 and 628 ± 9 per microhabitat and tree species, respectively, while the average number of oomycete OTUs was 155 ± 40 and 245 ± 21 per microhabitat and tree species. Overall, 651 and 272 OTUs were detected in the canopy stratum, for Cercozoa and Oomycota respectively. Whereas, 631 OTUs (Cercozoa) and 276 OTUs (Oomycota) were obtained from the ground habitats. In Cercozoa only ca. 1% of detected OTUs showed a sequence similarity of less than 70%, and only 7% of OTUs could not be assigned to any order (Fig. 1A). Cercozoan OTUs were dominated by bacterivores and omnivores of the orders Glissomonadida, Euglyphida, Cercomonadida and Cryomonadida. In Oomycetes, approximately 50% of all OTUs showed a sequence similarity of less than 70% to any known reference sequence, which lead to no taxonomic annotation at the order level for half of the detected oomycete OTUs (Fig. 1B). Taxonomic and functional annotation of oomycete OTUs revealed the order of Peronosporales with mainly obligate biotrophic species, as well as saprotrophic and hemibiotrophic genera within the order of Pythiales to be predominantly present in the investigated microhabitats.

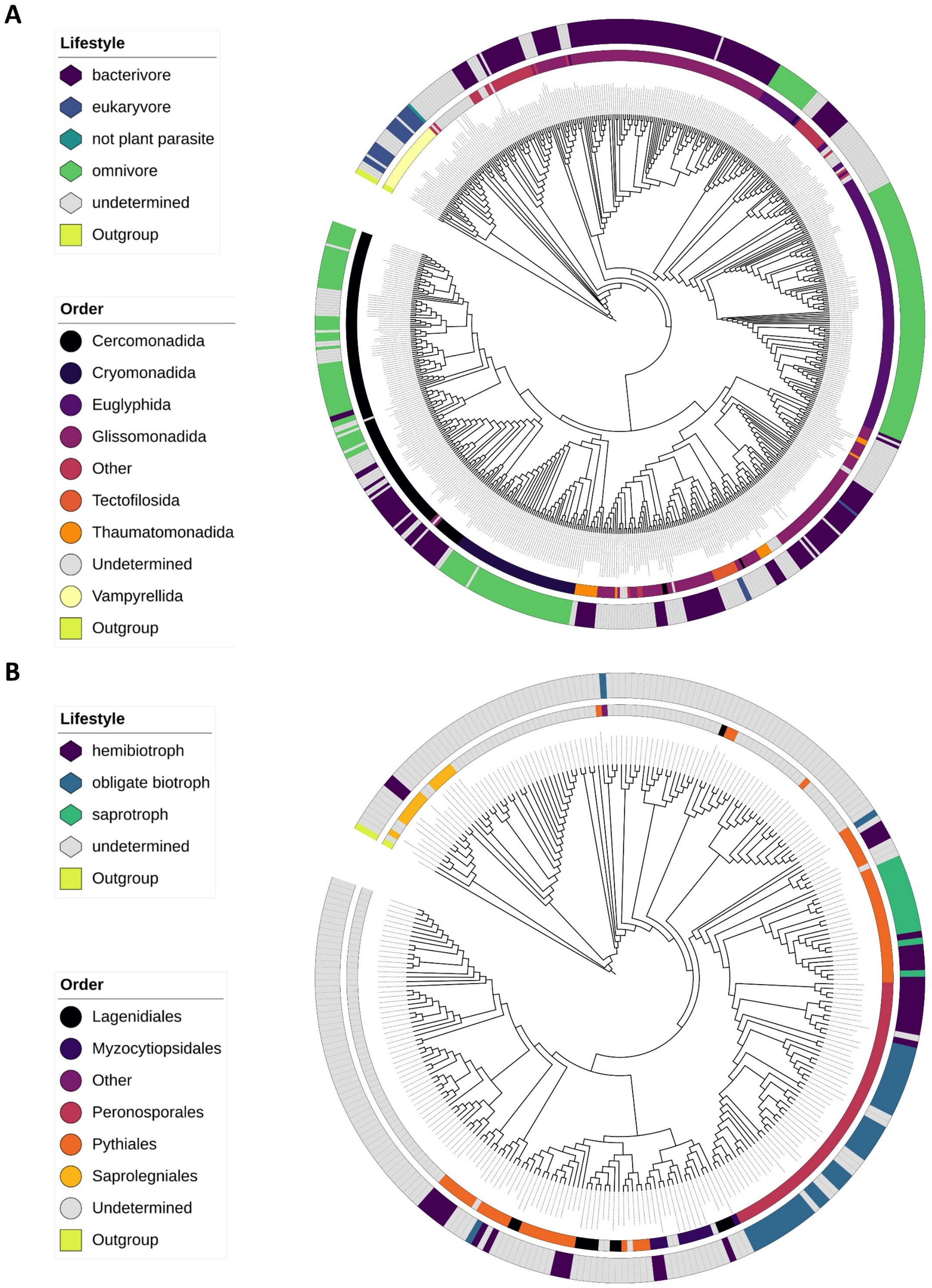

Figure 1: Phylogenetic diversity and functional trait assignment of detected cercozoan (A) and oomycete (B) OTUs. For Cercozoa 652 OTUs and for Oomycota 283 OTUs were detected from forest soil to the canopy. Detected Oomycota comprised a larger proportion of unknown diversity, compared to OTUs derived from Cercozoa.

Taxonomic and functional diversity

The used taxon-specific primers thoroughly recovered the OTU richness of canopy and ground samples as indicated by rarefaction analyses (Supplementary Figure 1). In Cercozoa, alpha diversity was higher in the ground stratum compared to the tree canopy. Whereas, no significant differences could be detected between both strata for the Oomycota. However, alpha diversity of the canopy samples showed higher variance for both phyla, but more pronounced in Oomycota, due to greater variation of the sampled microhabitats within this stratum (Supplementary Figure 2, 3). Investigation into protistan beta diversity reflected a strong separation of community profiles between canopy and ground strata for Cercozoa and Oomycota, respectively (Supplementary Figure 4; Goodness of Fit test; Cercozoa: R^2 0.45, $p = 0.001$; Oomycota: R^2 0.28, $p = 0.001$). Further, beta diversity of both phyla differed between tree species with a small, but significant proportion of explained variance (Goodness of Fit test; Cercozoa: R^2 0.09, $p = 0.035$; Oomycota: R^2 0.12, $p = 0.024$).

Taxonomic annotation of the OTUs detected in these two distinct strata revealed strong community differences at the order level when taking relative read abundances into account (Figure 2A). Indicative value analysis revealed >83% OTUs to be representative for the ground stratum (Supplementary Figure 5). Further, assignment of functional traits, i.e. feeding mode (Cercozoa) and lifestyle (Oomycota), reflected this pattern of disparity between the canopy and ground strata (Figure 2B). For Cercozoa, bacterivorous Glissomonadida dominated the canopy region, representing 64% of all reads detected in this stratum, followed by omnivorous Cryomonadida (11%) and Cercomonadida (8%). In contrast, omnivorous Euglyphida prevailed over other orders by representing 48% of all reads in the ground region, representing >100 indicative OTUs in the ground (Supplementary Figure 5A). Further, eukayvorous Vampyrellida and Cercomonadida were detected in 10% and 18% of the reads in the ground, respectively. For Oomycota, 83% of reads detected in the canopy could not be assigned to any taxonomic order, followed by 12% of mainly obligate biotrophic Peronosporales. In the ground stratum larger proportions of saprotrophic and hemibiotrophic Pythiales (31%) as well as hemibiotrophic Myzocytiopsidales (12%) and Peronosporales (12%) were detected. Still, 39% of reads could not be assigned to any functional trait in the ground.

