Soil protists in tropical systems: From morphological variation to trophic groups

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"Take the risk of thinking for yourself,
much more happiness, truth, beauty,
and wisdom will come to you that way."

Christopher Hitchens

General Introduction

The soil system

Understanding life in soil is of utmost importance as soil is the habitat in which key processes such as decomposition and nutrient mineralisation take place. These processes are essential for the functioning of both forests and agricultural systems. Across the world, but especially in tropical regions, where biodiversity is threatened by deforestation and conversion of rainforest into plantations, soil processes need closer attention (Gibbs et al., 2010). Key processes in soils are primarily carried out by different groups of organisms inhabiting the soil. These soil organisms, mostly invisible to the eye, include bacteria, archaea, fungi and protists as well as animals of different size classes from micro- to meso- to macrofauna (Swift et al., 1979). In particular the rhizosphere in soil plays a key role in this interconnected system (Nielsen, 2019).

Compared to the aboveground system, understanding of the belowground system is more challenging because direct observations are difficult or impossible due to the opaqueness of the soil. In addition, the soil is a very heterogeneous system with many microenvironments, due to aggregates formed by particles of different sizes ranging from 2 mm (coarse sand) to <0.5 µm (clay). Together with intermixed organic particles different soil horizons form along a vertical axis, each with individual properties, e.g. water holding capacity, pH, light availability, temperature and amount of nutrients. These microenvironments can differ significantly, even between individual soil aggregates. For example, the concentration of oxygen can vary from 20 % at the outside to nearly 0 % at the inside of an aggregate, allowing taxa with contrasting physiologies, i.e. aerobic vs. anaerobic metabolism, to thrive in close proximity (van Elsas, 2019). Such differences in

microenvironments can be particularly pronounced in old soils such as those typically dominating in nutrient limited tropical soils (Chesworth et al., 2008).

For long the exploration of the tiniest organisms of soils such as microorganisms was based on cultivation and microscopy approaches only, skewing observed richness and diversity numbers due to limited cultivation success of certain taxa. However, the advent of molecular techniques and the remarkable advances during recent years made soil microorganisms much more accessible. While the first screenings were applied to aquatic systems (López-García et al., 2001), early approaches focusing on specific groups in the soil system showed a plethora of new forms (Lara et al., 2007; Lejzerowicz et al., 2010; Fiore-Donno et al., 2016). High-throughput sequencing as well as amplicon sequencing revealed a much broader range of soil microorganism diversity than known before (Bates et al., 2013; Geisen et al., 2015; Mahé et al., 2017). Information on main soil taxa and their respective functional traits is growing at an increasing rate (van Elsas et al., 2019). Despite these advances, understanding the structure and functioning of soil systems remains challenging, with the inhabiting organisms interacting on multiple trophic levels.

Protists – Small but important soil organisms

Protists are the virtually invisible majority of all eukaryotic life on earth and occur in every biome and reaching abundances of tens of thousands individuals per gram of soil (Finlay, 2002; Geisen et al., 2018; Bonkowski et al., 2019). They are predominantly unicellular and include all eukaryotes outside plants, animals and fungi, spanning the entire eukaryotic tree of life (Adl et al., 2019). They comprise taxa smaller than bacteria (Massana et al., 2002) and up to multiple meters (Lamouroux, 1809). Armoured forms such as diatoms, testate amoebae or foraminiferans exist next to naked forms with flexible bodies such as naked amoebae or slime molds (Adam et al., 2017). In the hard bodied taxa

morphology plays a major role, as their remains can be studied in detail, while in the soft bodied taxa motion is important (Hausmann et al., 2003).

The classification of protist diversity changed markedly through time and is revised regularly (Adl et al. 2005, 2019; Burki et al. 2020; Honigberg et al. 1964; Levine et al. 1980). Until the early 2000s, taxonomy and classification of protists focused primarily on morphology and mode of nutrition. Photoautotrophs were separated from heterotrophs, which were further separated by mode of food intake, e.g. phagocytosis (ingestion of particles in a food vacuole), pinocytosis (ingestion of nutrients in solution in a food vacuole) and osmotrophy (absorption of nutrients through the cell membrane) (Bonkowski et al., 2019). With advances in light and scanning electron microscopy, molecular insights from cultured protists, sequences obtained through culture independent approaches, and the usage of metabarcoding and phylogenomics, classification of protists moved gradually from purely morphological thinking to mostly molecular data-driven systematics. Honigberg (1964) and Levine (1980) used the traditional three kingdom model with animals, plants and fungi, where protists were seen as a subkingdom of the Animalia. It was later recognised that it is the other way around and animals, plants and fungi nest within protists. This lead to a model of six supergroups with Opisthokonta (including animals and fungi), Amoebozoa, Rhizaria, Archaeplastida (including plants), Excavata and Chromalveolata (Adl et al., 2005). It was later refined into a five supergroup model with Opisthokonta, Amoebozoa, SAR (Stramenopiles, Alveolata, Rhizaria), Archaeplastida and Excavata (Adl et al., 2019). The supergroup model became quite popular as it conveniently and efficiently includes virtually all eukaryotes, has at least one ancestral biological characteristic for most groups, e.g. a free-living amoeboid form in Amoebozoa, a particular feeding grove in Excavata or the presence of primary

photosynthetic plastids in Archaeplastida. With more phylogenomic data and advances in next generation sequencing as well as (re-)discoveries of major taxa, the supergroup model has been transformed into a virtually purely molecular classification without integration of cell-biological evidence (Burki et al., 2020). Some supergroups remained nearly unchanged, i.e. TSAR (Telonemia and SAR) and Archeaplastida, or were grouped together, such as Amoebozoa and Opisthokonta with Breviates and Apusomonada formed the Amorphea, while new ones were formed, i.e. Haptista, Cryptista, Hemimastigophora and CRuMs (Collodictyonida, Rigifilida and Mantamonas), or comprised a single "orphan" taxon, i.e. Ancoracysta, Picozoa, Discoba, Metamonada, Malawimonadida and Ancryomonadida (Figure 1).

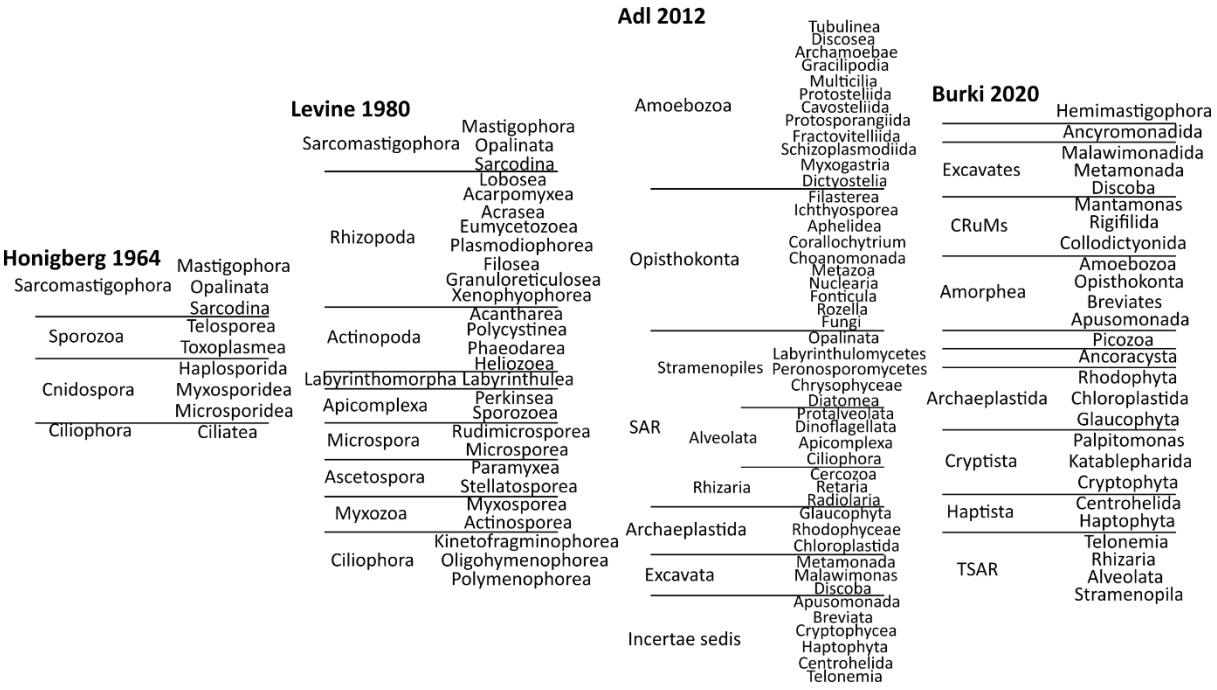

Figure 1: Major changes and revisions of the classification of protists.

Functional diversity - What protists do

Of all soil microorganisms, protists are arguably the most important and functionally divers group in the soil food web, seen in the different trophic groups of soil protists, e.g. phagotrophs, photoautotrophs, mixotrophs, but also parasites (Singer et al., 2021). A

large part of protists is phagotrophic and acquires their nutrients via consumption of other living organisms. Bacterivores are probably the most prominent phagotrophs in soil and are important in nitrogen cycling and can regulate the composition of bacterial communities. They occur in most groups. Examples for bacterivorous soil protists are glissomonads (Rhizaria) and colpodids (Alveolata). Probably more widespread than previously thought are fungivores, which feed on fungal spores and hyphae (Kramer et al., 2016). However, strict fungivores such as *Platyreta* (Rhizaria) are rarer than omnivorous taxa such as several members of the myxomycetes (Amoebozoa) or *Acanthamoeba* (Amoebozoa), which feed on multiple trophic levels, e.g. consume bacteria, fungi and other protists (Geisen et al., 2018). Algivores such as *Chilodonella* (Ciliophora) and Viridiraptoridae (Rhizaria) are specialised to consume algae, often by piercing the cell wall of their prey and sucking its content or entering their prey, consuming them from the inside (Hess and Melkonian, 2013). Examples of more general predators of other protists, eukaryovores, are hyalosphenid testate amoebae (Rhizaria) and ravenous *Lacrymaria* (Ciliophora), consuming basically everything (Rosati et al., 2008). Photoautotrophic protists, traditionally termed algae, are mostly known from marine and freshwater systems, but are present in the upper sunlit layer of soils, forming biological crusts (Belnap et al., 2001). Although they comprise only a small proportion of soil protists, photoautotrophs contribute significantly to soil organic carbon input. They are mainly found in the Stramenopila, e.g, diatoms and Xanthophyceae, and Archaeplastida, e.g. Chloroplastida and Rhodophyta. In-between photoautotrophs and phagotrophs are the mixotrophs, which can switch between both modes of life. This often depends on environmental conditions and availability of prey. Dinophyacea (Alveolata) or Cryptophycea (Cryptista), whose importance in planktonic systems is increasingly

recognized (Flynn et al., 2019), also occur in soils, and can live like photoautotrophs in the sunlit layer, but also thrive in the deeper layers employing a phagotrophic lifestyle (Selosse et al., 2017). Some protists are saprotrophs and degrade dead organic matter. This feeding mode occurs in some myxomycetes (Amoebozoa) and peronosporomycetes (Stramenopiles) (Beakes et al., 2014). However, protists can also parasitize animals, plants or other protists. In recent years, it has become obvious that gregarine apicomplexans (Alveolata) constitute a large part of soil protists (Mahé et al., 2017), while Oomycota (Rhizaria) and Phytomyxea (Rhizaria) can be severe plant pests in agricultural systems (Neuhauser et al., 2014), and Cryptosporida (Alveolata) infect other soil protists (Kinne and Lauckner, 1980). Infections of humans can occur as well, gaining more attention in recent years (Angelici et al., 2021).

Study area

Tropical rainforests are rich in endemic plant and animal species, many are seen as hotspots of biodiversity (Myers et al., 2000). At the same time, they are highly threatened ecosystems, due to mankind's increasing demand for cropland (Margono et al., 2014). The soils of tropical rainforests are often nutrient limited due to longevity, leaching and geochemical processes (Chesworth et al., 2008). Microbial communities of tropical forests are generally limited by phosphorous, with an additional nitrogen limitation in montane tropical rainforests (Camenzind et al., 2018).

In the studies of this thesis, I investigated soil protist diversity and ecology in two tropical regions, the montane rainforest of Southern Ecuador and the lowland rainforest of Sumatra, Indonesia. The study sites used in Chapter 1 and 3 are located in the northern part of the Podocarpus National Park on the eastern slope of the Andes in Southern Ecuador and are part of the nutrition manipulation experiment (NUMEX) (Homeier et al.,

2012). In Chapter 1 I examined an altitudinal gradient from 1,000 m a.s.l. near the city of Bombuscaro (S 04°06’54’’, W 78°58’02’’), to 2,000 m a.s.l. near the research station Estación Científica San Francisco (S 03°58’18’’, W 79°4’45’’), and in the region of Cajanuma at 3,000 m a.s.l. (S 04°06’711’’, W 79°10’581’’). In Chapter 3 I focused on the site at 2,000 m a.s.l. near the research station Estación Científica San Francisco. The forest is classified as lower montane rainforest (Homeier et al., 2008) and part of the private reserve Reserve Biologica San Francisco. It is in close to pristine condition and well-studied in regard to vegetation and above- and belowground arthropods, with extraordinary richness in tree species as well as other plant and animal species (Beck et al., 2008; Homeier et al., 2010).

The sampling sites used in Chapter 2 were located in the tropical lowlands of the Jambi Province in Sumatra, Indonesia. Two landscapes were studied, Bukit Duabelas (S 2°0’57’’, E 102°45’12’’) and Harapan (S 1°55’40’’, E 103°15’33’’). At each landscape four typical land-use systems representing the conversion from rainforest into plantations with increasing land-use intensity were selected: secondary lowland rainforest (rainforest), rubber agroforest (jungle rubber), rubber plantation (rubber) and oil palm plantation (oil palm). All sites form part of the Collaborative Research Centre 990 “Ecological and Socio-economic Functions of Tropical Lowland Rainforest Transformation Systems” funded by the German Science Foundation (DFG) (EFForTS project, http://www.uni-goettingen.de/crc990) (Clough et al., 2016; Drescher et al., 2016; Grass et al., 2020).

Methods used to access soil protists

Since the 17th century, starting with the work of Antoni van Leeuwenhoek, optical microscopy was the only way to make protists visible. Optical or light microscopes were refined and optimised to increase magnification up to the practical limit of ~1,500, a

resolution of around 0.2 µm. For the often translucent protists, methods such as staining were helpful but contrast improving inventions such as phase contrast or differential interference contrast enabled microbiologist a better access to details of protists. This was further increased with transmission and scanning electron microscopy with magnifications up to ~500,000, a resolution of around 0.4 nm. However, preparation effort of samples increased as well, but, if done correctly, results in astonishingly detailed pictures of protists. The armoured and hard bodied soil protists, testate amoebae in particular, are well accessible via microscopy. Species identification relies heavily on morphology (Clarke, 2003; Bobrov and Mazei, 2004). By measuring the variability in morphological traits, testate amoebae can be linked to and are important indicators of environmental changes (Wanner and Meisterfeld, 1994; Marcisz et al., 2020). Furthermore, identified species and all their attached knowledge can be linked to DNA sequences.

With the advent of molecular environment sampling, soil protists could be assessed in large-scale biodiversity surveys, which was simply not possible with microscopy alone. Regularly used markers are sections of ribosomal genes (the variable regions V4 and V9 of 18S), internal transcribed spacers (ITS1 and ITS2) and mitochondrial markers (Bonkowski et al., 2019). However, primer selection can cause a bias for specific groups of protists and incompleteness of reference databases are problems to be aware of (Del Campo et al., 2014). Development of methods is fast paced and continuously improving (Lin and Peddada, 2020). Focal points of ongoing discussions and development are how to relate reads to individual organisms, how to compensate for technical challenges during sequencing, but also how sampling methods influence soil protist diversity assays (Santos et al., 2017; Weißbecker et al., 2017; Knight et al., 2018; Calle, 2019). Despite of

all these challenges, molecular environment sampling is a well-established tool to study functional groups of protist communities (de Vargas et al., 2015; Geisen, 2016; Mahé et al., 2017).

Approach and scope of this work

In the studies of this thesis, I used two different approaches to shed light on soil protist diversity and ecology in two tropical regions, the montane rainforest of Southern Ecuador and the lowland rainforest of Sumatra, Indonesia. In Chapter 1, I have used classic approaches of light and scanning electron microscopy to study the morphology of testate amoebae to delineate species and relate variability of morphological characteristics to environmental changes. I while in Chapters 2 and 3 I have used high throughput sequencing of environmental DNA to move from individual organisms to whole communities of soil protists. While the effects of changes in the environment of soil protists communities are the focus of Chapter 2, an experimental approach manipulating nutrients, fungi and mesofauna is used to investigate interactions of soil protists with other soil organisms.

In Chapter 1, I focus on the morphology of the genus *Trigonopyxis* (Arcellinida, Amoebozoa), a testate amoeba. These protists have a cell protected by their name giving shell, the test, which is characteristic in shape and composition for each species. Shells of testate amoebae can persist over long periods of time and even fossilise in moist and acidic environments, such as the montane rainforests of the Andes (Charman, 2001). Ecological preferences can be inferred from the morphology of testate amoebae shells and related to past and present environmental condition and ecosystem functions (Marcisz et al., 2020). A focal characteristic is the shell opening, called pseudostome or aperture, through which the amoeboid cell has contact with its environment. The taxon *T. arcula* is

known for its large variety in shell size and pseudostome forms, which complicates the use of this widespread species in ecological studies. I hypothesized that pseudostome form is an appropriate character for taxa delineation of the genus *Trigonopyxis*, as different pseudostome types likely indicate different feeding types and point to trophic niche differentiation. Further, I hypothesized that shell size varies with environmental conditions, reflecting phenotypic adaptations and therefore being of limited value for taxa delineation. I identified five stable morphotypes in *Trigonopyxis arcula* based on pseudostome morphology, showing that this trait can indeed be used for species delineation. The often-used shell size as well as the correlated pseudostome diameter are strongly influenced by temperature and humidity, highlighting their limited use in species delineation. However, as shell sizes of all morphotypes were affected uniformly it can be used as indicator for changes in environmental conditions, making it useful for palaeoecological studies, e.g. for reconstruction of climatic conditions of the past.

In Chapter 2, I compare protist communities using high-throughput sequencing of environmental DNA from four different land-use types in Indonesia; secondary lowland rainforests, rubber agroforests, rubber plantations and oil palm plantations. Protists assume key roles in microbial food webs of soils, e.g. as bacterial feeders, primary producers, fungal feeders and more, and are useful as bioindicators but also biofertilizers and biocontrol agents (Gao et al., 2018; Geisen et al., 2018). However, due to ever increasing demands for cropland by humans, large areas of natural systems such as rainforests or peatlands are transformed into arable systems (Gibbs et al., 2010; Margono et al., 2014). Such conversion strongly reduces the abundance and richness of animals and plants aboveground, but also affects the belowground system severely (Drescher et al., 2016). While this has been studied for plants, bacteria, archaea, litter invertebrates,

arboreal ants and birds (Barnes et al., 2017), effects on protists remain mostly unclear. In this study, I hypothesized that protists are less diverse in more intensively managed land-use systems, i.e. decline in species richness from rainforest to oil palm plantations, and that trophic groups of protists are differentially affected by land-use intensification. Species richness of protists was significantly reduced in rubber plantations, but remained on a similar level in rainforests, rubber agroforests and oil palm plantations. Contrasting this general effect on protists, trophic groups of protists were affected differently by land-use transformation. The results indicated that phagotrophic, photoautotrophic but also symbiotic protists, albeit to a lesser degree, increased in relative abundance and richness with increasing land-use intensity, while both groups of parasitic protists decreased. Notably, within trophic groups individual taxa generally responded in a similar way, suggesting that trophic groups of protists reflect general patterns in changes in the structure of the micro-decomposer food web with conversion of rainforest into plantation systems.

In Chapter 3, I manipulate nitrogen content, arbuscular mycorrhizal fungi (AMF) and the abundance of collembolans and mites in the tropical montane rainforest of Southern Ecuador. Although our knowledge of basic traits of soil protists is steadily increasing, our understanding of their interactions with other soil organisms or their reactions to changes in nutrient levels in natural environments is still limited. Experiments under laboratory conditions shed light on some interactions between specific soil protists and bacteria, animals but also fungi (Koller et al., 2013; Henkes et al., 2018; Erktan et al., 2020). However, field experiments on interactions between protists and other soil biota are rare. In this study, I investigated the impact of increased N, reduced AMF and mesofauna abundance on different trophic protists groups at taxonomic as well as

functional level across two soil layers, the L and F/H layer. I hypothesized that diversity indices, community composition and proportional abundance of trophic groups of protists will be altered by the addition of nitrogen, reduction in AMF and reduction in mesofauna. Nitrogen is a limiting nutrient in this nutrient depleted system (Camenzind et al., 2018), AMF are abundant fungi in soils, which may antagonistically interact with certain protist groups (Treseder and Cross, 2006; Krashevska et al., 2014), and mesofauna acts as host for parasitic protists and, as important microbivores, they may also trophically interact with protists. Additionally, I assumed treatment effects will be more visible on the functional level compared to the taxonomic level. Moreover, I assumed that individual trophic groups will be differently affected by each treatment. The results suggest that protist communities in the tropical montane rainforest of Southern Ecuador are taxonomically but also trophically complex responding sensitively to even moderate increase in nitrogen input as well as variations in mesofauna abundance and concentrations of AMF. Nitrogen addition strongly affected virtually all trophic groups of protists, highlighting the susceptibility of microbial food webs to human disturbances. Reduced mesofauna abundance resulted in increased relative abundance of phagotrophs, presumably competing with mites and collembolans for bacterial food, as well as reduced relative abundance of animal parasites. Significant correlations between AMF and photoautotroph, mixotroph and plant parasite protists suggest that at a wide range of protist trophic groups closely interacts with AMF. While the experimental treatments typically affected trophic groups of protists in the L and F/H layer in a similar way, some trophic groups differentially responded in the two layers suggesting that protist communities need to be studied across layers to fully understand their role in ecosystem functioning as well as their response to environmental changes.

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Chapter 1

Evaluation of morphological characteristics to delineate taxa of the genus *Trigonopyxis* (Amoebozoa, Arcellinida)

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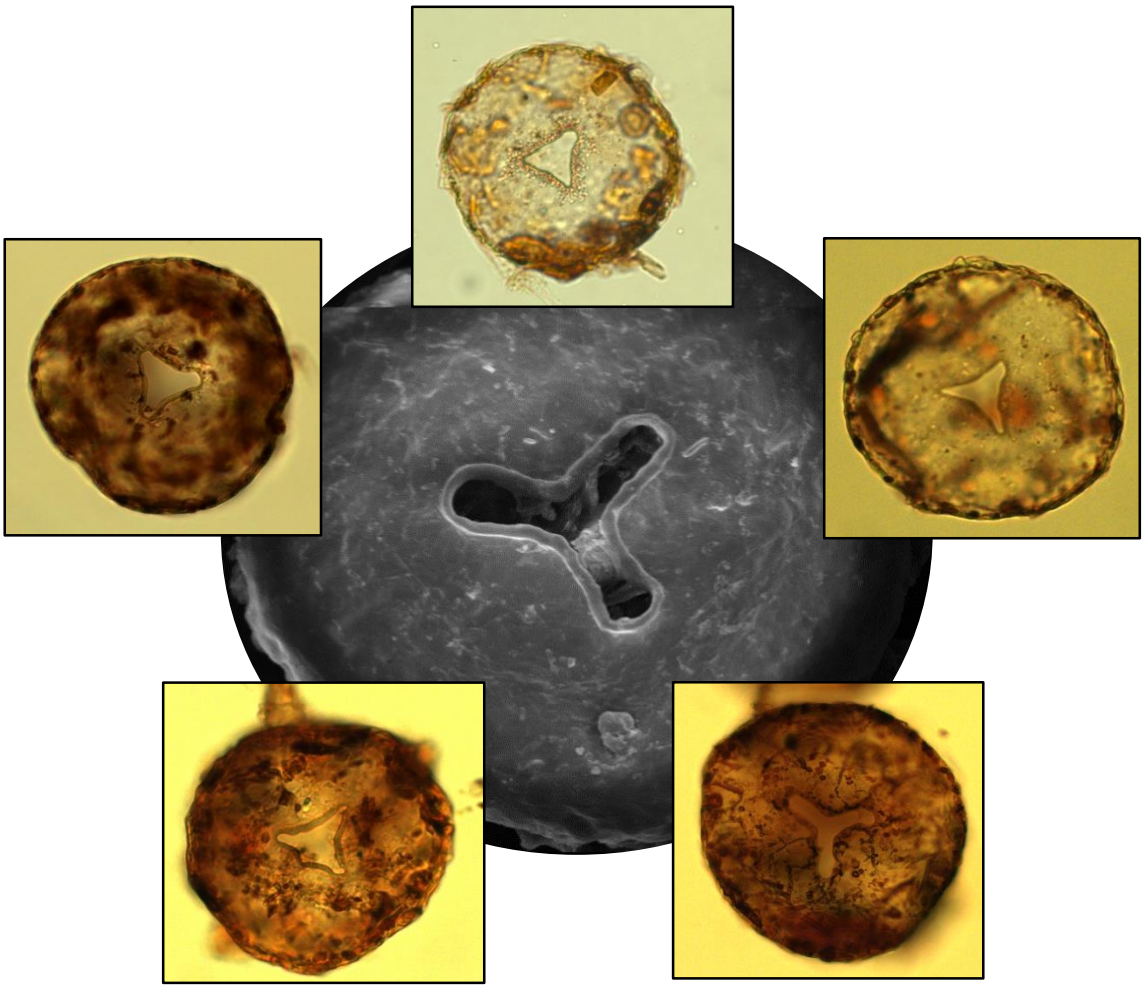

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Abstract

Morphological features are often the only characteristics suitable for identification of taxa in testate amoebae, especially in ecological and palaeoecological studies. However, whereas the morphology of some species is rather stable it may vary considerably in others. Within the order Arcellinida the genus *Trigonopyxis* with the type species *Trigonopyxis arcula* is morphologically highly variable. To identify reliable characteristics for morphology-based taxon delineation we investigated variations in shell size, pseudostome diameter and pseudostome form in *T. arcula* from three different sites of the Ecuadorian Andes, where these characteristics vary even more than previously described. Further, we investigated if morphological characteristics in *Trigonopyxis* varied with changes in environmental factors. We studied 951 shells of *Trigonopyxis* collected along an altitudinal gradient with varying abiotic factors. We established a method for characterization of the pseudostome form, which lead to five different morphotypes. Our results suggest that shell size alone is not an appropriate character for taxon delineation but can be used as an indicator for changes in environmental conditions. In contrast, the pseudostome form might be used for taxon delineation, but likely also varies considerably within taxa. Overall, the study provides an overview of the morphological variability of the genus *Trigonopyxis*.

Introduction

Testate amoebae are a morphologically diverse group of unicellular eukaryotes with worldwide aquatic and terrestrial distribution (Smith et al. 2008; Wylezich et al. 2002). The great majority of the approximately 2,000 described species are differentiated by morphological features of the shell and pseudopods (Leidy 1879; Ogden and Hedley 1980; Penard 1902). Although there are hints of sexual processes in amoebae (Lahr et al. 2011; Rhumbler 1898; Tekle et al. 2014), their typical mode of reproduction is asexual binary fission (Ogden 1981). Therefore, the widely used biological species concept, based upon sexual reproduction (Mayr 1942), is not applicable. Due to these limitations, the morphological species concept is widely adopted in testate amoebae (Cronquist 1978).

Since testate amoebae reproduce asexually, there is no common gene pool of the "morphospecies". Instead, each "individual" is subject to natural selection and accumulates deleterious or beneficial mutations. However, the morphology of asexual species usually is rather stable, indicating that environmental constraints maintain a coherent morphological species despite the lack of genetic exchange (Mayr 1942). Overall, testate amoebae species can be readily distinguished using morphological characteristics. However, phylogenetic relationships can be difficult to derive from morphology alone (Lahr et al. 2013). Moreover, some morphological plasticity likely exists in these asexual lineages (Oliverio et al. 2014).

The morphology of testate amoeba shells is influenced by abiotic and biotic environmental conditions (Heal 1963; Price et al. 2003; Wanner 1999), e.g. shell size and form of the pseudostome may indicate different levels of humidity or feeding types (Bobrov et al. 1999; Jassey et al. 2013; Lamentowicz et al. 2013). Moreover, morphological traits may vary with altitude or latitude, proxies for a suite of co-varying environmental

conditions (Wanner and Meisterfeld 1994). This variation complicates delineation of taxa in some testate amoebae (Bobrov et al. 1995) or may lead to erroneous species determination (Roland et al. 2017). Wrong species identification may result in incorrect conclusions in ecological and palaeoecological studies in particular if species are used to indicate certain environmental conditions (Mitchell et al. 2008). However, variation in morphological characteristics within and between testate amoebae morphospecies has not yet received wide attention hampering species delineation.

Variation in shell size of testate amoebae can be addressed using a thorough biometric characterization followed by construction of an "ideal" individual for each analyzed taxon (Schönborn et al. 1983). Another approach links changes in shell size with other characteristics (e.g., pseudostome diameter) or seeks to identify independent changes of characteristics (e.g., increase in shell length but constant shell width) (Bobrov and Mazei 2004). Neither approach has been satisfactory; therefore, morphological variability remains a challenging problem for testate amoeba taxonomy.

A highly variable and therefore ideal model taxon for investigating morphological variability and taxon delineation is the genus *Trigonopyxis* Penard, 1912. This cosmopolitan genus (Ogden and Hedley 1980) is abundant in mosses, litter and soil of tropical rainforests in southern Ecuador (up to 1,100 individuals per g dry material; Krashevska et al. 2007, 2010). Morphological variability in *Trigonopyxis* includes shell size varying from 60 to 210 µm. Further, the pseudostome is highly plastic in form and size, changing independently of other characters such as shell size (Bobrov and Mazei 2004, Lüftenegger et al. 1988, Swindles et al. 2014). The form of the pseudostome varies from rounded irregular to tri- or in few cases to quadrangular form (Hoogenraad and De Groot 1937; Leidy 1879; Penard 1902).

Molecular methods have been used to shed light on the validity of morphological characteristics for taxa delineation, as done for the *Nebela collaris* group (Kosakyan et al. 2012; Singer et al. 2015). This may result in identifying 'classic' groups as being paraphyletic (Kosakyan et al. 2012; Lara et al. 2008; Nikolaev 2005) or to comprise a set of cryptic species (Heger et al. 2013). For the genus *Trigonopyxis* no molecular data exist so far since cultivation of taxa of this genus is difficult and living specimens are highly contaminated by fungi (Vohník et al. 2011). The only known sequence of *Trigonopyxis arcula* (Genbank AY848967) likely is a *Bullinularia* contaminant (Gomaa 2013; Kosakyan et al. 2016).

Changes in diversity of testate amoebae along altitudinal gradients have been investigated, e.g. in Bulgaria (Todorov 1998), Italy (Mitchell et al. 2004) and southern Ecuador (Krashevska et al. 2007, 2010). However, these studies investigated the community level, whereas here we investigated the morphology and abundance of the genus *Trigonopyxis*, especially *T. arcula* sensu lato, in mosses along a three-point altitudinal transect at 1000, 2000 and 3000 m a.s.l. in a tropical montane rainforest in southern Ecuador. Altitude thereby served as proxy for changing environmental conditions such as humidity, precipitation and temperature.

We analyzed relationships between morphological characteristics, including shell size, pseudostome diameter and pseudostome form, to evaluate the reliability of morphological taxa delineation in this genus. Further, we measured environmental factors in the moss habitat such as pH, water content and microbial biomass at each altitude and included temperature and precipitation (Beck et al. 2008) to evaluate if the variability in morphological characteristics is related to changes in environmental factors. Thereby, we evaluated the suitability of instable characteristics for species delineation.