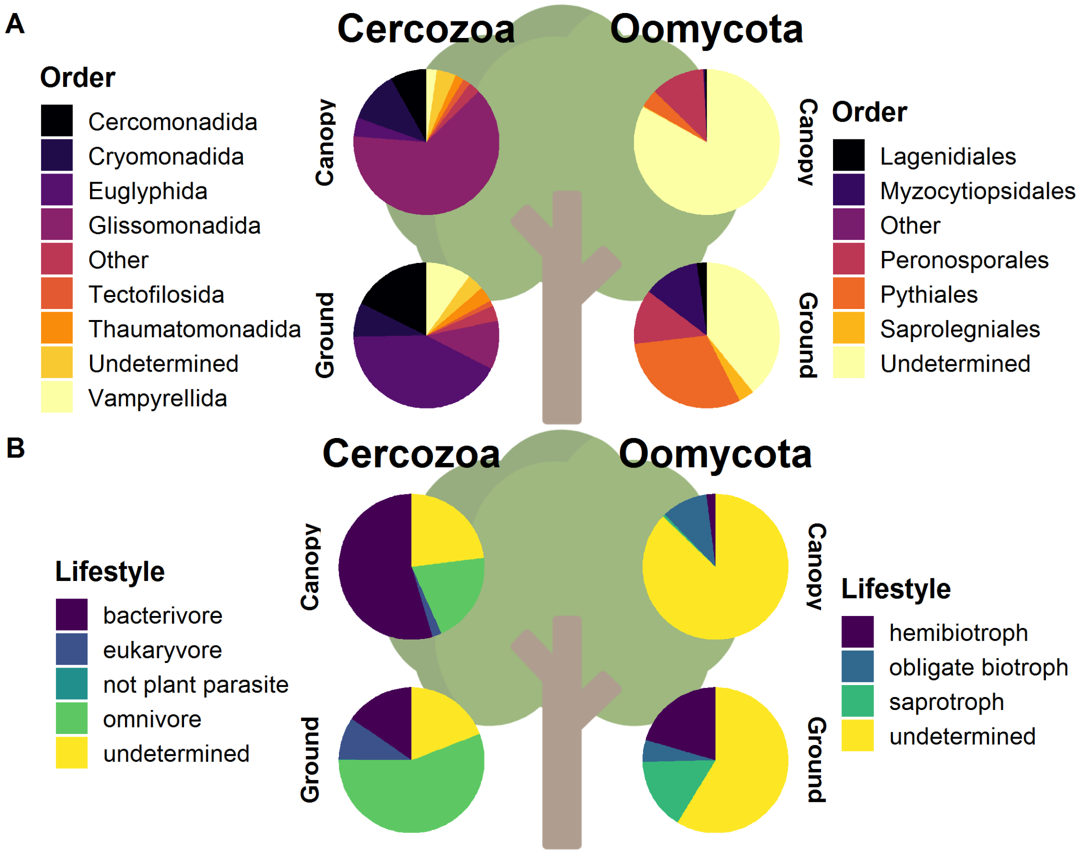

Figure 2: Relative proportion of orders (A) and functional traits (lifestyles) (B) for cercozoan and oomycete OTUs partitioned on the two strata of canopy and ground. In the canopy, Cercozoan OTUs were mainly assigned to the Glissomonadida and a bacterivorous lifestyle, while the vast majority of Oomycete OTUs could not be assigned to any order or lifestyle. Ground habitats were mainly dominated by Euglyphida and omnivorous OTUs for cercozoan communities and Pythiales for oomycete OTUs, while here again most OTUs could not be assigned to an order or lifestyle.

Cluster analysis and co-occurrence correlations of oomycetes

The cluster analyses of all oomycete OTUs revealed 44 distinct phylogenetic clusters (Supplementary Figure 6A). Of these, eight clusters were exclusively composed of undetermined OTUs with significantly strong co-occurrence correlations with other OTUs. (Figure 3, Supplementary Figure 6B). A schematic overview of the relative phylogenetic positions of these novel clusters is given in Figure 3A. The overall relative abundance in canopy habitats was quite low for these clusters. In contrast, some clusters, in particular clusters 6 and 15, showed high relative abundances in ground habitats. Despite their comparatively low relative abundances in the canopy region, most clusters showed several positive correlations with other OTUs in this stratum (Figure 3B). Especially cluster 6 showed more than 70 positive correlations, mostly with OTUs assigned to saprotrophic and hemibiotrophic taxa. This indicates this cluster to occupy similar ecological niches. In ground habitats, the novel undetermined clusters showed rather negative correlations.

This might be due to a consistently higher diversity within the respective ground samples and accordingly a higher competition in this stratum (Supplementary Figure 2B).

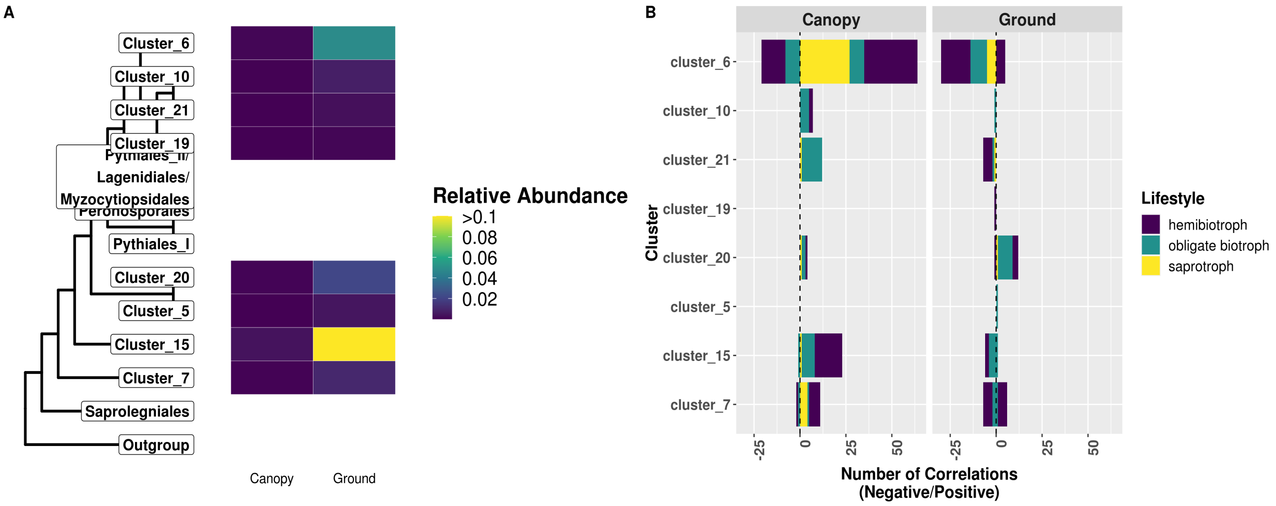

Figure 3: Simplified schematic position of the novel clusters in the oomycete phylogenetic tree with relative abundance of clusters in canopy and ground habitats (A) and number of positive and negative co-occurrence correlations in canopy and ground habitats for these novel clusters (B). The majority of positive correlations were detected for cluster 6 in the canopy stratum with OTUs assigned to saprotrophic and hemibiotrophic species. Whereby all clusters appeared to show low relative abundances in the canopy.

DISCUSSION

Unknown diversity in tropical tree canopies

Tropical tree canopies have been a hitherto neglected habitat for microorganisms and truly represent one of the “last biotic frontiers" in the field of environmental sequencing studies. Our results give a leading insight into protistan diversity of palaeotropical tree canopies. We detected a vast unknown diversity within the Oomycota, while the majority of cercozoan OTUs appeared to be well acquainted. Further, highly distinct canopy and ground communities were detected, reflecting the adaptation prospects prevailing in these physicochemically different habitats within the tropics. Similar to multicellular organisms, neotropical rainforests were recently shown to harbour a vast diversity of various microorganisms in the soils (Mahé *et al.* 2017). However, OTU richness of both Cercozoa and Oomycota in our study was at least an order of magnitude higher than in the described study by Mahé et al (2017), where general eukaryotic primers were applied. Several hypotheses have been discussed why the tropical diversity is much higher than temperate diversity, e.g. a higher mutation rate due to higher temperatures (Rensch 1959; Rohde

1992; Orton *et al.* 2019), lack of glacial processes (Fischer 1960; Hortal *et al.* 2011) or older land masses resulting in a longer evolutionary history (Wiens and Donoghue 2004). Either way, we wanted to elucidate if these processes shaping multicellular diversity might also be reflected in eukaryotic unicellular diversity. Interestingly, oomycetes showed a lower OTU richness with 283 OTUs in tropical biomes compared to their temperate counterpart with 330 OTUs (Jauss *et al.* 2020). However, our dataset contained more undetermined OTUs compared to the temperate forest (tropical forest: 50%, temperate forest: 17%), indicating different communities and lineages to be exclusively present in either biome, while the tropical forest harboured a higher proportion of unknown diversity in the canopy compared to the ground stratum. Cercozoa on the other hand showed a higher OTU richness in Papua New Guinea (652 OTUs) compared to the German temperate forest (550 OTUs), whereas the low number of undetermined OTUs is comparable in both biomes (tropical forest: 0.9%, temperate forest: 0.6%). Together our results lead to the conclusion that certain cercozoan OTUs and lineages are exclusively present in tropical biomes.