As a starting point, we provide an overview of the described morphospecies of the genus *Trigonopyxis* and identified characteristics that need to be considered.

In total, eight species of the genus *Trigonopyxis* have been described; however, the quality of the descriptions varies. In the described species the shell size ranges from 40 µm in *T. arboricola* up to 190 µm in *T. arcula* var. *major*. We summarized the characteristics of these taxa in a structured way based on the original descriptions (Table 1).

a) *Trigonopyxis arcula* (Leidy 1879)

   Shell hemispherical, brown to yellow color; pseudostome invaginated, trilobed with thickened rim. No living cells were observed.

b) *Trigonopyxis microstoma* (Hoogenraad and De Groot 1948)

   Compressed spheroid shell in lateral view contrasting the typical hemispherical form of *Trigonopyxis*; color ranging from yellowish to dark brown; pseudostome invaginated, sometimes only slightly; with thickened rim around the pseudostome; pseudostome form irregular round to polygonal but never trilobed. No living cells were observed.

c) *Trigonopyxis arcula* var. *major* (Chardez 1960)

   Shell hemispherical, dark colored from yellow-brown to green-brown; pseudostome irregular, triangular to quadrangular or nearly circular; edges often with irregular denticulation and thickened rim; larger than *T. arcula*.

d) *Trigonopyxis bathystoma* (Bartos 1963)

   Shell flattened hemispherical, brown; pseudostome strongly invaginated, triangular with rounded tips or also irregular, rarely quadrangular; no thickened rim around the pseudostome.

e) *Trigonopyxis foliumicola* (Chardez 1974)

Typical hemispherical shell with few xenosomes, dark brown to black color; pseudostome wide open, triangular with thickened rim; pseudopods surrounded the shell. No further characters are given.

f) *Trigonopyxis arboricola* (Decloitre 1978)

Shell dark almost black with white particles; pseudostome triangular. No further information about the shell and pseudostome is given.

g) *Trigonopyxis leidyi* (Couteaux and Chardez 1981)

Pseudostome trilobed, forming three outwards bent teeth-like structures with thickened rim. No information about shell composition or color is given.

h) *Trigonopyxis minuta* (Schönborn and Peschke 1988)

Hemispherical shell, brownish color; pseudostome circular to irregularly oblong; rim rarely or not thickened. In specimen with a damaged shell an internal shell is visible.

These descriptions vary strongly in quality, e.g. *T. arboricola* is only described in one line of text and a drawing without any details, while *T. microstoma* and *T. minuta* were described in detail including measurements of different morphological traits.

Table 1: Known Trigonopyxis taxa with dimensions (µm) and pictures of original description (L: Shell length, H: Shell height, P: Pseudostome diameter).

Species	Picture	L:	H:	P:
T. arcula (Leidy, 1879) Penard, 1912		112-144	N.A.	28-40
T. microstoma Hoogenraad and De Groot, 1948		90-127	52-60	10-17
T. arcula var. *major* Chardez, 1960		170-190	N.A.	N.A.
T. bathystoma Bartos, 1963		105-124	50-62	15-25
T. foliumicola Chardez, 1974		58-60	35-40	N.A.
T. arboricola Decloitre, 1978		40-45	25	10
T. leidyi Couteaux and Chardez, 1981		87-102	45-50	20-22
T. minuta Schönborn and Peschke, 1988		68.7-87.5	43.7-56.2	12.5-21.9

We hypothesized that (1) pseudostome form is an appropriate character for the delineation of taxa of the genus *Trigonopyxis*, as different pseudostome types likely indicate different feeding types and point to trophic niche differentiation. Further, we hypothesized that (2) shell size varies with environmental conditions, reflecting phenotypic adaptations and therefore being of limited value for the delineation of taxa of the genus *Trigonopyxis*.

Materials & Methods

Sampling site

Samples were collected in the Podocarpus National Park (PNP) in southern Ecuador at three study sites along an altitudinal transect: at 1,000 m a.s.l. near the city of Bombuscaro (S 04° 06′ 54′′, W 78° 58′ 02′′), at 2,000 m a.s.l. near the research station Estacion Cientifica San Francisco in the Reserva Biológica San Francisco (S 03° 58′ 18′′, W 79° 4′ 45′′), and in the region of Cajanuma at 3,000 m a.s.l. located in the north-west of the PNP (S 04° 06′ 711′′, W 79° 10′ 581′′). Six moss samples per site were collected in patches of 20 by 20 cm randomly from the bottom of tree trunks resulting in 18 samples in total. Moss pH ($CaCl_2$) was measured using a digital pH meter, water content of moss was determined gravimetrically. Microbial biomass was determined by substrate-induced respiration using an automated O_2 micro-respirometer system (Anderson and Domsch 1978; Scheu 1992).

Preparation of samples

The samples were washed and subsequently filtered through 250 µm and 15 µm mesh. *Trigonopyxis* shells were collected from the 15 µm mesh. Every shell was washed again and permanent microscope slides were prepared using Canada balsam (Serva, Heidelberg). About 20 cleaned shells were placed in a drop of water upon a coverslip. The shells were turned with the pseudostome to the bottom. After evaporation of the water one drop of Canada balsam was placed on the microscope slide and the cover slip turned around and carefully placed on the Canada balsam. When the cover slip adhered to the microscope slide, they were turned upside down, placed on a rack for seven to 20 days to let the Canada balsam harden. In total 951 shells of *Trigonopyxis* were inspected.

Pictures of each *Trigonopyxis* shell were taken with an Axio Lab A1 microscope equipped with an ICc1 camera at 200-400x magnification. The size of the shell and the diameter of the pseudostome were measured using the software Axiovision V4.8.2.0 (Carl Zeiss AG, Oberkochen, Germany). For scanning electron microscopy clean shells were then transferred to coverslips, dried and sputter-coated with platinum and examined using a Zeiss Sigma FE-SEM scanning electron microscope. Photos were selected based on their quality for illustrative purposes (Figure 1).

Shell size was measured between the most distant points of the shell. The pseudostome diameter was measured as the diameter of a circle enclosing all characteristics of the pseudostome.

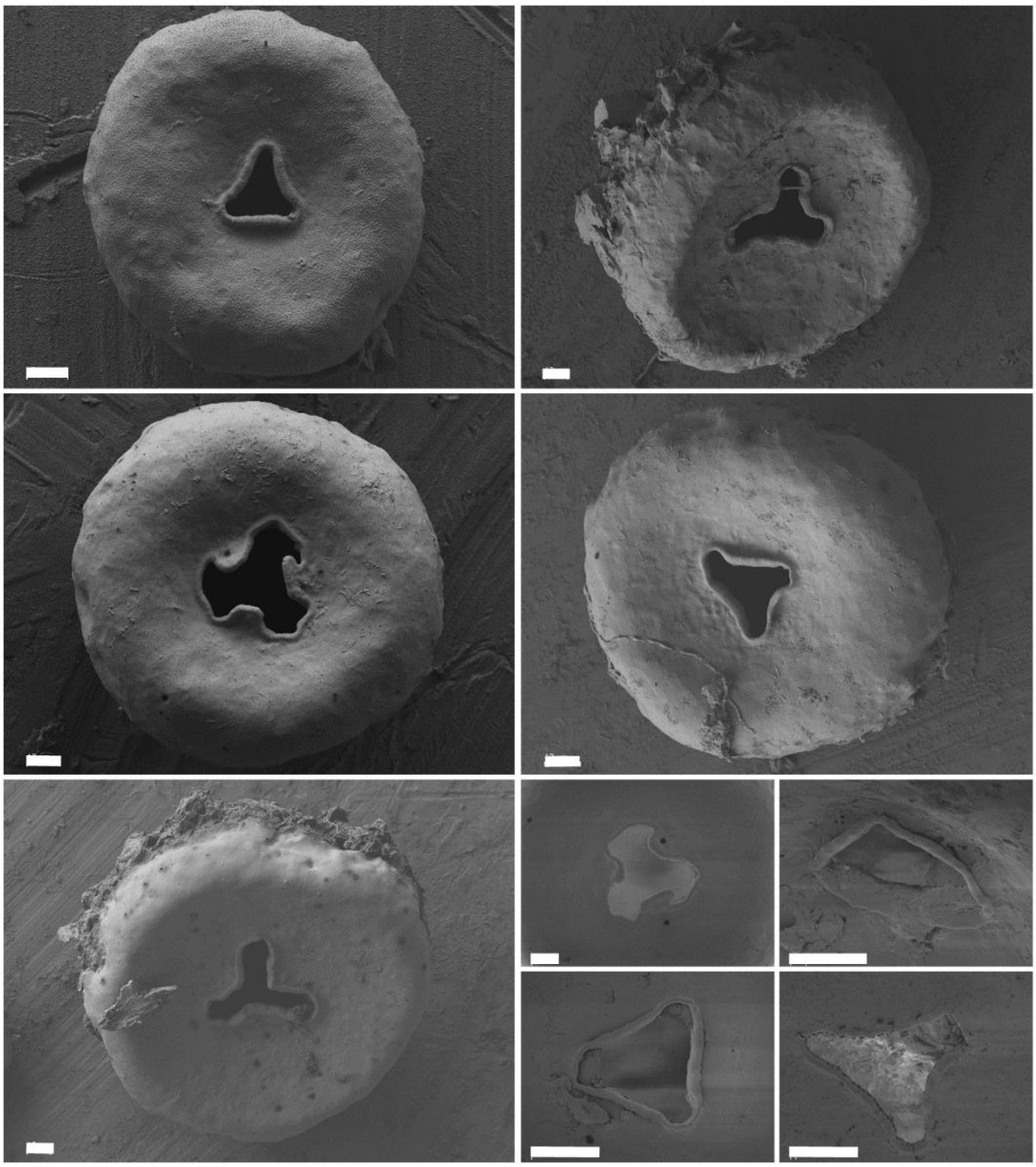

Figure 1: Scanning electron microscope images of Trigonopyxis shells. Scale bar = 10 µm.

Statistical analysis

For the characterization of the pseudostome form of every shell four traits were considered: number of concave edges (0-4), number of pointed angles (0-3), number of propeller-like edges (0-4) and number of a toothed edges (0-2), e.g. a shell with one concave edge, two sharp angles without toothed edges and without propeller-like edges was coded 1-2-0-0 (Table 3). However, there are two special cases: 4-4-0-0 codes for a pseudostome form with an irregular rim while 3-0-4-0 codes for pseudostome form with

very rounded lobes (Table 2). The correlation between shell size and pseudostome diameter was assessed using Pearson's correlation coefficient. Cluster analysis (Neighbor Joining, complete linkage) of the standardized abundance of the 36 observed pseudostome forms was performed to group pseudostome forms into morphotypes.

Data on shell abundance and shell size were analyzed by two-factorial analysis of variance (ANOVA) with the fixed factors altitude (1000, 2000, 3000 m a.s.l.) and morphotype (A, B, C, D and E). Means were compared using Tukey's honestly significant difference (HSD) test. Data on shell abundance were log-transformed prior to statistical analysis. The analyses were performed using STATISTICA 12.5 for Windows (StatSoft, Tulsa, USA).

Relationships between shell size of *Trigonopyxis* morphotypes (A, B, C, D, E) and environmental factors were analyzed using redundancy analysis (RDA) as implemented in CANOCO 5.02 (Ter Braak and Šmilauer 2012). Abiotic environmental variables included water content, pH, temperature and precipitation with the latter two taken from Beck et al. (2008). Microbial biomass was used as biotic environmental factor (Appendix 2).

Table 2: Characters for description of the pseudostome from the shell of taxa of the genus *Trigonopyxis*.

	Pseudostome form	Concave edges	Pointed angles	Propeller-like edges	Toothed edges	Number of shells		Pseudostome form	Concave edges	Pointed angles	Propeller-like edges	Toothed edges	Number of shells
1		0	0	0	0	82	19		2	0	3	0	2
2		0	0	0	1	5	20		2	1	0	0	22
3		0	1	0	0	9	21		2	1	0	1	6
4		0	1	0	1	2	22		2	1	1	0	1
5		0	2	0	0	10	23		2	2	0	0	13
6		0	2	0	1	1	24		2	2	0	1	1
7		0	3	0	0	12	25		2	3	0	0	1
8		1	0	0	0	77	26		3	0	0	0	287
9		1	1	0	0	20	27		3	0	1	0	2
10		1	1	0	1	4	28		3	0	3	0	58
11		1	1	0	2	3	29		3	0	4	0	53
12		1	2	0	0	10	30		3	1	0	0	31
13		1	2	0	1	3	31		3	1	3	0	1
14		1	3	0	0	5	32		3	2	0	0	21
15		2	0	0	0	90	33		3	2	3	0	1
16		2	0	0	1	40	34		3	3	0	0	19
17		2	0	1	0	3	35		3	3	3	0	5
18		2	0	2	0	5	36		4	4	0	0	24

Results

Correlation of shell size and pseudostome diameter

The two morphological traits, shell size and pseudostome diameter, were closely correlated (Pearson's correlation coefficient, $r = 0.91$; $R^2 = 0.83$, $F_{1,927} = 4417$, $p \leq 0.001$). Thus, further statistical analysis were performed based on shell size only.

Cluster analysis of pseudostome forms

The cluster analysis of the pseudostome forms resulted in five clusters at a linkage distance of 3.5 (Figure 2). These clusters represent five morphotypes: (A) a regular triangular form, (B) a trilobed form with inwards bent edges, (C) a trilobed form with pointed tips and inwards bent edges, (D) a triangular form with a toothed edge and (E) a trilobed, propeller-like form (Table 3). While morphotypes A, B, C and D resembled the trilobed morphotype described by Leidy (1879), morphotype E was different. However, although morphotypes A and D were triangular with only slightly bent inwards edges, in morphotype D this was modified by a small dent with sharp point at the edge resembling a small tooth. Morphotypes B, C and E did not have such triangular pseudostome but stronger inward bound edges. These transformed the triangle into a form with three lobes in morphotype B. While in morphotype C the tips between the lobes were sharply pointed, in morphotype E the edges bent further inwards, resulting in a pseudostome form with a smaller opening resembling three slits or a propeller. Further, there was a pseudostome in which the edges were strongly bent outwards forming a pseudostome with a very big opening. However, this is considered as special case of morphotype E.

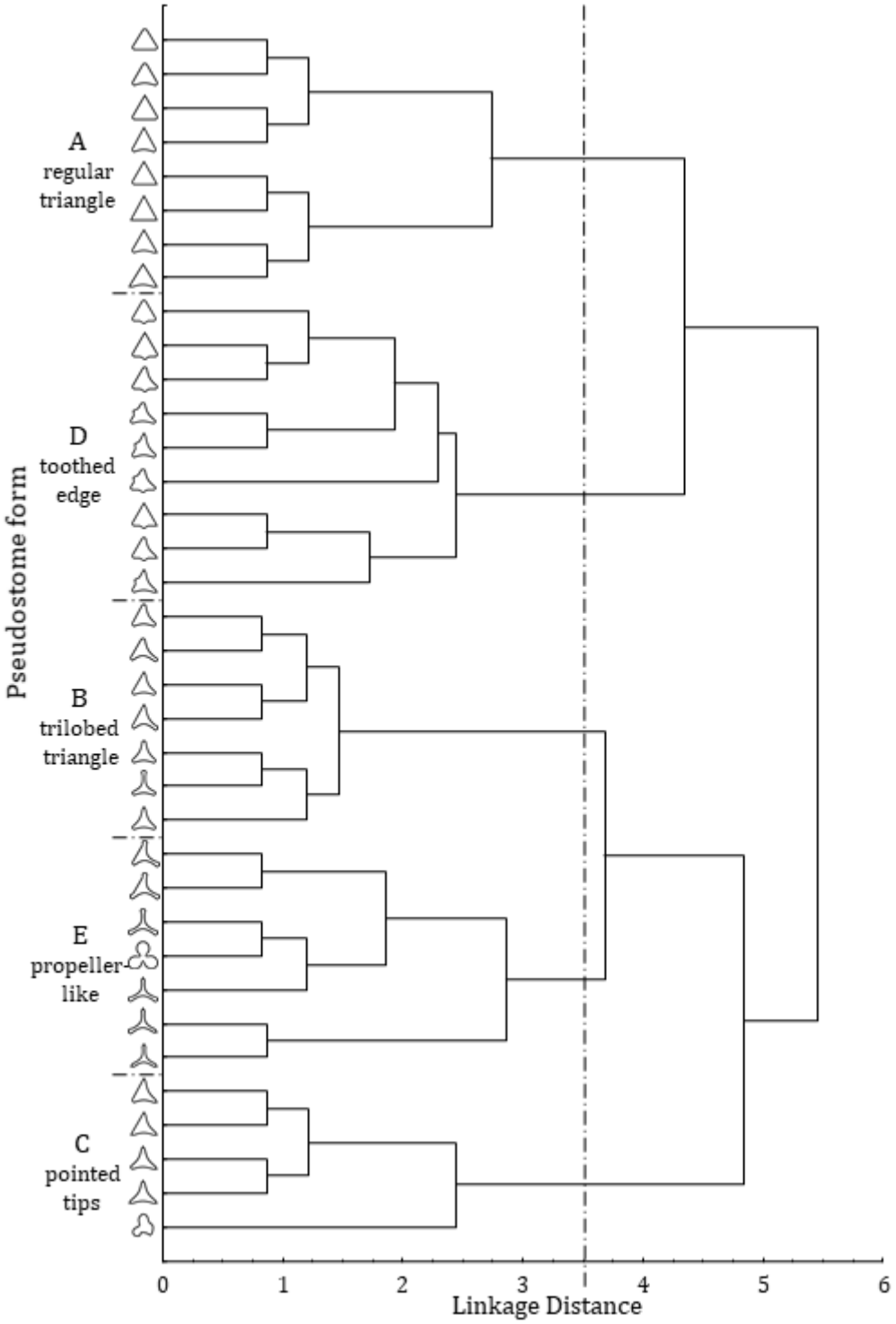

Figure 2: Cluster analysis (Neighbor Joining, complete linkage) of 36 different pseudostome forms resulting in five distinct clusters (morphotypes A, B, C, D and E). Dashed line indicates linkage distance chosen for separation of clusters.

Table 3: Five morphotypes of *Trigonopyxis arcula* from moss samples based on pseudostome characters (cluster analysis).

Cluster	Pseudostome form	Picture	Number of shells
A			225
B			436
C			78
D			65
E			125

Variation of morphotype abundance and shell size with altitude

Shell abundance varied significantly between morphotypes ($F_{4,55} = 11.7$, $p < 0.001$; Figure 3). Morphotype B (46.9 % of total, 76.4 ± 12.2 ind. per sample) and A (24.2 %, 43.8 ± 10.6 ind. per sample) were most abundant, followed by morphotype E (13.5 %, 27.1 ± 7.10 ind. per sample), C (8.40 %, 17.6 ± 4.90 ind. per sample) and D (7.00 %, 19.9 ± 2.65 ind. per sample). Shell abundance per sample differed significantly between 2,000 and 3,000 m ($F_{2,55} = 7.06$, $p < 0.001$; Tukey's HSD test, $\alpha < 0.05$). Shell abundance per sample was highest at 2,000 m (17.4 ± 14.7), followed by 1,000 m (10.4 ± 7.94) and 3000 m (9.95 ± 11.7). Generally, shell size of morphotypes decreased in the order E (140.5 ± 11.4 µm) > B (118.5 ± 16.2 µm) > C (112.6 ± 18.4 µm) > D (111.4 ± 17.1 µm) > A (105.1 ± 10.8 µm) ($F_{4,62} = 27.7$, $p < 0.001$; Figure 4). Further, shell size increased with altitude from an

average of 109.1 ± 15.2 µm at 1,000 m to 113.1 ± 18.8 µm at 2,000 m to 133.5 ± 13.8 µm at 3,000 m ($F_{2,62}$ = 43.5, $p < 0.001$). Also, shell size at 3,000 m varied significantly from shell size at 1,000 m and 2,000 m (Tukey's HSD test, $\alpha < 0.05$). However, variations in shell size of morphotypes depended on altitude (Altitude × Morphotype interaction, $F_{8,62}$ = 3.8, p = 0.001). Shells of morphotypes A, B, C and D were small at 1,000 m and 2,000 m, but significantly larger at 3,000 m, whereas shell size of morphotype E did not vary significantly with altitude (Figure 4).

Variation of morphotypes with environmental factors

Among the five quantitative environmental variables considered (pH, precipitation, temperature, water content, microbial biomass) only temperature and precipitation significantly correlated with the shell size of morphotypes (RDA, forward selection procedure, $p < 0.05$, Appendix 1). Together, these variables explained 76.9 % of the variation. Temperature accounted for 57.9 % of total variation in species data (F = 16.5, p = 0.002), while precipitation accounted for only 10.4 % (F = 3.6, p = 0.044). Axis 1 was significant and explained 65.4 % of the variation (F = 15.2, p = 0.012), while axis 2 explained only 10.2 % of the variation (F = 5.3, p = 0.006). With decreasing temperature and increasing precipitation the shell size of morphotypes increased.

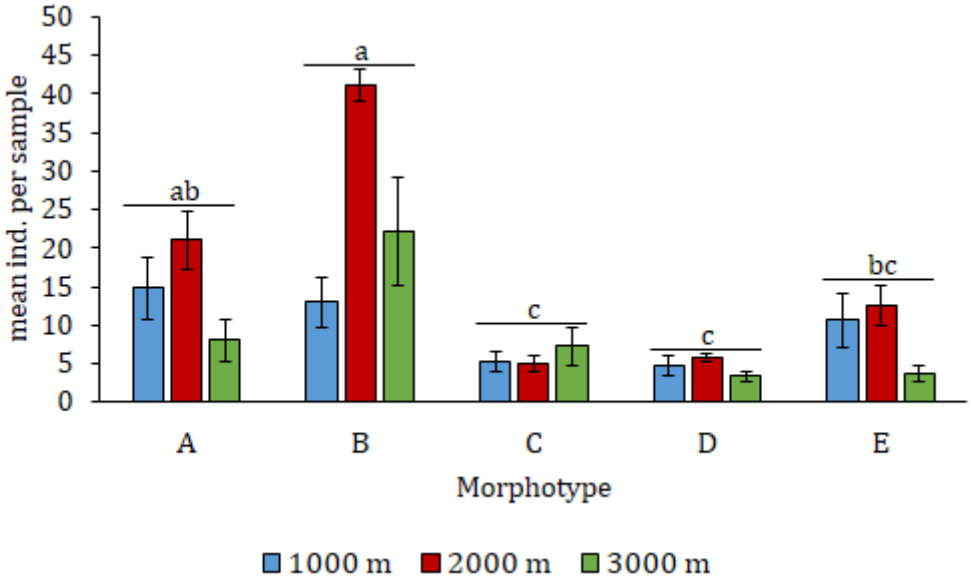

Figure 3: Abundance of shells of five morphotypes of Trigonopyxis (A, B, C, D, E) along an altitudinal transect from 1,000 to 2,000 to 3,000 m in the Southern Andes of Ecuador. Bars sharing the same letter do not differ significantly (Tukey's HSD test, $p < 0.05$).

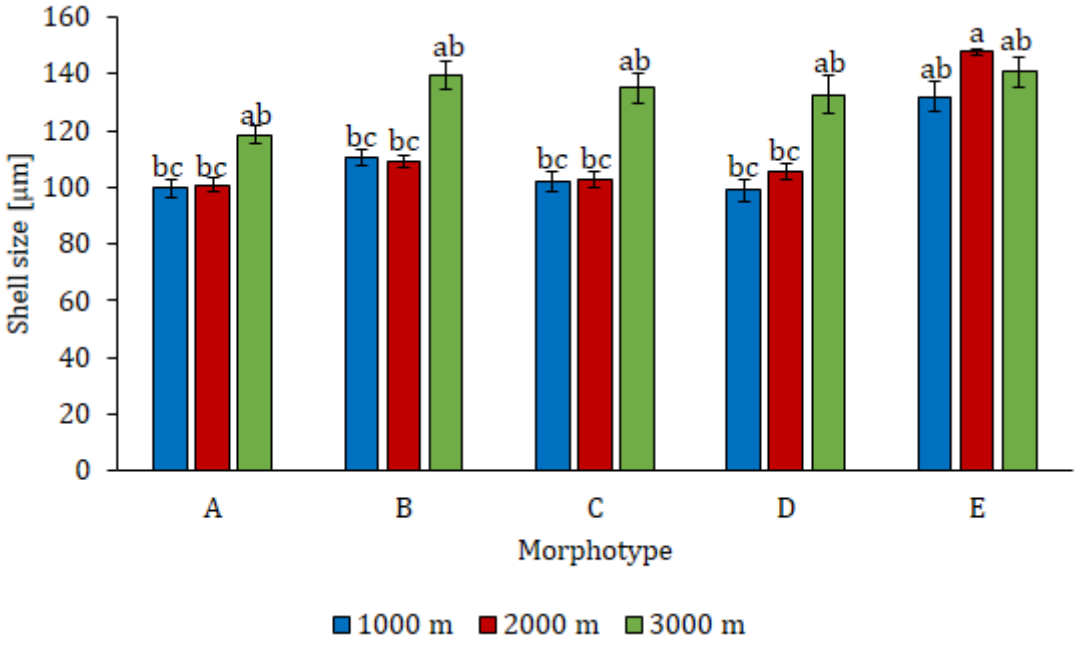

Figure 4: Shell size of five morphotypes of Trigonopyxis (A, B, C, D, E) along an altitudinal transect from 1,000 to 2,000 to 3,000 m in the Southern Andes of Ecuador. Bars sharing the same letter do not differ significantly (Tukey's HSD test, $p < 0.05$).

Discussion

A comprehensive description of the known taxa of *Trigonopyxis* is crucial to understand the problems and uncertainties in the delineation of taxa in this genus. This is especially true for studies using testate amoebae as environmental or palaeoenvironmental indicators. As stated above, wrong determination may lead to erroneous conclusions on species – environment interrelationships. Thus, prior to discussing the results of the present study we summarize the original descriptions and provide an overview of the status of the genus.

Overview of the genus *Trigonopyxis*

The genus *Trigonopyxis* forms part of the family Trigonopyxidae Loeblich & Tappan, 1964 which also includes the genera *Cornuapyxis*, *Cyclopyxis* and *Geopyxella* (Kosakyan et al. 2016). The type-species *T. arcula* was first described as *Difflugia arcula* (Leidy 1879): "Shell hemispheroidal fundus convex; base inverted, shallow infundibuliform; mouth inferior, central, trilobed. Structure of shell usually of yellowish chitinoid membrane, mostly with more or less adherent dirt or scattered particles of quartz-sand or diatoms, especially occupying the fundus." It was placed close to *D. globulosa*, *D. lobostoma*, *Centropyxis* and *Arcella*. After closer examination of this species by Penard (1902, 1910, 1912) *Trigonopyxis* was separated from the genus *Difflugia* based on, e.g. the shell being not as rigid but thinner and more flexible. The type species, *T. arcula*, at this time was the only species of the genus (Penard 1912). Additionally, some variability in the form of the pseudostome was noted. The pseudostome was characterized as being irregular forming a square or having an irregular contour. Even the placement within the Rhizopoda was questioned as no pseudopods could be observed, neither by Penard (1912) nor by others (Cash and Hopkins 1909). However, in further thorough studies of this species the cellular structure including lobose pseudopods has been described (Volz 1929). While no epipods

were observed, it was recognized that the cell is encased in a secondary thin and elastic inner shell. In a series of samples from different locations (i.e., the Netherlands, New Zealand, Indonesia, Russia) *Trigonopyxis* taxa of varying size and different pseudostome forms were reported (Hoogenraad and De Groot 1937, 1942, 1952). The polygon analysis of *T. arcula* from the Netherland samples showed different size classes within and between different locations that raised speculations about different taxa (Hoogenraad and De Groot 1937). However, the different pseudostome forms could not be correlated to these size classes. In samples from Indonesia specimens of different size were documented too (Hoogenraad and De Groot 1942). However, the size varied in one morphotype from 80 - 160 µm, while it was rather constant in another morphotype (50 - 80 µm) within one sample (Hoogenraad and De Groot 1942). In Russia specimens from one location were rather constant in size (130 - 160 µm), while at another location they varied more (110 - 160 µm) (Hoogenraad and De Groot 1952). The comparison of populations from Canada and Russia showed that in one location the pseudostome diameter was closely correlated with shell size, while in another it was not (Bobrov et al. 1995). Shell size varied in both populations form Russia (50 - 185 µm) and Canada (50 - 145 µm), which lead the authors to separate the populations into *T. minuta* (< 70 µm), *T. arcula* (70 - 140 µm) and *T. arcula* var. *major* (> 140 µm) (see Table 1). However, the authors concluded that *T. arcula* comprises a highly polymorphic taxon, *T. arcula* sensu lato, since no morphological characters except shell size were identified for taxa delineation. Furthermore, Bobrov et al. (1999) suggested that environmental factors may be responsible for the high variability in this taxon. An overview of described pseudostome forms is given in Figure 5.

Taxonomy of organisms is based on different types of traits, i.e. morphological, ecological and molecular. A high degree of variation in these traits is problematic, especially if the taxonomy is based on a single type of traits. In case of *Trigonopyxis* the morphological trait used for taxa delineation mostly is shell size while the pseudostome form typically is seen as variable (Leidy 1879; Penard 1902). In contrast, we hypothesized shell size to vary with environmental factors and therefore be less suited for taxa delineation than the pseudostome form, which is less variable as generally assumed.

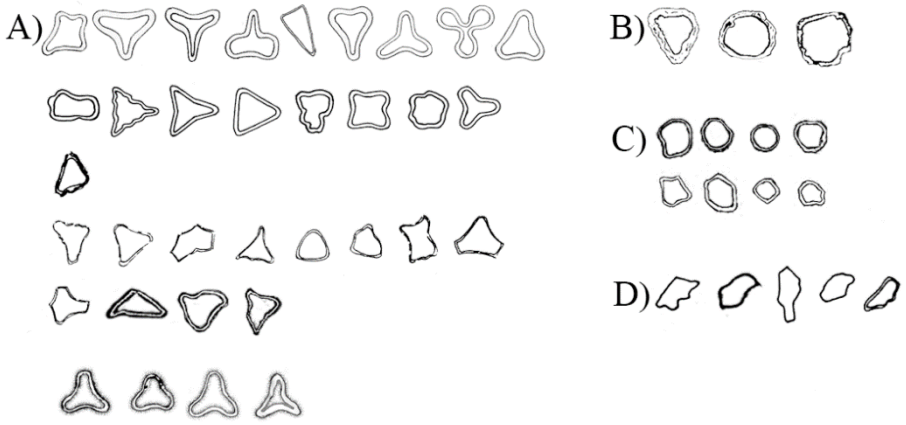

Figure 5: Overview of pseudostome forms of (A) *Trigonopyxis arcula* (Bartos 1963; Bobrov et al. 1995; Chardez 1968; Hoogenraad and De Groot 1937, 1942; Leidy 1879; Schönborn and Peschke 1988), (B) *Trigonopyxis arcula* var. *major* (Chardez 1960), (C) *Trigonopyxis microstoma* (Hoogenraad and De Groot 1942, 1948) and (D) *Trigonopyxis minuta* (Schönborn and Peschke 1988).

Pseudostome form

We found five distinct pseudostome forms in samples of *Trigonopyxis* from montane rainforests in Ecuador (Figure 3). Although there were some in-between forms, all pseudostome forms have been described earlier but were seen as highly variable (Bartos 1963; Bobrov et al. 1995; Hoogenraad and De Groot 1937; Penard 1902). Only the easy

recognizable form of *T. leidyi* (Table 2) separated from the broad range of roughly triangular forms.

The types of pseudostome forms only became evident after examination of a large number of shells, nearly 1,000 shells were examined in this study. Morphotypes A, B and C resemble the pseudostome forms typical for *T. arcula*, *T. bathystoma*, *T. foliumicola* and *T. arboricola*. Morphotype D shows an entirely new feature of the pseudostome form in the genus *Trigonopyxis*, a small fourth tooth-like lobe extrudes from one edge. Morphotype E is similar to *T. leidyi* and in fact *T. leidyi* clustered within this morphotype.