Distinct diversity patterns in canopy and ground habitats

Our results indicate a strong discrepancy between investigated protistan communities dwelling ground and canopy strata (Supplementary Figure 4), which was also observed for the temperate biome (Jauss *et al.* 2020, 2021; Walden *et al.* 2021). Here, however, the species richness increased with increasing habitat diversity to a greater extent than in temperate canopies (Supplementary Figure 3). Surprising was the relatively high diversity in the tropical soil and leaf litter samples, which were characterized by multitude of indicative species (Supplementary Figure 5), rejecting our hypothesis that the rapid mineralization of litter layer on the soil surface lead to a reduced complexity (protistan diversity) compared to relatively thick organic layers in temperate forests. Our samples were taken during the dry season in Papua New Guinea, and the comparatively low humidity in the ground region might have slowed the mineralization of the ground habitats and lead to higher diversity. The cercozoan order of Euglyphida dominated the ground habitat (Figure 2), while in ground samples of the temperate forest Glissomonadida prevailed (Jauss *et al.* 2020; Walden *et al.* 2021). Euglyphida are omnivorous testate amoeba that feed on both, bacteria and small eukaryotes, such as yeast, algae and other protists (Dumack *et al.* 2020). Whereby the morphological feature of bearing a silica or organic shell (Supplementary Figure 7), might be conducive to the survival in tropical soils during dry seasons by providing physical protection against desiccation (Booth 2001). In contrast, the canopy dominating bacterivorous Glissomonadida are naked flagellates, which might benefit from the comparatively high atmospheric humidity prevailing in the tropical rainforest of Papua New Guinea throughout the year (~80%, ~24 g/m^3, Worlddata.info).

Oomycetes however showed similar patterns in temperate and tropical biomes. Apart from the large proportion of undescribed species, Pythiales dominated the ground habitats, while more Peronosporales could be found in the canopy stratum. Even though Pythiales can display all lifestyles, from saprotrophy to hemibiotrophy and obligate biotrophy (Fawke, Doumane and Schornack 2015; Marano *et al.* 2016; Fiore-Donno and Bonkowski 2021), most Pythiales detected in the ground stratum were assigned to hemibiotrophic species. Hemibiotrophy is characterized by an initial biotrophic phase followed by a necrotrophic mode of feeding (Fawke, Doumane and Schornack 2015; Pandaranayaka *et al.* 2019). Soils and leaf litter therefore offer more suitable habitats for necro- and saprotrophic species compared to the living tissues of the sampled canopy habitats. Peronosporales dominated the canopy in both tropical and temperate biomes (Jauss *et al.* 2021), which indicates also tropical tree canopies to be a reservoir for parasitic oomycetes.

Putting the unknown diversity into a taxonomic and functional context

The high number of oomycete OTUs showing a sequence similarity of less than 70% to any known sequence indicates tropical tree canopies to be a reservoir for hitherto molecularly undescribed oomycetes, and perhaps other protists. The phylogenetic analyses revealed some of the novel clusters to be nested within, some apart from known oomycete orders, which therefore may represent so far unknown novel oomycete lineages. This makes it difficult to draw conclusions on their lifestyle and potential impact on ecosystem functioning. In order to extract at least some information from these novel clusters we were eager to put our data into a taxonomic and functional context by co-occurrence analysis. The detected positive co-occurrence correlations of some clusters may indicate similar ecological niches with well-known oomycete lineages, yet comprehensive assessments on their morphology and function are still lacking.

Conclusions

Overall, our study shows that palaeotropical forests indeed still contain a substantial unknown diversity of microbial eukaryotes that needs to be unraveled. The application of group specific primers revealed the biased state of knowledge in terms of different taxonomic groups within protists, especially in unexplored areas, like the tree canopies of palaeotropical rainforests. Whereby, the currently available molecular database appeared to provide better coverage for the phylum of Cercozoa compared to Oomycota. Tentative approaches of assigning a taxonomy and function to these novel undetermined oomycete clusters pointed towards similar ecological niches, which are occupied by well-known lineages in the tree canopies. Interestingly, the increased habitat diversity in the canopy region of tropical trees also increased the OTU richness, which is a pattern hitherto only observed at a macroscopic scale. Together, our results show how different functional traits, e.g. feeding modes and lifestyles, contribute to the establishment of taxonomically diverging protistan communities dwelling the canopy and ground strata of a tropical forest. The diversity, however, is comparably high for both, canopy and ground habitats. Whereby the ground stratum was characterized by a large number of indicative OTUs, elucidating the hitherto unknown complexity of microeukaryote food webs in tropical soils.

Conflict of Interest

None declared.

Funding

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Author contributions

MB and MS designed the study. SW, R-TJ and StS conceived and conducted the sampling and DNA extraction. KM assisted the sampling and determined tree species. SW, R-TJ and KF conducted the PCRs. SW and R-TJ performed the bioinformatic and statistical analyses and drafted the manuscript. All authors contributed to and approved the final version.

Data Accessibility

Raw sequence data have been submitted to the European Nucleotide Archive (ENA) database under the Bioproject number PRJEB37525, with accession numbers ERR4911998 (Cercozoa) and ERR5214402 (Oomycota), respectively.

Tables, figures, codes and detailed bioinformatic/statistical methods used in this study will be available at https://github.com/RJauss/ProtistsInPaupua, once the article is published.