Overall, the results suggest that the pseudostome form of *Trigonopyxis* is not as variable as previously assumed and can be used for distinguishing morphotypes supporting our first hypothesis. However, taxa delineation based solely on the pseudostome may not be justified, but morphotypes may be used for revising the taxa of *Trigonopyxis*. Re-descriptions and revisions of taxa have been done earlier, e.g. *Cyclopyxis kahli* has been synonymized with ten species and *C. aerophila* has been proposed to form a complex of taxa (Foissner and Korganova, 1995, 2000).

Shell Size

The shell size of the specimens of *Trigonopyxis* examined in this study was highly variable and exceeded the size range of described taxa. Comparing the shell size of *Trigonopyxis* taxa from Ecuador with those given in Hoogenraad and De Groot (1937) and Volz (1929) indicates that the use of shell size for taxa delineation is limited (Figure 6).

Delineation of taxa based on shell size only generally appears not to be possible. The German samples from Volz (1929) in part resemble the later described *T. minuta* but the shell size of 50-110 µm reached beyond the size of this species. Additionally, Volz (1929) described the pseudostome consistently as roughly triangular or trilobed never as

irregular as described for *T. minuta*. The same is true for the shells from the Netherlands (Hoogenraad and De Groot 1937); the sizes of some fit to later described taxa, e.g. *T. bathystoma*, *T. microstoma*, *T. minuta* and *T. leidyi*, but exceed the size range of these taxa. Although the studied shells were described as *T. arcula*, the authors suspected some new taxa but could not delineate them clearly. Similarly, in the samples from Ecuador some small shells fit to the descriptions of *T. minuta*, *T. leidyi* and *T. microstoma*, while the larger shells fit better to the description of *T. arcula* and *T. arcula* var. *major* but in part exceeded the size range of these taxa. Overall, the results document the high variability of shell size in *Trigonopyxis* and suggests, that shell size is of limited use for the delineation of species.

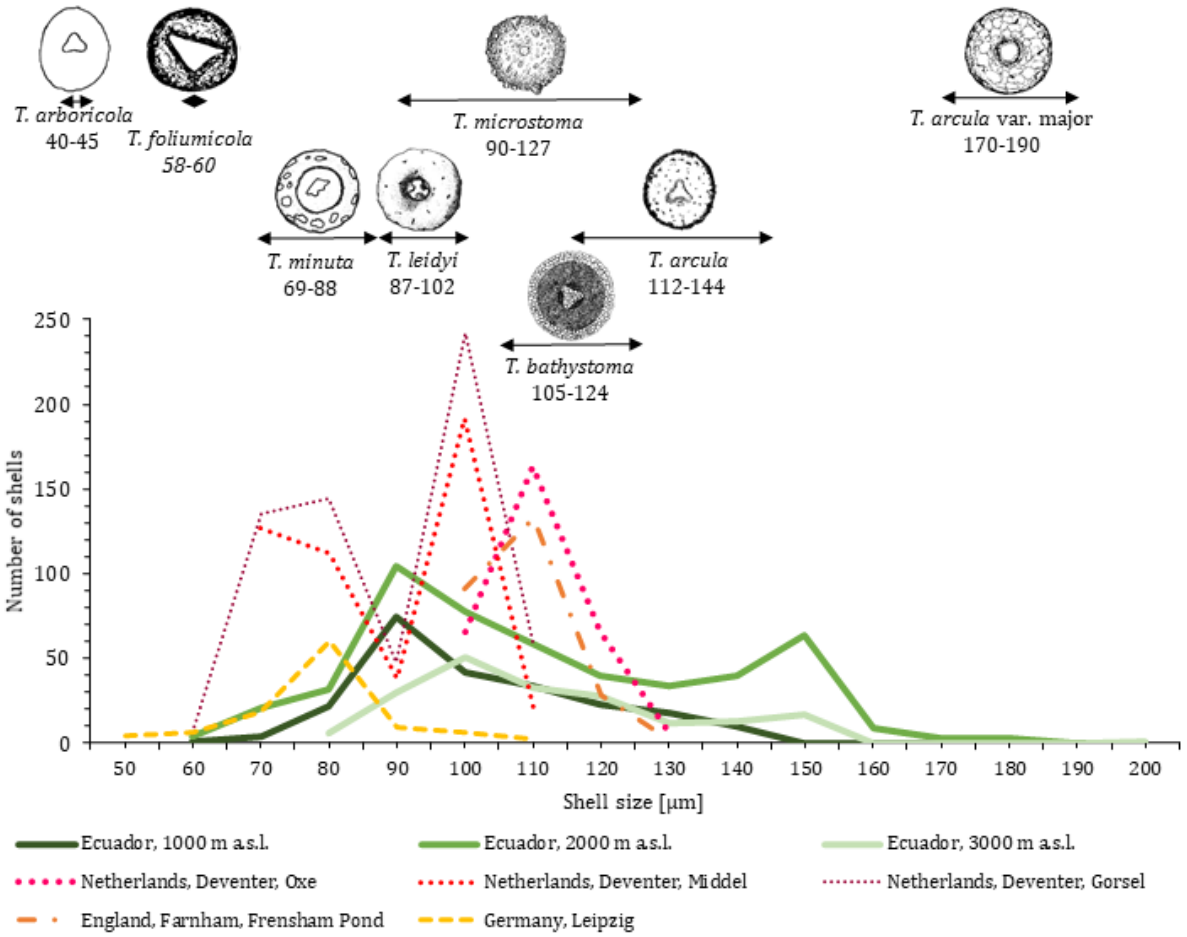

Figure 6: Comparison of shell sizes of described species of *Trigonopyxis* with data from Ecuador (solid lines) (this study), Netherlands (dotted lines), England (dashed-dotted line) (Hoogenraad and De Groot 1937) and Germany (dashed line) (Volz 1929).

Correlation of shell size and pseudostome diameter

In the Ecuadorian samples of *Trigonopyxis* the shell size and the pseudostome diameter were strongly correlated. The same has been found in samples from Canada (Bobrov et al. 1995). However, this was not the case in samples of *Trigonopyxis* from the Tomsk region in West Siberia (Bobrov et al. 1995). Additionally, in two samples from Russia the variability in shell size differed strongly, i.e. in samples from the Sakhalin region shell size was rather constant while in samples from the Tomsk region it was rather variable (Hoogenraad and De Groot 1952). In *Trigonopyxis* from Ecuador the pseudostome diameter was tightly correlated with shell size, resembling the samples from Canada. Notably, the shell size increased with altitude whereas the variability in the shape of the pseudostome stayed rather constant. This indicates that shell size is more variable than pseudostome diameter, however, both may also be correlated at some locations. In natural populations of testate amoebae general patterns for the correlation of morphological traits have been proposed (Bobrov and Mazei 2004). However, in the genus *Trigonopyxis* this only applies to some populations. Again, this supports our second hypothesis that shell size alone is not appropriate for taxa delineation.

Environmental influence

Shell size of the morphotypes A, B, C and D significantly increased by up to 30 µm along the altitudinal gradient (Figure 4). This suggests that shell size varies with changes in environmental factors. Results of RDA suggested that both temperature and precipitation contribute to this variability with the former being more important than the latter. The mean annual air temperature decreases with altitude from 19.4 to 15.7 to 9.4 °C while the annual precipitation increases from 2,200 to 3,500 to 4,500 mm $year^{-1}$ from 1,000 to 2,000 to 3,000 m, respectively (Beck et al. 2008). Although temperature nearly halves while precipitation doubles along the altitudinal gradient shell size of *Trigonopyxis*

morphotypes was similar at 1,000 and 2,000 m but larger at 3,000 m (except in morphotype E). Consistent with this pattern, the size of protists generally has been shown to decrease with increasing temperature (Atkinson et al. 2003). Additionally, it has been observed that the morphology of testate amoebae varies with moisture conditions with shell size increasing at wetter conditions (Wanner 1999). Also, in laboratory cultures shell size and morphology of the testate amoebae *Cyclopyxis kahli* (Amoebozoa, Trigonopyxidae) and *Trinema lineare* (Cercozoa, Trinematidae) have been shown to vary with temperature, chemical composition of the medium and food concentration (Wanner and Meisterfeld 1994). In *C. kahli* the shell size increased by up to 10 µm at lower temperature. At the studied altitudinal gradient the increase in shell size at 3,000 m (as compared to 1,000 and 2,000 m) was even more pronounced and averaged 28 µm for morphotypes A, B, C and D.

In addition to shell size, the abundance of morphotypes varied significantly with altitude (see Figure 3). The variation in abundance suggests that morphotypes are either adapted to the specific environmental conditions at a certain altitude, e.g. morphotype B at 2,000 m, or have a broader spectrum of optimal environmental conditions, e.g. morphotypes C and D. For culturing testate amoebae typically it is advised to use temperatures close to the optimum growth temperature of the species as it becomes increasingly difficult to interpret the effect of environmental factors on protist morphology if the conditions deviate from this temperature, e.g. along a temperature gradient shell size may first increase but then abruptly decrease (Wanner and Meisterfeld 1994). Unfortunately, nothing is known on optimum temperature conditions of *Trigonopyxis* as no laboratory cultures exist. However, presence of each of the morphotypes at each altitude suggests that they are adapted to a wide range of temperature and the rather uniform increase in

shell size at 3,000 m argues against sudden changes in shell size at less favorable temperature conditions.

Overall, the co-occurrence of each of the five morphotypes at the studied altitudinal transect and the uniform response of four of the five morphotypes further adds to the conclusion that shell size is an insufficient characteristic for taxa delineation in the genus *Trigonopyxis*. Rather, in agreement with our second hypothesis, larger shells of testate amoebae point to environments with lower temperatures and wetter conditions. Shell size thereby might be used as bioindicator reflecting changes in temperature rather than taxa delineation.

Conclusions

We showed that, despite commonly assumed to be variable, the pseudostome form in the genus *Trigonopyxis* can be used to delineate morphotypes. The shell size, which is widely used for taxa delineation (Mazei and Tsyganov, 2006), varies markedly with environmental conditions such as temperature and humidity, and therefore is of limited value for characterizing taxa. However, as shell size uniformly increases with decreasing temperature it might be used as indicator for changes in environmental conditions. Based on the documented variability in characteristics used for taxa delineation in the genus *Trigonopyxis* and the co-occurrence of each of the morphotypes at the studied montane rainforests we suggest not to separate different taxa of *Trigonopyxis,* but rather to view them as different morphotypes of *T. arcula* as suggested earlier (Bobrov et al. 1995). One potential exception might be *T. leidyi* with its unique pseudostome; however, considering the variability in this characteristic and the co-occurrence of morphotype E including this species with each of the other morphotypes in the studied montane rainforests we refrain from considering it as separate taxon. Therefore, the genus *Trigonopyxis* may best be

considered comprising a single polymorphic species, i.e. *T. arcula*. However, thorough morphological characterization allows distinguishing different morphotypes with varying response to changes in environmental conditions, e.g. along altitudinal gradients. Advances in cultivation techniques and further molecular work are needed to establish the validity of the five morphotypes for the genus *Trigonopyxis*.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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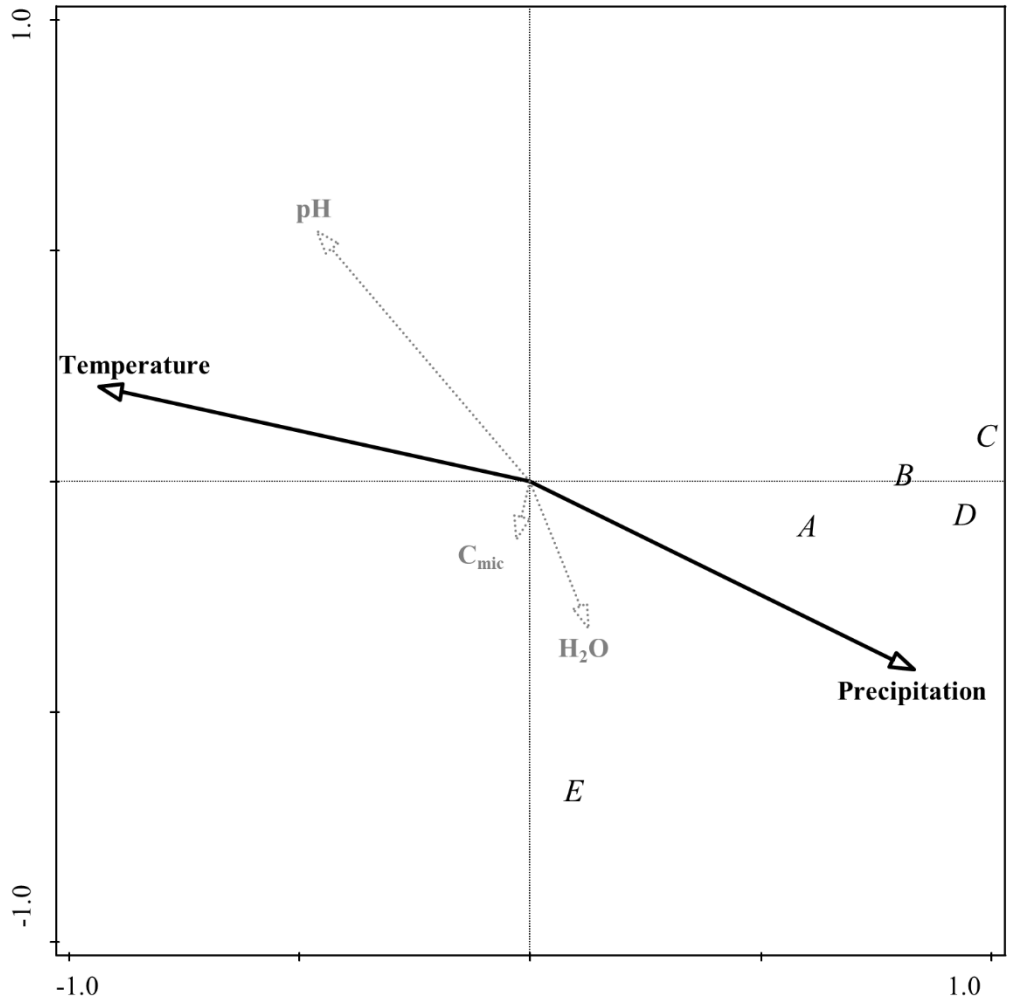
Appendix Chapter 1

Appendix 1: RDA of the shell size of five morphotypes of *Trigonopyxis arcula* (A, B, C, D and E) constrained by five environmental variables (pH, precipitation, temperature, water content = H_2O, microbial biomass = C_{mic}). Environmental variables with significant effect are in black. Axis 1 was significant and explained 65 % of the variation (F = 15.2, p = 0.012), while axis 2 explained only 10 % of the variation (F = 5.3, p = 0.006).



Appendix 2: Environmental data along an altitudinal transect from 1,000 to 2,000 to 3,000 m in the Southern Andes of Ecuador.