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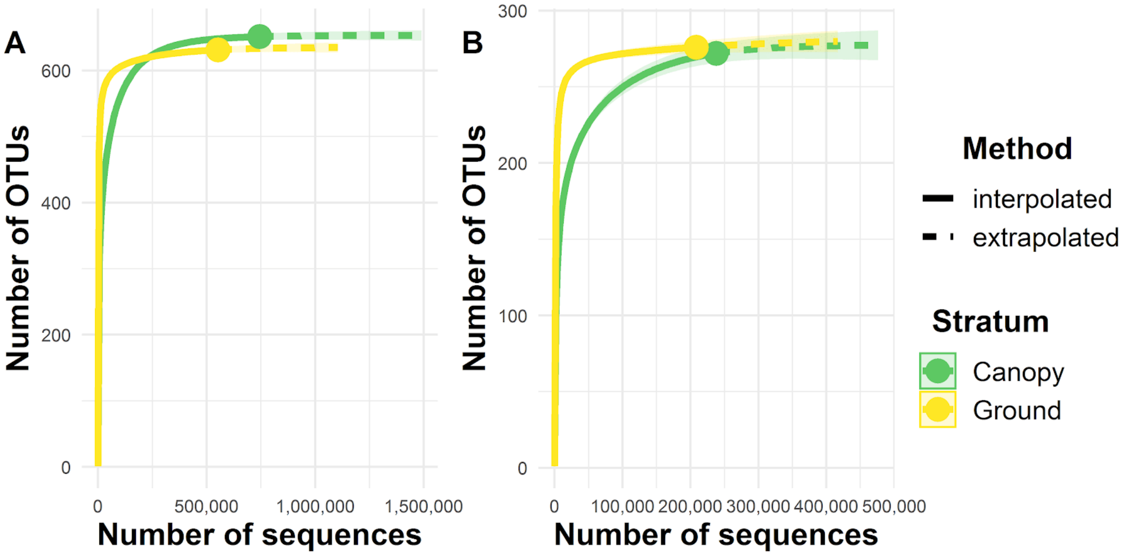
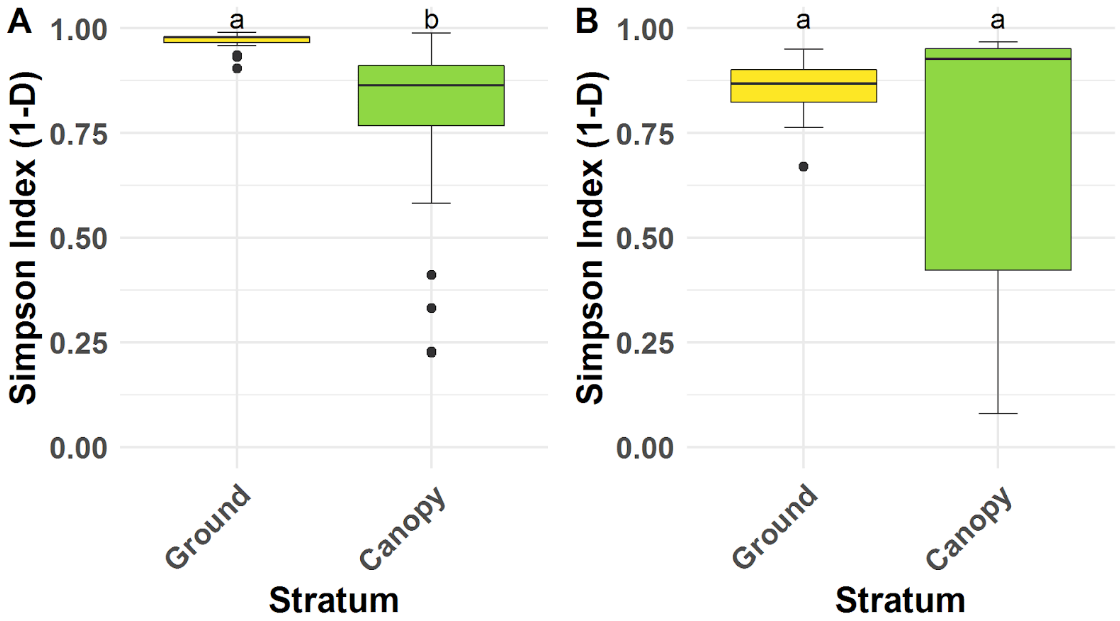
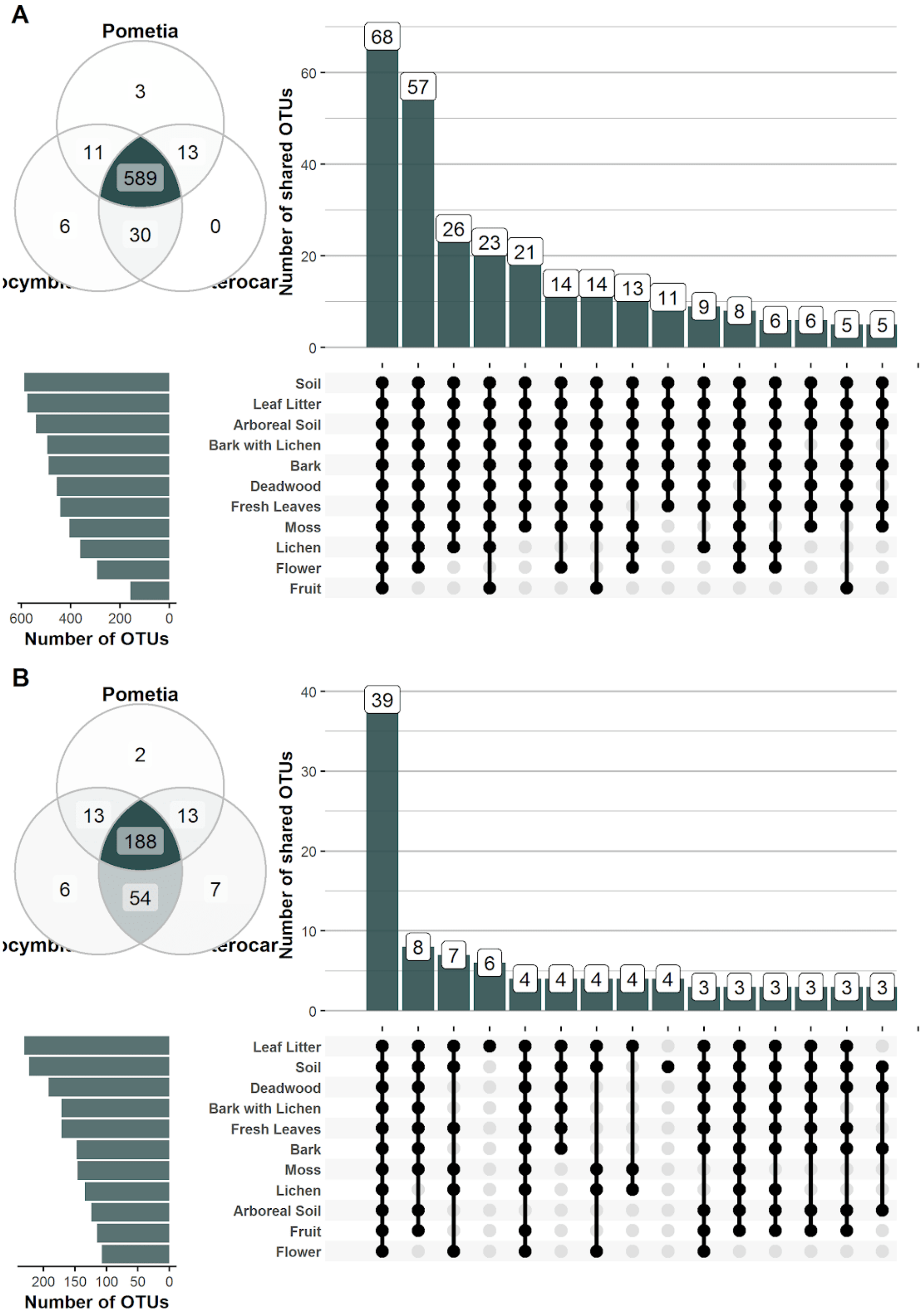
Supplementary Material



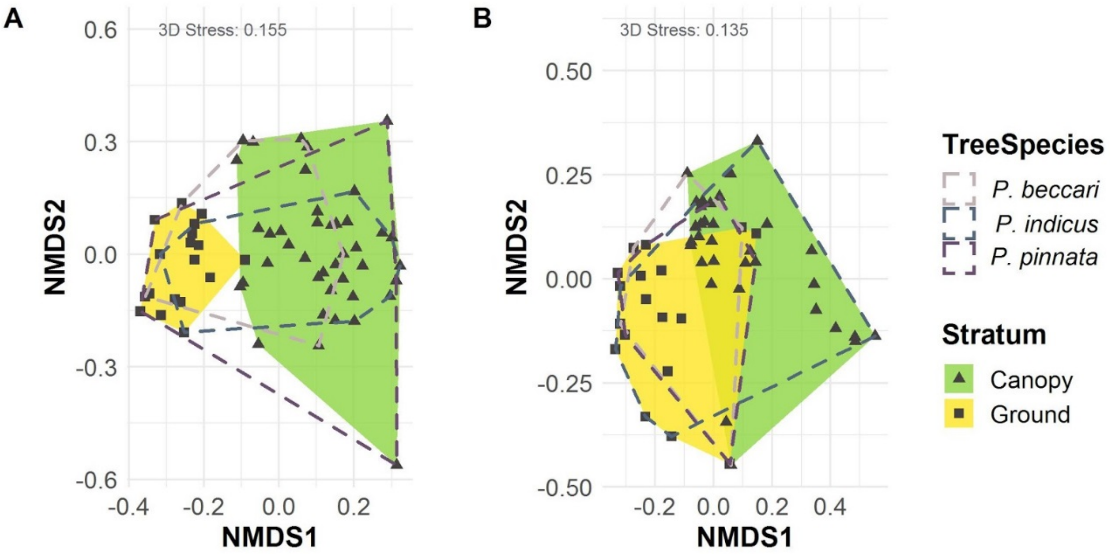
Supplementary Figure 1: Rarefaction curves of cercozoan (A) and oomycete (B) datasets. Solid lines give the interpolated number of OTUs from subsampled sequences; dashed lines represent extrapolated number of OTUs with increasing number of sequences. Shaded areas give the 97% confidence intervals.



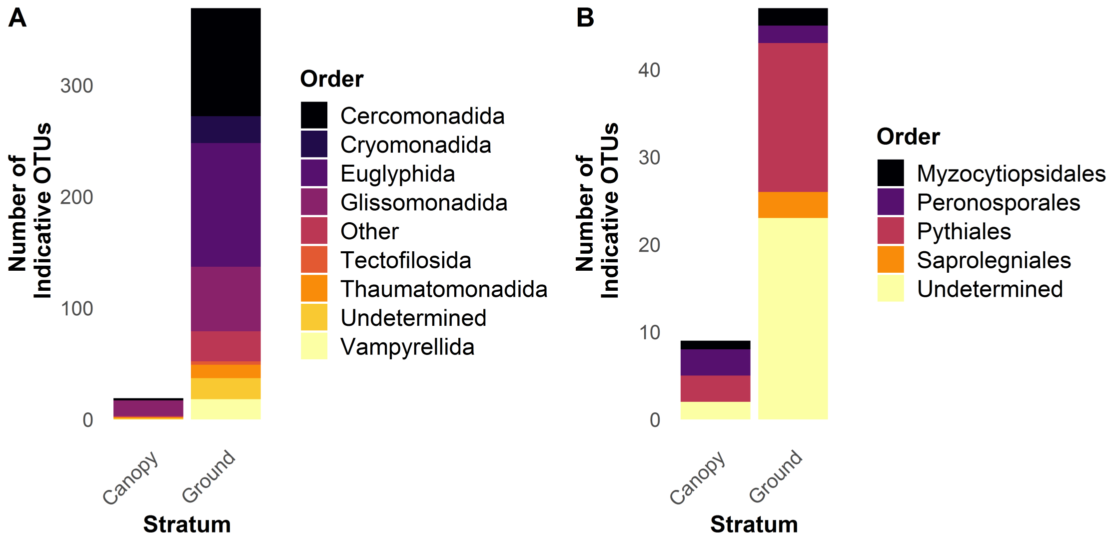
Supplementary Figure 2: Alpha diversity of canopy and ground strata for cercozoan (A) and oomycete (B) communities. Boxplots describe the Simpson Index of the samples grouped by strata; outliers are given by dots. Letters correspond to results from inferential statistics (t-test), with strata not sharing any letter having significantly different means. Simpson Index revealed high alpha diversity irrespective of the investigated protistan group, with higher variance of alpha diversity the canopy.



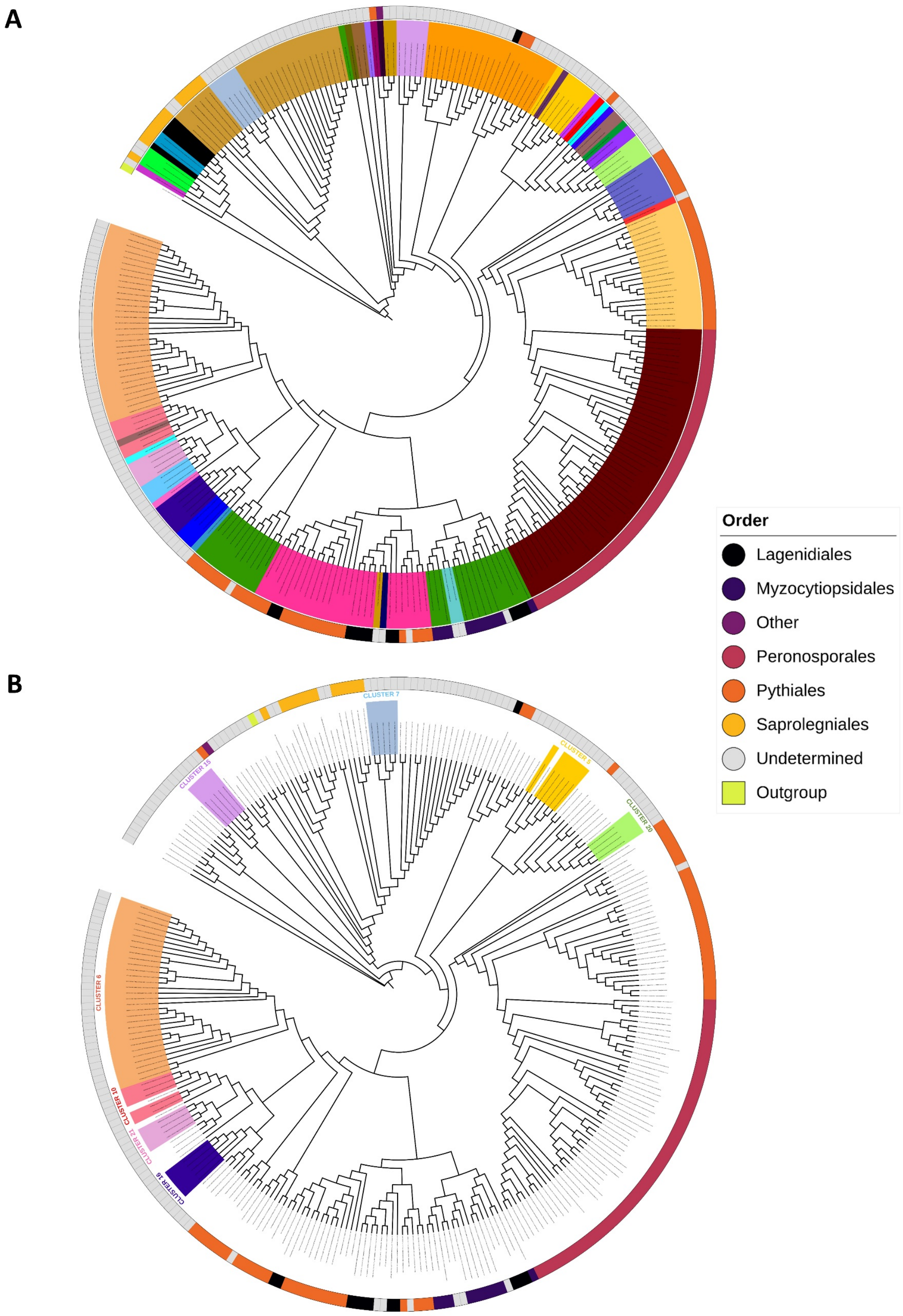
Supplementary Figure 3: Shared OTUs of Cercozoa (A) and Oomycota (B) between microhabitats (bar charts and combination matrix) and tree species (Venn diagrams). Top bar chart represents the sum of the number of shared OTUs resulting from the combination of microhabitats in the matrix below. Only the 15 combinations with the highest numbers of shared OTUs are shown. Left bar chart gives the number of OTUs for the corresponding microhabitat. Venn diagrams give the number of shared OTUs between the tree species.



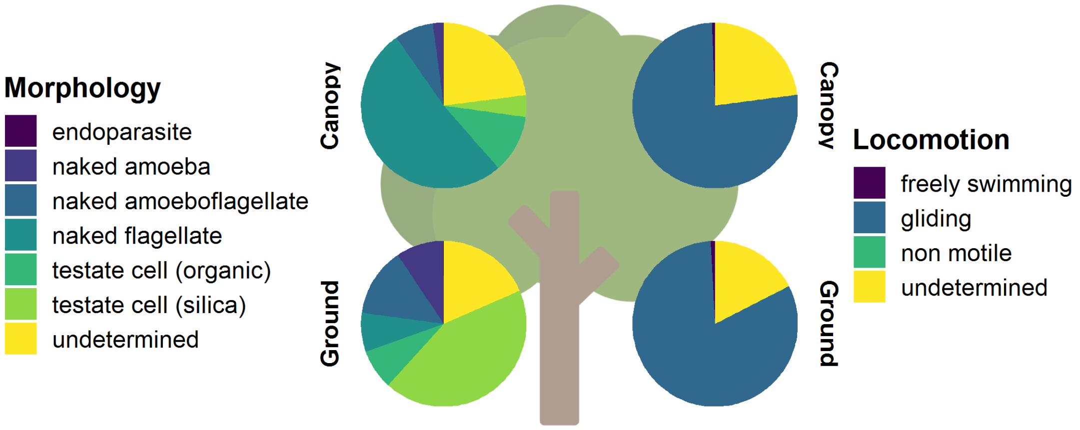
Supplementary Figure 4: Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities of cercozoan (A) and oomycete (B) communities among microhabitats. Cercozoan communities showed distinct canopy (green) and ground (yellow) communities compared to oomycetes, while the latter showed an overlap of two ground samples with canopy communities. Stress values of NMDS are shown in the upper left of each graph.



Supplementary Figure 5: Indicative OTUs of Cercozoa (A) and Oomycota (B) in canopy and ground habitats. For Cercozoa 365 indicative OTUs were found in the ground stratum, while Cercomonadida and Euglyphida represented the majority of indicative OTUs in the ground, with ca. 100 OTUs each. Only 20 OTUs appeared to be indicative for Cercozoa in the canopy region. For Oomycota 48 and 9 indicative OTUs were detected in the ground and canopy strata, respectively. Seventeen OTUs within the order of Phytiales were indicative for the canopy stratum. Whereas, 24 indicative OTUs could not be assigned to any taxonomic order. Orders represented by less than 1% of all reads were concatenated to "Other" for the sake of clarity.



Supplementary Figure 6: Phylogenetic cluster analyses of oomycete OTUs. Clustering of all oomycete OTUs revealed 44 distinct clusters (left). Investigation of novel undetermined oomycete clusters (comprising OTUs with a sequence similarity of less than 70% to any known reference sequence) revealed eight phylogenetic clusters (right).



Supplementary Figure 7: Relative proportion of cercozoan morphology (left) and locomotion modes (right) assigned to the OTUs, partitioned on the two strata canopy and ground. In the canopy, Cercozoan OTUs were mainly assigned to naked flagellates, while the majority of OTUs in the ground could be assigned OTUs representing taxa wearing a testate shell made from silica. The overall dominating locomotion mode in both strata were mainly dominated by OTUs that could be assigned to a "gliding" locomotion. Relative proportion of undetermined OTUs for canopy and ground strata were approximately the same.