Altitude [m]	Cmic [µg per g dry weight]	pH	Watercontent [%]	Temp [°C]	Precipitation [mm per year]
1,000	14,569	6.53	595	19.4	2,200
1,000	15,610	6.43	287	19.4	2,200
1,000	11,828	5.52	256	19.4	2,200
1,000	24,912	6.26	440	19.4	2,200
1,000	30,741	6.05	498	19.4	2,200
1,000	12,916	6.64	367	19.4	2,200
2,000	14,411	3.55	299	15.7	3,500
2,000	13,281	3.94	220	15.7	3,500
2,000	10,713	3.08	246	15.7	3,500
2,000	12,793	3.36	197	15.7	3,500
2,000	5,500	3.25	129	15.7	3,500
2,000	33,594	4.23	235	15.7	3,500
3,000	15,087	3.95	194	8.9	4,500
3,000	12,630	3.08	291	8.9	4,500
3,000	19,262	3.55	345	8.9	4,500
3,000	13,095	3.48	223	8.9	4,500
3,000	13,305	4.32	348	8.9	4,500
3,000	14,551	3.63	286	8.9	4,500

Chapter 2

Changes in trophic groups of protists with conversion of rainforest into rubber and oil palm plantations

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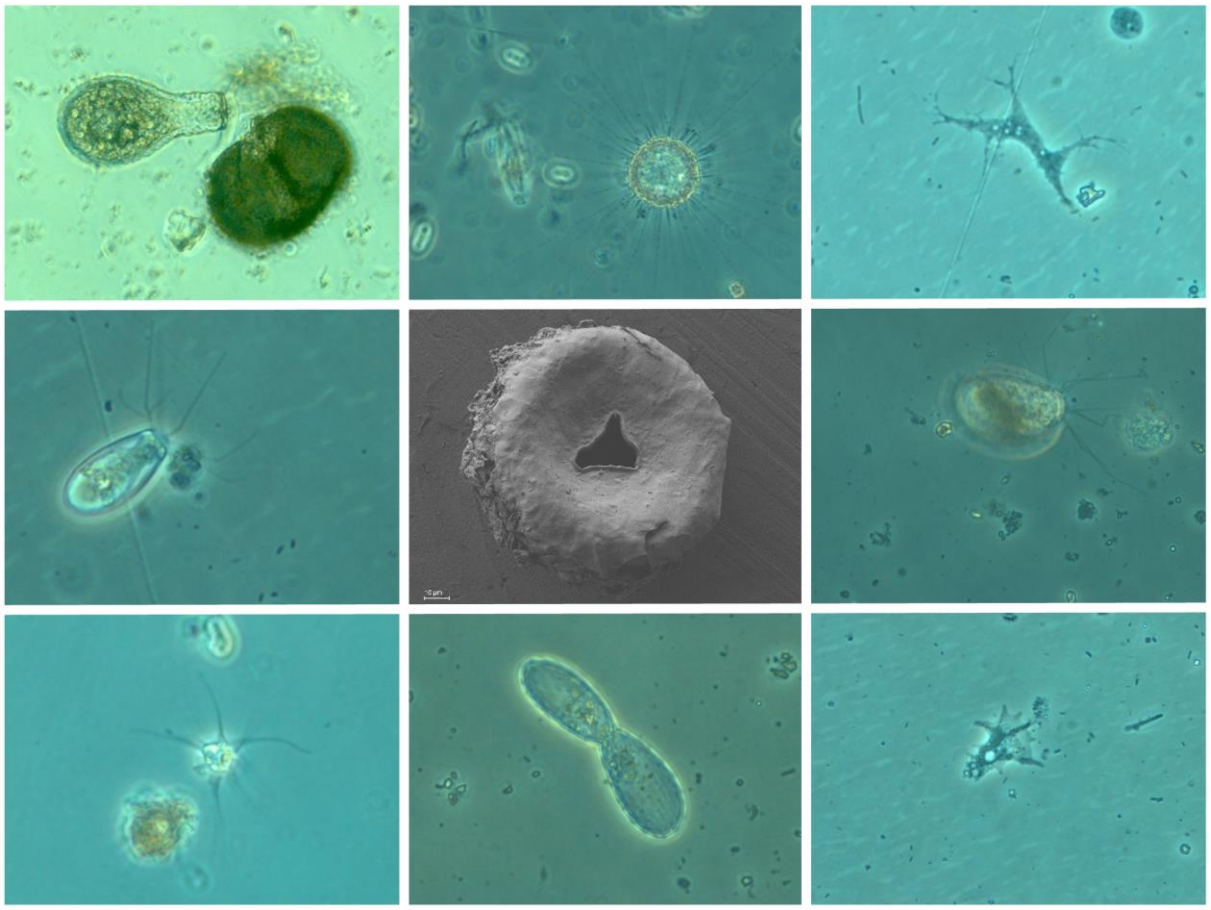

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Abstract

Protists, abundant but enigmatic single-celled eukaryotes, are important soil microbiota providing numerous ecosystem functions. We employed high-throughput sequencing of environmental DNA, targeting the V4 region of the 18S rRNA gene, to characterise changes in their abundance, species richness, and community structure with conversion of lowland rainforest into rubber agroforest (jungle rubber), and rubber and oil palm plantations. We identified 5,204 operational taxonomic units (OTUs) at 97% identity threshold of protists from 32 sites. Protists species richness was similar in rainforest, jungle rubber and oil palm plantations but significantly lower in rubber plantations. After standardisation, 4,219 OTUs were assigned to five trophic groups, and inspected for effects of land-use change, and potential biotic and abiotic driving factors. The most abundant trophic group was phagotrophs (52%), followed by animal parasites (29%), photoautotrophs (12%), plant parasites (1%), and symbionts (<1%). However, the relative abundance and OTU richness of phagotrophs and photoautotrophs increased significantly with increasing land-use intensity. This was similar, but less pronounced, for the relative abundance of symbionts. Animal and plant parasites decreased significantly in abundance and species richness with increasing land-use intensity. Community compositions and factors affecting the structure of individual trophic groups differed between land-use systems. Parasites were presumably mainly driven by the abundance and species richness of their hosts, while phagotrophs by changes in soil pH and increase in Gram-positive bacteria, and photoautotrophs by light availability. Overall, the results show that relative species richness, relative abundance, and community composition of individual trophic groups of protists in tropical lowland rainforest significantly differ from that in converted ecosystems. This is likely associated with changes in ecosystem functioning. The study provides novel insight into protist communities and their changes

with land-use intensity in tropical lowland ecosystems. The results support earlier suggestions that protists are powerful indicators reflecting changes in the functioning of ecosystems with conversion of rainforest into monoculture plantations.

Introduction

Tropical rainforests are one of the most threatened ecosystems in the world (Koh et al., 2011; Wilcove et al., 2013). Through high demand for cropland due to an increasing human population, large areas suffer from deforestation or peatland degradation and are converted into agricultural systems (Gibbs et al., 2010; Margono et al., 2014; Miettinen et al., 2013). This is especially true for Indonesia, one of the world's top producers and exporters of palm oil and rubber (Koh et al., 2011; Marimin et al., 2014). On Sumatra alone, approximately 12 million ha of tropical rainforest have been converted into oil palm and rubber plantations since the 1980s (Laumonier et al., 2010). Conversion of rainforest into agricultural land strongly reduced the abundance and species richness of animals and plants (Barnes et al., 2014; Drescher et al., 2016; Rembold et al., 2017). As a result, ecosystem functions of the converted tropical systems, such as carbon storage, air quality, flood and drought prevention, decomposition, and nutrient cycling, are changing (Dislich et al., 2017; Guillaume et al., 2018; Krashevska et al., 2018; Sala et al., 2000).

Ecosystem functions depend to a large extent on the functional diversity of the belowground system (Bardgett and van der Putten, 2014; De Deyn et al., 2003; Fierer, 2017). Virtually all biogeochemical cycles are driven by soil microbial communities, and microorganisms play a key role in decomposing soil organic matter and mineralising the nutrients therein (Delmont et al., 2012; Falkowski et al., 2008). Despite the importance of microorganisms in the belowground system, their diversity and functions are still poorly studied. Microorganisms in soils are represented by archaea, bacteria, fungi and protists,

but the latter often are overlooked in soil ecology studies. Protists, eukaryotic single-celled organisms, are neither animals, nor plants, nor fungi, but make up the majority of all eukaryotic life forms (Adl et al., 2012; Geisen et al., 2018). They are not only highly diverse in species richness but also in life cycles, trophic interactions, and cellular structures. They can obtain carbon photoautotrophically and heterotrophically, form symbiotic relationships with animals, plants and fungi, parasitise and be parasitised by other protists (Geisen et al., 2018; Kinne and Lauckner, 1980). They can reach densities of up to 100,000 individuals per gram of soil (Geisen and Bonkowski, 2017). However, their diversity in soil is very different to that of aquatic ecosystems, where they are best studied, but still is underestimated; protist diversity has been seen as "near imponderable" (Foissner, 1999b). This has been confirmed recently by high throughput sequencing (HTS) methods (Bates et al., 2013; Geisen, 2016; Geisen et al., 2015c; Grossmann et al., 2016; Richardson et al., 2014). HTS approaches are promising in multiple ways. First, they improve access to the cryptic belowground biodiversity. They avoid the problem in single-celled organisms that the majority of taxa are difficult to extract and cultivate, and that enrichment cultures introduce a bias in abundance and species richness estimates (Geisen et al., 2015c). HTS of soil environmental DNA (eDNA) has successfully been used to examine the biodiversity and structure of protist communities along environmental gradients in Canada (Heger et al., 2018), Switzerland (Seppey et al., 2017), Costa Rica, Panama and Ecuador (Mahé et al., 2017), and the results suggest that protists, but not arthropods, are the most diverse eukaryotes in tropical rainforests. However, in spite of the fast increase in HTS data and information in reference databases, knowledge on protists in Southeast Asia is scarce and studies on protists of tropical land-use systems are lacking entirely.

To assess the effect of rainforest conversion on protists and to identify driving factors for their community composition, we used Illumina MiSeq HTS of soil eDNA to measure abundance and species richness of protists in tropical rainforest, rubber agroforests (jungle rubber), rubber plantations and oil palm plantations in Sumatra, Indonesia. To understand community functions of the vast diversity of protists recovered by this method, OTUs were categorised into five trophic groups; phagotrophs, photoautotrophs, animal parasites, plant parasites, and symbionts. Using these broad categories, we compared community composition between land-use systems. Finally, we inspected potential explanatory factors driving the distribution of the different trophic groups between land-use systems, including biodiversity of plants and animals, and soil phospholipid fatty acids as proxies for bacterial and fungal communities, and abiotic environmental factors (Drescher et al., 2016; Krashevska et al., 2015).

Based on previous studies on macro-, meso- and microfauna, as summarised in Clough et al. (2016) and Drescher et al. (2016), we hypothesised that (1) protists are less diverse in more intensively managed land-use systems, i.e. decline in species richness from rainforest to oil palm plantations, and that (2) trophic groups of protists are differentially affected by land-use intensification. In more detail, we expected that in oil palm plantations, with higher relative bacterial abundance (Schneider et al., 2015), phagotrophs increase in relative species richness and relative abundance, and with increasing canopy openness in plantations (Drescher et al., 2016), photoautotrophs increase. Further, we expected the relative abundance and species richness of parasites and symbiotic protists to follow their host availability and therefore decrease in plantations.

Materials & Methods

Study sites and sampling

The sampling sites were located in the tropical lowlands of the Jambi Province in Sumatra, Indonesia. Two landscapes were studied, Bukit Duabelas (2° 0' 57" S, 102° 45’ 12" E) and Harapan (1° 55' 40" S, 103° 15' 33" E). At each landscape four typical land-use systems representing the conversion from rainforest into plantations with increasing land-use intensity were selected: secondary lowland rainforest (rainforest), rubber agroforest (jungle rubber), rubber plantation (rubber) and oil palm plantation (oil palm). Each land-use system was replicated four times in each landscape, resulting in 32 sampling sites with three sub-plots each. The rainforest was used as reference system of low anthropogenic influence. Jungle rubber is a traditional managed agroforest system, where rubber trees (*Hevea brasiliensis*) are interspersed with native tree species. Both plantation systems, rubber and oil palm (*Elaeis guineensis*), represent intensively managed monocultures with high fertilizer input including liming, for details see Kotowska et al. (2015) and Drescher et al. (2016).

Samples were taken in October/November 2012 (rubber jungle, rubber plantations and oil palm plantations) and November/December 2013 (rainforest) as described in Sahner et al. (2015). In short, in each subplot five soil cores (4 cm diameter and 20 cm depth) were taken. Coarse roots and stones (> 5 mm) were removed by consecutive sieving through 10 and 5 mm mesh. The samples from each subplot were pooled and homogenized resulting in one bulk soil sample per subplot resulting in 96 samples in total. Reaction tubes (50 ml, Sarstedt, Nümbrecht, Germany) with bulk soil were opened, a gauze was added to avoid soil loss during freeze drying and the samples were precooled for at least three hours in a -80 °C freezer. Freeze drying was conducted in a VirTis

Benchtop K Freeze Dryer (SP Industries, Warminster, USA) with a dual stage rotary vane vacuum pump (Trivac E2, Leybold Vakuum GmbH, Köln, Germany) for approximately 32 hours. After freeze drying, three perforated Eppendorf tubes filled with 5 g of silica gel (Carl Roth, Karlsruhe, Germany) were added to the reaction tubes to keep the soil samples dry before shipping to the University of Goettingen.

Permission

The Ministry of Research and Technology RISTEK (Kementrian Ristek dan Teknologi, Jakarta, Indonesia) provided the research permit (Kartu Izin Peneliti Asing, permission number: 333/SIP/FRP/SM/IX/2012). The Research Center for Biology of the Indonesian Institute of Science LIPI (Lembaga Ilmu Pengetahuan Indonesia, Jakarta, Indonesia) recommended issuing a sample collection permit (Rekomendasi Ijin Pengambilan dan Angkut (SAT-DN) Sampel Tanah dan Akar, number: 2696/IPH.1/KS:02/XI/2012). The collection permit (number: S.16/KKH-2/2013) and an export permit (reference number: 48/KKH-5/TRP/2014) were provided by the Directorate General of Forest Protection and Nature Conservation PHKA (Perlindungan Hutan dan Konservasi Alam, Jakarta, Indonesia) under the Ministry of Forestry of the Republic of Indonesia. The Chamber of Agriculture of Lower Saxony (Plant Protection Office, Hannover, Germany) provided the import permits (Letter of Authority, numbers: DE-NI-12-69 -2008-61-EC, DE-NI-14-08-2008-61-EC).

DNA extraction and amplification

DNA was extracted using the MoBio PowerSoil isolation kit (Dianova, Hamburg, Germany) as recommended by the manufacturer. The hypervariable V4 region of the 18S rRNA gene was amplified using the general eukaryotic primers TA-Reuk454FWD1 (5'-CCAGCASCYGCGGTAATTCC-3') and TA-ReukREV3 (5'-ACTTTCGTTCTTGATYRA-3')

(Stoeck et al., 2010) paired with the MiSeq-Adapters Forward overhang (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and Reverse overhang (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). For amplification, the Phusion High Fidelity DNA Polymerase kit (ThermoFisher Scientific, Germany) was used. The PCR reaction mixture contained 10 µl of 5-fold Phusion GC Buffer, 1 µl of the forward and reverse primers (10 µM), 1 µl $MgCl_2$ (50 mM), 1 µl dNTPs (10 mM), 2.5 µl DMSO, 0.5 µl Phusion Polymerase (1 U) and 1 µl template DNA. The following thermocycling scheme was used for amplification: initial denaturation at 98 °C for 1 min, 35 cycles of denaturation at 98 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min, followed by a final extension period at 72 °C for 5 min. Amplicon length was approximately 400 bp. All amplicon PCRs were performed three times and pooled equimolar for sequencing. The University of Goettingen Genomic Laboratory facility determined the sequences of the 18S amplicons using MiSeq.

Sequence data deposition

The 18S rRNA gene sequences were deposited in the European Bioinformatics Institute (EMBL-EBI) European Nucleotide Archive (ENA) under the study accession number PRJEB23943.

Bioinformatic analysis of 18S rRNA gene sequences

The resulting 18S rRNA gene sequences were processed and analysed employing PEAR, cutadapt, USEARCH 9.24 and QIIME 1.9.1 (Caporaso et al., 2010). Initially, sequences shorter than 250 bp, containing unresolved nucleotides, exhibiting an average quality score lower than 20, were removed with split_libraries_fastq.py. Additionally, we used cutadapt (Martin, 2011) with default settings for efficient forward and reverse primer

removal. Chimeric sequences were removed using UCHIME2 (Edgar et al., 2011) with SSU SILVA 128 as a reference dataset.

Operational taxonomic unit (OTU) determination was performed at a genetic divergence of 3% (species level) with USEARCH. Taxonomic classification was performed with parallel_assign_taxonomy_blast.py against the same database. OTU tables were created using USEARCH. Singletons, bacteria, archaea chloroplasts, metazoa, Streptophyta, fungi and unclassified OTUs were removed from the table by employing filter_otu_table.py (quality-filtered data). In order to homogenise the differences in the number of reads per sample, we randomly selected 2,300 sequences for each sample (standardised data with 4,219 OTUs). Diversity estimates and rarefaction curves were generated by employing alpha_rarefaction.py.

Data analysis

Data handling and transformation was done with the packages *dplyr* (Wickham et al., 2017), *reshape2* (Wickham, 2007), and *tidyr* (Wickham and Henry, 2017) in R (R Core Team, 2017). Graphics were implemented in R with the additional packages *ggplot2* (Wickham, 2009), *ggrepel* (Slowikowski, 2017), *ggpubr* (Kassambara, 2017), *ggsn* (Santos Baquero, 2017), *rgdal* (Bivand et al., 2018), *rworldmap* (South, 2011), *rworldxtra* (South, 2012), *scales* (Wickham, 2017), *vegan* (Oksanen et al., 2017), and viridis (Garnier, 2018).

After data standardisation procedure, 4,219 OTUs were categorised into five trophic groups (symbionts, photoautotrophs, phagotrophs, plant parasites and animal parasites) based on the work of Adl and Gupta (2006), Geisen et al. (2018) and Seppey et al. (Seppey et al., 2017) (Supplementary Table 1). All OTUs that could not be ascribed to one of these groups were categorised as undetermined. As the HTS approach is based on extracted

DNA of soils, our data might include OTUs derived from extracellular DNA or encysted cells.

MANOVA as implemented in the *stats* package in R (R Core Team, 2017), including all five trophic groups showed that the relative abundance (Wilks' $\lambda = 0.19$, $F_{3,91} = 10.5$, $p < 0.001$) and relative species richness (Wilks' $\lambda = 0.05$, $F_{3,91} = 29.6$, $p < 0.001$) of trophic groups varied with land-use changes. Therefore, the effect of forest conversion on each of the trophic groups was analysed separately using linear mixed-effects models with landscape (Harapan, Bukit Duabelas) as block, land-use (rainforest, jungle rubber, rubber, oil palm) as fixed effect and replicate plots and subplots fitted as random effect (Crawley, 2007), as implemented in the *nlme* package in R (Pinheiro et al., 2017). Tukey's HSD test, as implemented in the *multcomp* package in R (Hothorn et al., 2008), was used to identify significant differences between means.

Discriminant function analysis (DFA) as implemented in STATISTICA 13.1 for Windows (StatSoft, Tulsa, USA) was used to identify effects of the land-use system on overall protist communities (based on abundances of quality filtered data) and for each individual trophic group (based on relative abundance). Squared Mahalanobis distances (MD^2) between group centroids were determined to identify significant differences in protist community structure between land-use systems.

Relationships between OTUs (based on relative abundance) and environmental factors were analysed using distance-based redundancy analysis (db-RDA) with Bray-Curtis dissimilarity as distance measure as implemented in CANOCO 5.02 (Ter Braak and Šmilauer, 2012). RDA was chosen as the length of gradient of OTUs data was 3.10 SD units (Leps and Smilauer, 2003). The forward selection procedure of db-RDA allowed to relate OTUs (dependent variables) to a set of environmental factors (independent variables) by

direct ordination. Environmental factors included water content, microbial basal respiration, microbial biomass, pH, C concentration, N concentration, air temperature, humidity, canopy openness, plant abundance, plant richness, soil fauna abundance, soil fauna richness, the sum of phospholipid fatty acids (PLFAs) relative markers for gram-positive bacteria (i15:0, a15:0, i16:0, i17:0), gram-negative bacteria (16:1ω7, cy17:0, cy19:0), fungi (18:2ω6,9, 18:3ω6, 18:3ω3) and neutral lipid acid marker for arbuscular mycorrhizal fungi (16:1ω5c). The data for the analyses were taken from Krashevska et al. (2015), Drescher et al. (2016) and A. Potapov (unpublished data), for details see Supplementary Table 2. Monte Carlo tests (999 permutations) were performed to evaluate the significance of individual axes (Ter Braak, 1996).

Results

Sequencing and quality filtering resulted in 2,433,278 high-quality 18S rRNA gene sequences from all subplots. After removal of singletons, bacteria, archaea chloroplasts, metazoa, Streptophyta, fungi and unclassified OTUs, the dataset comprised 5,204 OTUs at 97% genetic identity and 220,800 sequences (quality-filtered data). After subsampling (2,300 sequences per sample), the dataset comprised 4,219 OTUs, with an average number of 269 ± 65 OTUs per plot ranging from 132 (HR1) to 396 OTUs (BO1).

Overall protist species richness and abundance

Protist OTU richness (based on quality-filtered data) differed significantly between land-use systems ($F_{3,27} = 5.39$, $p = 0.005$). It was similar in oil palm plantation (492 ± 145), jungle rubber (482 ± 156) and rainforest (459 ± 195) but significantly lower in rubber plantation (285 ± 94). Also, mean OTU richness was significantly higher in Bukit Duabelas (481 ± 154) as compared to Harapan landscape (378 ± 174; block effect $F_{3,27} = 6.07$, $p = 0.02$). In contrast to richness, total OTU abundance did not differ significantly between

land-use systems with an overall mean of 25,347 ± 1,900 sequences ($F_{3,27} = 0.33$, $p > 0.05$). Neither Shannon nor Simpson diversity index differed between land-use systems (overall mean of 4.42 ± 0.44, $F_{3,27} = 1.53$, $p = 0.23$, and 0.96 ± 0.04, $F_{3,27} = 0.89$, $p = 0.46$, respectively).

DFA separated protist community composition along land-use systems (Wilks' $\lambda = 0.12$, $F_{12,235} = 23.5$, $p < 0.001$; Figure 1). The three linear discriminant functions explained 68.7%, 16.6% and 14.6% of the variation, respectively. Protist communities in rainforest were separated from those in oil palm ($MD^2 = 38.6$, $p < 0.001$) and rubber plantations ($MD^2 = 27.7$, $p < 0.001$), and less pronounced also from those in jungle rubber ($MD^2 = 4.52$, $p < 0.001$). Protist community composition in jungle rubber was most similar to that in rubber plantations ($MD^2 = 10.6$, $p < 0.001$) and more separate from that in oil palm plantations ($MD^2 = 17.5$, $p < 0.001$). The communities in rubber and oil palm plantations were also distinct ($MD^2 = 1.08$, $p < 0.001$).

Relative abundance, species richness and community composition of trophic groups

Overall, the relative abundance of trophic groups declined in the order phagotrophs (52.08 %), animal parasites (28.74 %), photoautotrophs (12.17 %), plant parasites (0.82 %), and symbionts 0.09 %); based on standardised data. About 6 % of all sequences could not be assigned to any trophic group and were grouped as "undetermined". Generally, in rainforest animal parasites and phagotrophs were the dominating groups, followed by undetermined, photoautotrophs, plant parasites and symbionts (Figure 2). In jungle rubber, phagotrophs were dominating, followed by animal parasites, photoautotrophs, undetermined, plant parasites and symbionts. Also, in rubber plantation phagotrophs dominated, followed by animal parasites, photoautotrophs, undetermined, plant

parasites and symbionts. In oil palm plantation phagotrophs were dominant, followed by photoautotrophs, animal parasites, undetermined, plant parasites and symbionts.

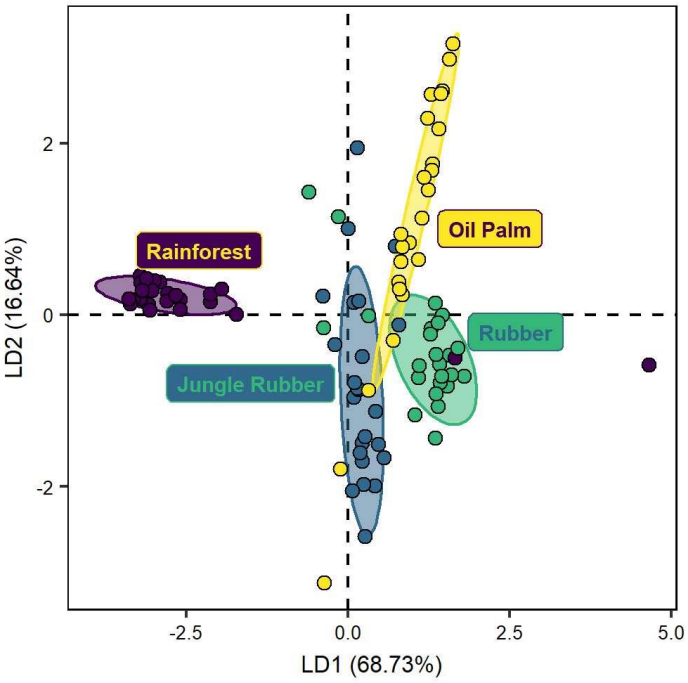

Figure 1: Discriminant function analysis of protist communities of four land-use systems (rainforest, jungle rubber, rubber plantation and oil palm plantation; Wilks' λ = 0.12, $F_{12,235}$ = 23.5, $p < 0.001$) based on quality-filtered data. Eigenvalues: LD1 = 0.60, LD2 = 0.25. Ellipses drawn for better visualisation of the respective land-use systems include 75 % of the respective plots.

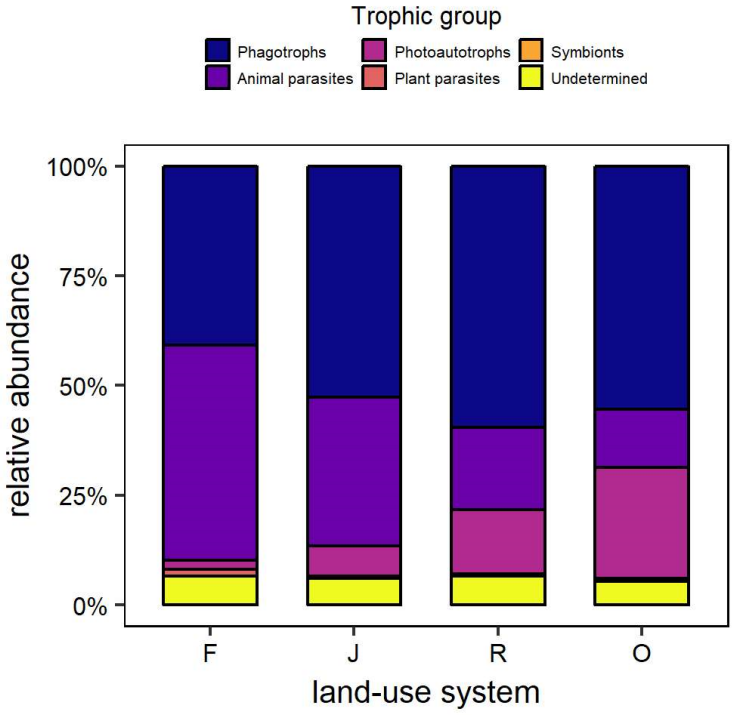

Figure 2: Relative OTU abundance of trophic groups of protists in the soil of rainforest (F), jungle rubber (J), rubber plantations (R) and oil palm plantations (O), based on standardised data.

Phagotrophs

The relative OTU richness of phagotrophs was high in oil palm plantations, low in rubber plantations and intermediate in rainforest and jungle rubber ($F_{3,27} = 4.10$, $p = 0.02$; Figure 3A). By contrast, the relative OTU abundance of phagotrophs was similar in jungle rubber, and rubber and oil palm plantations, but significantly lower in rainforest ($F_{3,27} = 9.17$, $p < 0.001$; Figure 3B).

DFA separated the communities of phagotrophs of the four land-use systems (Wilks' $\lambda = 0.18$, $F_{12,235} = 17.5$, $p < 0.001$, Figure 4A). The community of phagotrophs in rainforest was separated from that in oil palm ($MD^2 = 21.0$, $p < 0.001$) and rubber plantations ($MD^2 = 15.6$, $p < 0.001$), and less pronounced also from that in jungle rubber ($MD^2 = 3.24$, $p < 0.001$). Similar to total protists, the community composition of phagotrophs in jungle rubber was similar to that in rubber plantations ($MD^2 = 4.74$, $p < 0.001$) and more distinct from that in oil palm plantations ($MD^2 = 9.02$, $p < 0.001$). The communities in rubber and oil palm plantations also differed significantly ($MD^2 = 1.87$, $p < 0.001$), but in part communities overlapped widely.

The dominance of phagotrophic species changed between land-use systems. Across all land-use systems the most dominant OTU was assigned to *Ischnamoeba* sp. (Amoebozoa *incertae sedis*). It represented 8.07 % of the protists relative abundance overall and reached 11.1 % in rainforest, 10.0 % in jungle rubber, 5.77 % in rubber, and 6.42 % in oil palm plantations. In rainforest *Bodomorpha* sp. (Glissomonadida, Cercozoa) was the second dominant OTU followed by *Palpitomonas* sp. (Palpitia *incertae sedis*), BOLA868 (Euamoebida, Amoebozoa), *Lacrymaria* sp. (Haptoria, Ciliophora), *Telonema* (*incertae sedis*), and different *Heteromita* spp. (Glissmononadida, Cercozoa). In jungle rubber, the dominance of OTUs differed from that in rainforest. Following *Ischnamoeba* sp,

Discicristoidea (Nucletmycea) species were as abundant as *Palpitomonas* sp., followed by *Bodomorpha* sp., BOLA868, *Schizoplasmodium* sp. (Schizoplasmodida, Amoebozoa), *Platyophrya* sp. (Colpodea, Ciliophora), *Vermamoeba* sp. (Arcellinida, Tubulinea), *Acanthamoeba* sp. (Longamoebia, Amoebozoa) and *Heteromita* sp. Similarly, in rubber plantations *Ischnamoeba* sp. was followed by Discicristoidea species, BOLA868, *Heteromita* spp., *Palpitomonas* sp., *Telonema* sp., another Glissmononadida, and a different Discicristoidea species. In oil palm plantations *Ischnamoeba* sp. was followed by *Heteromita* spp., *Telonema* sp., a member of Discicristoidea (different species than in jungle rubber), *Palpitomonas* sp., *Cercomonas* sp. (Cercomonadidae, Cercozoa), *Eocercomonas* sp. (Cercomonadidae, Cercozoa), and *Ceratomyxella* sp. (Schizoplasmodida, Amoebozoa).

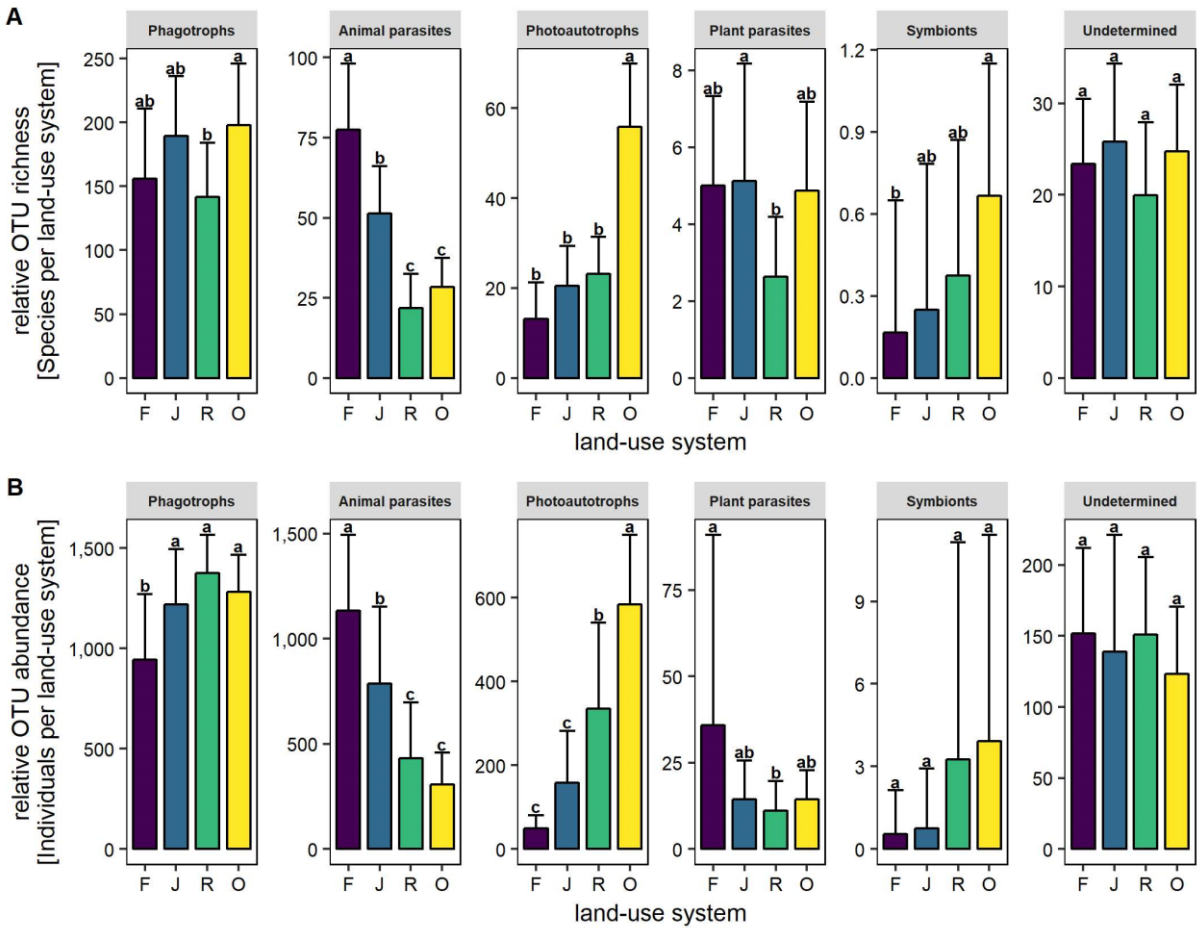

Figure 3: Relative OTU richness (**A**) and relative OTU abundance (**B**) of trophic groups of protists in soil of four land-use systems: rainforest (F), jungle rubber (J), rubber plantations (R) and oil palm plantations (O). Bars sharing the same letter do not differ significantly (Tukey's HSD test, $p < 0.05$).