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CHAPTER II

Forest Canopies – A Reservoir for Cryptic Species

What Drives the Diversity of the Most Abundant Terrestrial Cercozoan Family (Rhogostomidae, Cercozoa, Rhizaria)?

Reference:

Öztoprak H, **Walden S**, Heger T, Bonkowski M, Dumack K. What Drives the Diversity of the Most Abundant Terrestrial Cercozoan Family (Rhogostomidae, Cercozoa, Rhizaria)? *Microorganisms* 2020, DOI: 10.3390/microorganisms8081123.

CHAPTER III

On the Aerobiology of Microbial Eukaryotes

To the Canopy and Beyond: Air Dispersal as a Mechanism of Ubiquitous Protistan Pathogen Assembly in Tree Canopies

Reference:

Jauss R-T, Nowack A, **Walden** S *et al.* To the Canopy and Beyond: Air Dispersal as a Mechanism of Ubiquitous Protistan Pathogen Assembly in Tree Canopies. *European Journal of Protistology* 2021, DOI: 10.1016/j.ejop.2021.125805.

CHAPTER IV

How Seasonality Affects Protistan Communities and their Functionality in Tree Canopies

Part 4.1: On the Phenology of Protists: Recurrent Patterns Reveal Seasonal Variation of Protistan (Rhizaria: Cercozoa, Endomyxa) Communities in Tree Canopies

Reference:

Walden S, Jauss R-T, Feng K *et al*. On the Phenology of Protists: Recurrent Patterns Reveal Seasonal Variation of Protistan (Rhizaria: Cercozoa, Endomyxa) Communities in Tree Canopies. *FEMS Microbiology Ecology* 2021, DOI: 10.1093/femsec/fiab081.

CHAPTER IV

How Seasonality Affects Protistan Communities and their Functionality in Tree Canopies

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Part 4.2: A Parasite's Paradise: Biotrophic Species Prevail Oomycete Community Composition in Tree Canopies

Reference:

Jauss R-T, **Walden S**, Fiore-Donno AM *et al*. A Parasite's Paradise: Biotrophic Species Prevail Oomycete Community Composition in Tree Canopies. *Frontiers in Forests and Global Change* 2021, DOI: 10.3389/ffgc.2021.668895.

CONCLUSIVE SUMMARY & GENERAL DISCUSSION

Conclusive Summary and General Discussion

The studies described in this PhD thesis aimed to contribute to the knowledge on eukaryotic microbial communities and their diversity in the structurally highly heterogeneous ecosystem of tree canopies. The major aim of this thesis was to provide first insights into the hitherto unknown taxonomic and functional diversity prevailing in communities of the phylum Cercozoa across all major microhabitat compartments, from forest soil to the canopy region, in a temperate floodplain forest (Chapter I). To gain an overall view on cercozoan diversity in tree canopies, we compared community data derived from two different biomes (temperate and tropical forest), and uncovered the unknown cercozoan diversity in the canopy region of a palaeotropical rainforest in Papua New Guinea (Chapter I). By applying highly group-specific primers we were able to elucidate a putative cryptic-species-complex in the highly abundant family of Rhogostomidae (Cryomonadida), of which, to this day, only a handful of species have been described (Chapter II). A further objective was to shed light on the abiotic forces that contribute to the assembly of cercozoan canopy communities. We therefore investigated their potential dispersal by air in tree canopies, and found that passive air-dissemination of cercozoan taxa across tree canopies appears common (Chapter III). Finally, we investigated the seasonal variability of cercozoan communities in tree crowns (Chapter IV).

Diversity of Canopy Associated Cercozoa Across Biomes

Hitherto, knowledge on protistan diversity existing in the highly heterogeneous habitat of tree canopies was almost entirely limited to reports obtained from microscopical observations and broader morphological classifications (Foissner 2003; Bamforth 2007; Duarte *et al.* 2013; Buosi *et al.* 2014; Durán-Ramírez *et al.* 2015). While no standardized protocol for sampling canopy protists existed, we developed a protocol to take randomized samples of cercozoan communities across all major ecological compartments (microhabitats) in the forest canopy. Further, by applying a well-established metabarcoding protocol, we were the first to assess quantitative and qualitative informations on canopy associated cercozoan communities. The most decisive advantage, however, was the application of highly taxon-specific primers, which ensured an exhaustive coverage of the investigated cercozoan diversity. In comparison to other metabarcoding studies on protistan diversity in terrestrial systems, where general eukaryotic primers were applied (Lentendu *et al.* 2014; Geisen *et al.* 2015a; Mahé *et al.* 2017), we were able to obtain an OTU richness which was at least an order of magnitude higher, confirming our first hypothesis (**H1**) on a hitherto unknown taxonomic diversity of Cercozoa in tree canopies. In the temperate forest we observed that the majority of OTUs were present in all sampled microhabitats and therefore concluded that the data obtained by this approach was neither subject to undersampling nor to erroneous classification of taxa.

In Chapter I, we were able to demonstrate how discrete microhabitat niches in tree canopies led to specific differences in the composition of cercozoan communities, and ultimately within the entire forest ecosystem. Thus, most variation in cercozoan communities was explained by microhabitat differences, which supports our second hypothesis (**H2**) that postulates the presence of highly microhabitat-specific cercozoan communities. In particular, when comparing canopy communities to mineral soil communities on the ground high beta diversity was observed. However, the low beta diversity between the cercozoan communities of different cryptogamic epiphytes indicated that Cercozoa were likely depending on general microhabitat characteristics rather than on microhabitat identity. Therefore, our findings are in line with recent observations of Cregger et al. (2018) who reported highly specific microbial communities (i.e. bacteria, archaea and fungi) across broader ecological compartments (plant organ and tissue types) of poplar trees.

Only recently, Mahé and colleagues (2017) postulated the hypothesis that some protistan taxa could be 'a shadow of the tree-canopy communities from cells that have rained down from above' after analyzing soil samples in 279 locations in a variety of lowland neotropical forest types. Aforementioned hypothesis was subsequently corroborated by a study on endophytic fungi in 1-year-old litter by Guerreiro et al. (2018). Their findings revealed that a considerable proportion of previously endophytic fungi can be also found in fungal litter communities, where they contribute to the process of litter decomposition. Shortly afterwards, the term *viaphyte* or *viaphytism* ('by way of plant') was introduced by Nelson et al. (2020) to refer to the life cycle of certain fungi that undergo an interim stage as leaf endophytes and, after leaf fall, colonize the forest floor. Hence, one of our most exiting results was the high similarity between the communities of fresh leaves in the canopy and the litter layer on the ground, indicating that the phyllosphere may indeed contribute to the community assembly of Cercozoa in leaf litter on the ground. Thus, we were able to provide further support for our second hypothesis (**H2**) that foliar communities represent a subset of the communities dwelling in the forest floor. Surprisingly, we detected only a slight impact of differences between tree species on cercozoan community assembly. Giving the fact that taxa belonging to the phylum Cercozoa are mainly secondary consumers, the low plant-host specificity appears to be in striking difference to the repeatedly reported high host specificity of microbial primary consumers (i.e. bacteria, archaea and fungi) (Den Bakker *et al.* 2004; Redford *et al.* 2010; Wagner *et al.* 2016; Cregger *et al.* 2018; Taffner *et al.* 2018).

Interestingly, cercozoan beta diversity in the temperate forest was almost entirely driven by differences in the relative abundance of OTUs, suggesting an ubiquitous distribution of Cercozoa within this ecosystem – a pattern which was already reported from other metabarcoding studies on Cercozoa in temperate soil habitats (Fiore-Donno *et al.* 2018, 2019; Degrune *et al.* 2019). Therefore, differences in cercozoan communities among different microhabitats resulted from differences in species performance and were thus a consequence of habitat filtering (e.g. microhabitat-specific filters). By contrast, OTU richness increased with the number of structurally different microhabitat compartments in the palaeotropical tree canopies of Papua New Guinea, a pattern which has been hitherto only observed at macroscopic scales (MacArthur and MacArthur 1961; Hortal *et al.* 2009; Stein, Gerstner and Kreft 2014). Furthermore, Cercozoa showed considerably higher total OTU richness in the sampled canopies of the tropical compared to the temperate forest. According to reports of early naturalists, tropical rainforests are well known to be exceedingly species rich, at least in macroscopic species. Earlier investigations based on insect fogging of tree crowns across different tree species indicated that arthropods represent the most diverse eukaryotes in canopies of tropical forests (Erwin 1982). Our findings, however, suggest that also microbial eukaryotes show similar hyperdiversity patterns in tree crowns of the sampled palaeotropical forest. Thus, our data corroborate the results of Mahé et al. (2017) who suggested that protist in general are probably more diverse than arthropods in tropical terrestrial ecosystems. We further showed that certain cercozoan OTUs and lineages appeared to occur exclusively in the tropical biome, supporting the third hypothesis (**H3**) of this thesis, which postulates that tropical forest canopies harbour a specific, mainly undescribed diversity of Cercozoa compared to temperate forest biomes.

Taxonomical and Functional Diversity of Cercozoa

Detailed knowledge on the taxonomic composition and function of cercozoan canopy communities was obtained in Chapter I, III and IV. For the first time we obtained detailed insights on the diversity, taxonomical structure and functional attributes of taxa that allow Cercozoa to cope with the harsh environmental conditions prevailing in canopy microhabitats. Patterns of cercozoan diversity in tree canopies corresponded to earlier studies, which recognized Sarcomonadea (Glissomonadida and Cercomonadida) as the dominant class in terrestrial habitats (Geisen *et al.* 2015b; Bugge Harder *et al.* 2016; Fiore-Donno *et al.* 2018, 2020). In both investigated biomes – temperate and tropical forest – the small, gliding, bacterivorous flagellates of the order Glissomonadida (Howe *et al.* 2009) dominated in all canopy microhabitats, whereas one so-far undescribed glissomonadid OTU was identified as a clear canopy specialist in the temperate forest. The high abundances and uniform distribution of glissomonads across the canopy surface supported earlier findings of Ploch et al. (2016) who found that Glissomonadida were the dominant order of Cercozoa in the phyllosphere microbiome of four *Brassica* species. In tree canopies, the Glissomonadida were directly followed by a substantially smaller proportion of the mainly omnivorous testate amoebae of the orders Euglyphida and Cryomonadida. Within the Cryomonadida sequence reads assigned to the genus *Rhogostoma* in the Rhogostomidae (Thecofilosea), were particularly dominant (Chapter II), which is confirmed by earlier investigations on protistan diversity in a wide range of terrestrial habitats (Harder *et al.* 2016; Seppey *et al.* 2017; Degrune *et al.* 2019; Fiore-Donno *et al.* 2019).

Erected one century ago by Belar in 1921, the genus *Rhogostoma* accommodate omnivorous thecate amoebae with a characteristic cleft-like opening. Only recently, taxa of the genus *Rhogostoma* were associated with plant surfaces (Ploch *et al.* 2016; Dumack *et al.* 2017a), which triggered the description of *Rhogostoma epiphylla*, the first described plant surface-associated *Rhogostoma* species (Dumack *et al.* 2017a). However, aside from molecular surveys only a handful of Rhogostomidae have been described to the present day, and thus only little information exists on their morphology, phylogeny as well as their ecology (Howe *et al.* 2011; Dumack *et al.* 2017a, 2017b). Unfortunately, morphological identification of Rhogostomidae proved to be very difficult: their small size (<15 µm in diameter) and the absence of distinct morphological traits make it nearly impossible to conduct species delimitation without informations on their genetics, phylogeography and ecology (Howe *et al.* 2011; Dumack *et al.* 2017a). Chapter II thus revealed 23 new clades and extended the untapped diversity of Rhogostomidae by the description of eleven new *Rhogostoma* species. Integrative taxonomy, combining molecular, morphologic and environmental data (Riedel *et al.* 2013), thus allowed to identify the discrete morphological traits which are conducive for species delimitation in this family (i.e. cell length-to-width ratio and aperture invagination). Elucidating the hidden genetic diversity of this cryptic-species-complex with highly group-specific primer pairs led further support to our first hypothesis on the advantage of specific primers in environmental sequencing surveys (**H1**). Re-evaluating own environmental sequencing data and environmental metadata as well as environmental sequences deposited to the NCBI database from different terrestrial systems allowed us to identify various abiotic drivers that shape Rhogostomidae communities in terrestrial environments. The composition of Cryomonadida (Rhogostomidae and relatives) communities differed strongly between canopy and soil habitats, whereas no differences could be detected between canopy microhabitats, such as fresh leaves, bark, deadwood and arboreal soil. Thus, high beta diversity between canopy and soil communities indicated species turnover due to adaptations to specific environmental conditions in the prevailing strata (canopy and ground).

While omnivory and bacterivory were the dominant feeding strategies in the canopy, the litter and soil communities were markedly enriched by eukaryvores. The higher proportion of eukaryvores in ground habitats of the temperate forest could be related to significantly higher read numbers of vampyrellid amoebae that feed on a wide range of soil eukaryotes, such as algae, fungal spores as well as nematodes (Anderson and Patrick 1980; Pakzad and Schlösser 1998; Hess, Sausen and Melkonian 2012). In addition, taxa of undetermined nutrition type made up a larger proportion in litter and soil communities compared to canopy communities in the floodplain forest. In the tropics, on the other hand, omnivorous Euglyphida prevailed over all other orders in litter and soil communities. Taxa of the order Euglyphida have been reported to feed on both, bacteria and small eukaryotes, such as yeast, algae and other protists (Dumack *et al.* 2020).

One possible explanation for their dominance in tropical ground habitats could be their morphological characteristic of bearing a silica or organic shell (Wylezich *et al.* 2002), which, in turn, might be a crucial adaptation to ensure their survival in tropical soils even during dry season by providing physical protection against desiccation (Thomas 1958; Bonnet 1961; Booth 2001). In contrast, the canopy dominating naked flagellates of the order Glissomonadida might still benefit from the comparatively high atmospheric humidity prevailing in a tropical rainforest throughout the year. These observations corroborate our fourth hypothesis (**H4**) on niche selection for functionally better adapted species. It should, however, be mentioned that the relatively high cercozoan diversity in the tropical soil and leaf litter samples, was beyond our expectations, as we presumed that the increased mineralization of litter on the soil surface within a rainforest might induce reduction of habitat complexity compared to the relatively thick organic layers in temperate forests. However, since moisture seasonality might be responsible for forest floor decomposition rates in tropical forests (Wieder and Wright 1995), the communities detected in the ground habitats were characterized by higher alpha diversity compared to the canopy region, and further comprised a multitude of indicative species – elucidating the hitherto unknown complexity of microeukaryote food webs in tropical forest soils.

Stochasticity of Cercozoan Distribution in Tree Canopies

In Chapter I we investigated the diversity and community composition in canopy microhabitats and in litter and soil on the ground across two different biomes. Interestingly, within the temperate biome, the detected cercozoan OTUs were present in almost every microhabitat and only varied in their abundances between the sampled ecological compartments. Thus, we identified habitat filtering as one major deterministic process driving the cercozoan community assembly in terms of species performance in the tree crowns. One major explanation for the ubiquity of cercozoan OTUs across all microhabitats could be their high dispersal rate as well as the dominance of potential habitat generalists within this phylum. Building upon these findings, the question arises of how the canopy surface gets colonized by Cercozoa before habitat filtering induces species sorting within the respective microhabitat.

By analysing near-ground and canopy air samples at two different time points (March and May), we investigated potential air dispersal of Cercozoa in the study represented by Chapter III. A direct comparison of the total diversity described in the first part of Chapter I with the diversity of Cercozoa distributed by air revealed that 22% of the diversity from the examined forest stand could be detected in the air samples, as well. Here, too, Glissomonadida and Cryomonadida made up the largest proportion of detected OTUs. Further, our investigation revealed that neither OTU richness nor Shannon-diversity, or evenness of air samples were affected by the tree species or sampling location (near-ground and canopy). However, Shannon-diversity and evenness appeared to be higher during the second sampling in May after leaf unfolding, demonstrating the role of hitherto unexplored stochastic processes that are conducive to the distribution of cercozoan taxa across the canopy surface. According to our data, we suggest that the differences between the two different sampling time points could be either explained by temporal variations in the distribution of taxa or by the prevailing weather conditions during the respective sampling period. The atmospheric weather conditions appeared to be more favourable for dispersal of cercozoan propagules in May due to higher temperatures and increased humidity. Interestingly, wind speed in May was slower compared to March, suggesting that not only a single factor like the wind speed, but instead a whole set of environmental factors (e.g. wind speed, temperature, humidity, precipitation) must be considered when determining stochastic processes that drive OTU richness and community composition of Cercozoa in the canopy surrounding air.

Air dispersal is thought to be an important means by which plant pathogens can be spread to reach new susceptible host plants (West and Kimber 2015). In the first part of Chapter IV we provide first evidence for the role of forest canopies as physical filters and/or reservoirs for air dispersed plant pathogens of the order Plasmodiophorida within the phylum Endomyxa. By investigating cercozoan communities dwelling in the canopy during two consecutive spring and autumn seasons, we found several endomyxan root parasites to be exceptionally abundant across the entire canopy surface during autumn. This observed ubiquitous distribution further points towards the high potential of air dispersal of cercozoan propagules, and reflects the complex life cycle of these taxa with distribution via sporangia in autumn (Barr and Asher 1996). More importantly, this observation extends our knowledge on the ecosystem services tree canopies provide: since potential host plants of particular Plasmodiophorida were completely absent in the sampled floodplain forest, one can conclude that tree canopies may play an important role as a physical barrier for air dispersed microbial plant pathogens, and thus may partly prevent their further spread to the environment.

Examination of protistan seasonality over two consecutive years revealed phenology patterns in the abundance of certain cercozoan taxa and further explained a small but significant proportion of variation in the investigated communities (Chapter IV). Whereby a

Rhogostoma OTU was temporally the most abundant taxon in autumn, while a *Bodomorpha* OTU within the Glissomonadida dominated during spring samplings. Stochastic seasonal differences were particularly evident within communities dwelling the microhabitat of fresh leaves. We reported lower OTU richness of foliar communities in spring right after leaf emergence with higher variation between individual samples compared to autumn communities. In autumn, however, foliar communities showed less variation and became highly specific for this microhabitat and the respective autumn season. In this respect, it should be noted that microbial succession in the phyllosphere has been mainly reported for bacterial communities (Redford and Fierer 2009; Maignien *et al.* 2014; Copeland *et al.* 2015). Bacterial phyllosphere communities have been observed to initially mirror airborne communities and subsequently converge to distinct foliar communities, suggesting stochastic events in early stages of colonization to be responsible for bacterial community assembly (Maignien *et al.* 2014).

The fundamental goal of communitiy ecology is to shed light on the mechanisms that influence community assembly after habitat colonization (Mergeay *et al.* 2011). Especially, when organisms are not limited by their dispersal rates, environmental filtering of taxa according to their ecological traits is expected to be a highly deterministic process (Cottenie 2005). However, the foundation for community assembly lays, up to a certain point, in the so-called priority effect, which describes the impact that particular species can have on future community assembly due to prior arrival in a habitat (Drake 1991). The core thesis of this theory states that the order of species arrival is responsible for alterations in abiotic an biotic conditions that positively or negatively affect the establishment of species that arrive later (Weidlich *et al.* 2021). With our findings we were able to provide first evidence for the presence of ecological priority effects in tree canopies, which induced variable outcomes of cercozoan community assembly in autumn due to temporal stochasticity of pioneer communities during initial colonization of leaves in the respective spring season. In summary, it can be stated that the studies described in Chapter III and IV made significant contributions to the understanding on the balance between deterministic and stochastic processes in eukaryotic microbial systems and thus gave evidence for our last hypothesis (**H5**) on the contribution of stochastic processes to the distribution of Cercozoa in forest canopies. Finally, it can be said that a comprehensive understanding of the processes that govern distribution and selection of microbial eukaryotes in terrestrial systems might provide new insights into ecosystem functioning as well as the ecosystem services tree canopies provide. Moreover, the findings obtained in this thesis reflect the role of tree canopies as – to put it in William Beebe's words – another continent of life that awaits discovery, in particular with regard to the taxonomically and functionally highly diverse group of protists.

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I dedicate this thesis to my grandparents ∞

Subpublications and Records of Achievement

Publications mentioned in chapters:

Chapter I

(2,6)Jauss R-T & **Walden** S *et al.* From Forest Soil to the Canopy: Increased Habitat Diversity Does Not Increase Species Richness of Cercozoa and Oomycota in Tree Canopies. *Frontiers in Microbiology* 2020, DOI: 10.3389/fmicb.2020.592189.

(2,6)**Walden S** & Jauss R-T *et al.* Exploring the "Last Biotic Frontier" – Unraveling the Diversity of Cercozoa and Oomycota in Palaeotropical Tree Canopies. To be submitted to a high-ranking journal.

Chapter II

(4,5)Öztoprak H, **Walden S**, Heger T, Bonkowski M, Dumack K. What Drives the Diversity of the Most Abundant Terrestrial Cercozoan Family (Rhogostomidae, Cercozoa, Rhizaria)? *Microorganisms* 2020, DOI: 10.3390/microorganisms8081123.

Chapter III

(3)Jauss R-T, Nowack A, **Walden S** *et al.* To the Canopy and Beyond: Air Dispersal as a Mechanism of Ubiquitous Protistan Pathogen Assembly in Tree Canopies. *European Journal of Protistology* 2021, DOI: 10.1016/j.ejop.2021.125805.

Chapter IV

(1,6)**Walden S**, Jauss R-T, Feng K *et al*. On the Phenology of Protists: Recurrent Patterns Reveal Seasonal Variation of Protistan (Rhizaria: Cercozoa, Endomyxa) Communities in Tree Canopies. *FEMS Microbiology Ecology* 2021, DOI: 10.1093/femsec/fiab081.

(4,6)Jauss R-T, **Walden S**, Fiore-Donno AM *et al*. A Parasite's Paradise: Biotrophic Species Prevail Oomycete Community Composition in Tree Canopies. *Frontiers in Forests and Global Change* 2021, DOI: 10.3389/ffgc.2021.668895.

Further publications

(4,7) Siemensma F, Holzmann M, Apothéloz-Perret-Gentil L, Clauß S, Voelcker E, Bettighofer W, Khanipour Roshan S, **Walden S**, Dumack K, Pawlowski J. Broad Sampling of Monothalamids (Rhizaria, Foraminifera) Gives Further Insight into Diversity of Non-Marine Foraminifera. *European Journal of Protistology* 2020, DOI: 10.1016/j.ejop.2020.125744.

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(1) This study was designed and planned together with Prof. Dr. Michael Bonkowski and Prof. Dr. Martin Schlegel. Sampling, laboratory work, bioinformatics, taxonomic and statistical analyses were carried out mainly by the author, if not stated otherwise.
The article was mainly written by the author under guidance of Prof. Dr. Michael Bonkowski and Prof. Dr. Martin Schlegel.

(2) Both first authors performed the sampling, laboratory work, bioinformatic analyses, if not stated otherwise, and their complementary contributions merit equal credit. This study was designed and planned together with Prof. Dr. Michael Bonkowski and Prof. Dr. Martin Schlegel. The article was mainly written by both first authors under guidance of Prof. Dr. Michael Bonkowski and Prof. Dr. Martin Schlegel.

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(4) The author contributed in adjusting and refining the final version of the manuscript.

(5) The author contributed in sampling, laboratory work and bioinformatic analysis for this study.

(6) Sampling, DNA extraction and PCRs were mainly performed by the author and the co-author Robin-Tobias Jauss by assistance of Stefan Schaffer, Ronny Wolf and Kai Feng.

(7) The author sampled and isolated species material for this study.

(8) The author was partly involved in establishing the bioinformatic scripts for visualizing results of this study.

ERKLÄRUNG ZUR DISSERTATION

Gemäß § 7 Absatz 8 der Promotionsordnung vom 12. März 2020

Erklärung zur Dissertation

gemäß der Promotionsordnung vom 12. März 2020

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Teilpublikationen:

Jauss R-T & **Walden S** *et al.* From Forest Soil to the Canopy: Increased Habitat Diversity Does Not Increase Species Richness of Cercozoa and Oomycota in Tree Canopies. Frontiers in Microbiology 2020, DOI: 10.3389/fmicb.2020.592189.

Walden S & Jauss R-T *et al.* Exploring the "Last Biotic Frontier" – Unraveling the Diversity of Cercozoa and Oomycota in Palaeotropical Tree Canopies. To be submitted to a high-ranking journal.

Öztoprak H, **Walden S**, Heger T, Bonkowski M, Dumack K. What Drives the Diversity of the Most Abundant Terrestrial Cercozoan Family (Rhogostomidae, Cercozoa, Rhizaria)? Microorganisms 2020, DOI: 10.3390/microorganisms8081123.

Jauss R-T, Nowack A, **Walden** S *et al.* To the Canopy and Beyond: Air Dispersal as a Mechanism of Ubiquitous Protistan Pathogen Assembly in Tree Canopies. European Journal of Protistology 2021, DOI: 10.1016/j.ejop.2021.125805.

Walden S, Jauss R-T, Feng K *et al.* On the Phenology of Protists: Recurrent Patterns Reveal Seasonal Variation of Protistan (Rhizaria: Cercozoa, Endomyxa) Communities in Tree Canopies. FEMS Microbiology Ecology 2021, DOI: 10.1093/femsec/fiab081.

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