Animal parasites

The relative OTU richness of animal parasites was high in rainforest, lower in jungle rubber and lowest in rubber and oil palm plantations ($F_{3,27} = 43.8$, $p < 0.001$; Figure 3A). The relative OTU abundance of animal parasites followed a similar pattern ($F_{3,27} = 22.1$, $p < 0.001$; Figure 3B).

DFA separated animal parasite communities of the four land-use systems (Wilks' $\lambda = 0.13$, $F_{12,235} = 22.4$, $p < 0.001$, Figure 4B). Animal parasite communities in rainforest were most distinct from those in oil palm ($MD^2 = 30.8$, $p < 0.001$) and rubber plantations ($MD^2 = 28.2$,

$p < 0.001$), but less distinct from those in jungle rubber ($MD^2 = 5.28$, $p < 0.001$). The animal parasite community in jungle rubber was separated from that in both oil palm ($MD^2 = 11.8$, $p < 0.001$) and rubber plantations ($MD^2 = 9.33$, $p < 0.001$), while the animal parasite communities in rubber and oil palm plantations differed little ($MD^2 = 0.90$, $p = 0.04$).

In each of the land-use systems, the dominant animal parasites were species of the gregarines group (Apicomplexa, SAR). In rainforest the dominant OTUs included different *Gregarina* species. Another gregarine of the order Eugregarinorida were detected in rainforest but could not be identified further. Similar to rainforest, in jungle rubber gregarines were dominant. *Prismatospora* sp. and other not further identified species from the order Eugregarinorida were also detected. In rubber plantations an unspecified OTU belonging to Eimeriidae (Apicomplexa, SAR) was most abundant. Also, in rubber plantations different *Gregarina* spp. as well as *Syncystis* sp., *Psychodiella* sp. and *Monocystis* sp. were abundant. Similar to rubber plantations, the same unspecified OTU of the Eimeriidae family was most abundant in oil palm plantations, followed by *Monocystis* sp., *Psychodiella* sp. and other species of Eugregarinorida order but not *Gregarina* sp.

Photoautotrophs

The relative OTU richness of photoautotrophs was low in rainforest, jungle rubber and rubber plantations and significantly higher in oil palm plantations ($F_{3,27} = 60.3$, $p < 0.001$; Figure 3A). The relative OTU abundance followed a similar pattern but increased more linearly from rainforest to oil palm plantations ($F_{3,27} = 30.8$, $p < 0.01$; Figure 3B).

DFA separated photoautotroph communities of the four land-use systems (Wilks' $\lambda = 0.21$, $F_{12,233} = 15.9$, $p < 0.001$; Figure 4C). The community of photoautotrophs of rainforest was most distinct from the communities in rubber ($MD^2 = 17.9$, $p < 0.001$) and oil palm plantations ($MD^2 = 17.5$, $p < 0.001$), but less distinct from the community in jungle rubber

(MD^2 = 9.84, $p < 0.001$). The community of photoautotrophs in jungle rubber was more distinct from that in oil palm plantations (MD^2 = 2.02, $p < 0.001$) than from that in rubber plantations (MD^2 = 1.23, $p = 0.001$), with the latter differing only along the second axis (MD^2 = 1.47, $p = 0.003$).

The most dominant photoautotroph in rainforest was *Ceratium* sp. from the Ceratiaceae family (Dinoflagellata, SAR), followed by unspecified species of the family Cryptophycea, *Chrysochromulina* sp. from the Prymnesiaceae family (Prymnesiales, Haptophyta), *Rhizosolenia* sp. from the Rhizosoleniaceae family (Diatomea, SAR) and unspecified chlorophytes species. In jungle rubber the Chlorophyta classes Chlorophyceae and Trebouxiophyceae were most dominant, followed by *Prymnesium* sp. (Prymnesiaceae, Haptophyta), unspecified Dinoflagellata of the Dinophyceae family, *Bangia* sp. (Rhodophyaceae, Archaeplastida) and *Ochromonas* sp. (Ochrophyta, SAR). Rubber and oil palm plantations also were dominated by chlorophytes from the classes Chlorophyceae and Trebouxiophyceae. In the latter no species were identified, while Chlorophyceae included *Hylodesmus* sp., *Chlorosarcinopsis* sp. and *Bracteacoccus* sp.

Plant parasites

The relative OTU richness of plant parasites was similar in rainforest, jungle rubber and oil palm plantations but lower in rubber plantations ($F_{3,27}$ = 3.11, $p < 0.001$; Figure 3A). By contrast, the relative OTU abundance of plant parasites was similar in jungle rubber, rubber and oil palm plantations, but higher in rainforest ($F_{3,27}$ = 3.35, $p = 0.03$; Figure 3B).

DFA separated the plant parasite communities of the four land-use systems (Wilks' λ = 0.47, $F_{12,227}$ = 6.38, $p < 0.001$; Figure 4D). The plant parasite community in rainforest was most distinct from that in oil palm (MD^2 = 6.78, $p < 0.001$) and rubber plantations (MD^2 = 3.25, $p < 0.001$), but less from that in jungle rubber (MD^2 = 1.17, $p = 0.01$). The plant

parasite community of jungle rubber was distinct from that in oil palm plantations (MD^2 = 3.03, $p < 0.001$). Further, the plant parasite communities differed between rubber and oil palm plantations but differences were less pronounced (MD^2 = 0.95, p = 0.04). The plant parasite communities from jungle rubber and rubber plantation were not significantly distinct (MD^2 = 2.34, p = 0.06).

The rainforest was dominated by Peronosporomycetes, SAR: *Eurychasma* sp., *Pseudoperonospora* sp., *Olpidiopsis* sp. and *Phytophthora* sp. In jungle rubber, the dominating species were *Eurychasma* sp., *Olpidiopsis* sp. and *Pseudoperonospora* sp. followed by *Achlya* sp. and *Pythium* sp. (all Peronosporomycetes), as well as *Sorodiplophrys* sp. and *Thraustochytrium* sp. (both Labyrinthulomycetes, SAR). In rubber plantations *Eurychasma* sp. was most abundant, followed by *Pythium* sp., *Aphanomyces* sp., *Pseudoperonospora* sp., *Phytopythium sp.* and *Achlya* sp. In oil palm plantations *Eurychasma* sp. and *Aphanomyces* sp. (both Peronosporomycetes) dominated followed *Polymyxa* sp. (Cercozoa), as well as unspecified Peronosporomycetes and *Sorodiplophrys* sp. (Labyrinthulomycetes).

Symbionts

The relative OTU richness of symbionts increased continuously with increasing land-use intensity from rainforest to oil palm plantations ($F_{3,27}$ = 4.03, p = 0.02; Figure 3A). By contrast, the relative OTU abundance did not vary significantly between land-use systems ($F_{3,27}$ = 2.19 and $p > 0.05$; Figure 3B).

DFA separated the symbiont communities of the four land-use systems (Wilks' λ = 0.31, $F_{12,69}$ = 3.23, $p < 0.001$; Figure 4E). The symbiont community in rainforest was distinct from that in rubber (MD^2 = 13.9, $p < 0.001$) and oil palm plantations (MD^2 = 13.4, $p < 0.001$), but less from that in jungle rubber (MD^2 = 10.1, p = 0.009). Further, the symbiont

community in jungle rubber was distinct from that in oil palm plantations ($MD^2 = 3.91$, $p = 0.02$), but neither symbiont communities between jungle rubber and rubber plantations ($MD^2 = 2.44$, $p = 0.07$) nor between rubber and oil palm plantations differed significantly ($MD^2 = 0.10$, $p = 0.98$).

In each land-use system, the dominating symbiont OTUs were different. However, all of them belonged to unspecified Syndiniales (Alveolata, SAR) and in rainforest and jungle rubber also to *Saccinobaculus* sp. (Metamonada, Excavata).

Undetermined

The relative OTU richness of undetermined protists ($F_{3,27} = 1.59$, $p > 0.05$; Figure 3A) as well as the relative OTU abundance of undetermined protists did not vary significantly between land-use systems (overall mean 141 ± 62.7, $F_{3,27} = 0.51$, $p > 0.05$; Figure 3B).

DFA separated the undetermined OTUs communities of the four land-use systems (Wilks' $\lambda = 0.16$, $F_{12,235} = 19.6$, $p < 0.001$; Figure 4F). The undetermined community in rainforest was distinct from that in rubber ($MD^2 = 15.8$, $p < 0.001$) and oil palm plantations ($MD^2 = 15.6$, $p < 0.001$), but less from that in jungle rubber ($MD^2 = 1.94$, $p < 0.001$). Further, the community in jungle rubber was distinct from that in oil palm ($MD^2 = 8.31$, $p < 0.001$) and rubber plantations ($MD^2 = 8.21$, $p < 0.001$) with the latter two also differing significantly ($MD^2 = 3.63$, $p < 0.001$).

In each land-use system the undetermined protists were dominated by different Cercozoa, Stramenopiles and Amoebozoa. Neither of these could further be specified.

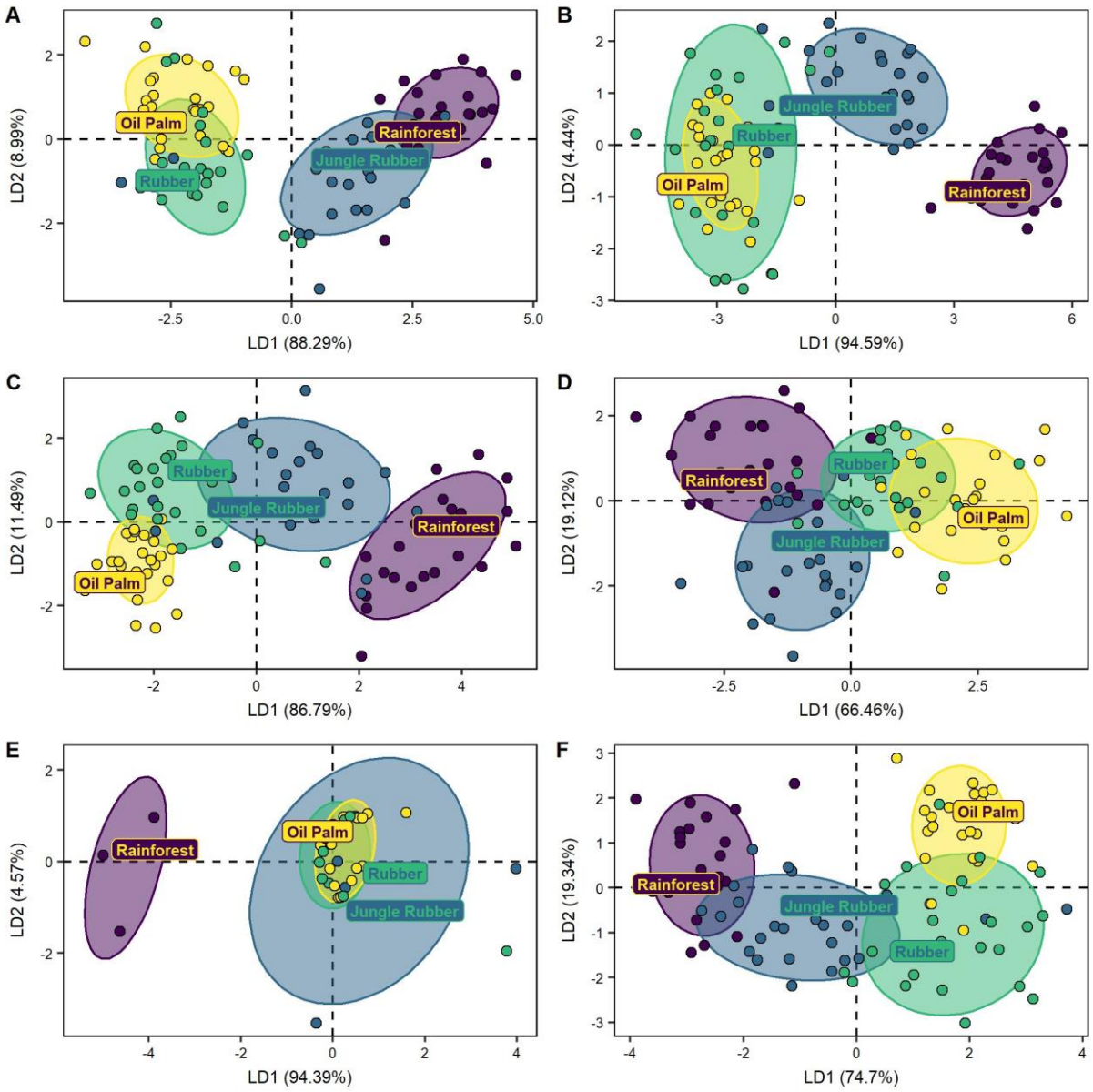

Figure 4: Discriminant function analyses of five trophic groups of protists (**A** phagotrophs, **B** animal parasites, **C** photoautotrophs, **D** plant parasites, **E** symbionts) and of undetermined protists (**F**) from four land-use systems (rainforest, jungle rubber, rubber plantation and oil palm plantation). Ellipses drawn for visualisation of the respective land-use systems include 75 % of the respective plots.

Environmental factors

In the forward selection procedure of the db-RDA, five of the 17 environmental variables were significant ($p < 0.05$). These five variables explained 49.5 % of the total variation: pH accounted for 33.5 % ($F = 47.3$, $p = 0.001$), canopy openness for 9.20 % ($F = 14.9$, $p = 0.001$), the sum of Gram-positive bacterial PLFAs for 3.20 % ($F = 5.5$, $p = 0.004$), plant abundance for 1.80 % ($F = 3.2$, $p = 0.021$) and soil fauna abundance for 1.80 % ($F = 3.1$, $p = 0.029$). The first axis explained 46.3 % of variation ($F = 86.3$, $p = 0.001$) and positively correlated with canopy openness, pH and the sum of PLFAs of Gram-positive bacteria but negatively with plant abundance and soil fauna abundance (Figure 5). The second axis only explained 2.48 % of the variation ($F = 6.3$, $p = 0.001$) and positively correlated with the sum of Gram-positive bacterial PLFAs, pH, soil fauna abundance and canopy openness but negatively with plant abundance. The RDA separated the different land-use systems as well as the different trophic groups of protists. Phagotrophs and symbionts clustered with pH and the sum of Gram-positive bacterial PLFAs, photoautotrophs clustered with canopy openness, animal parasites with soil fauna abundance and plant parasites and undetermined protists with plant abundance.

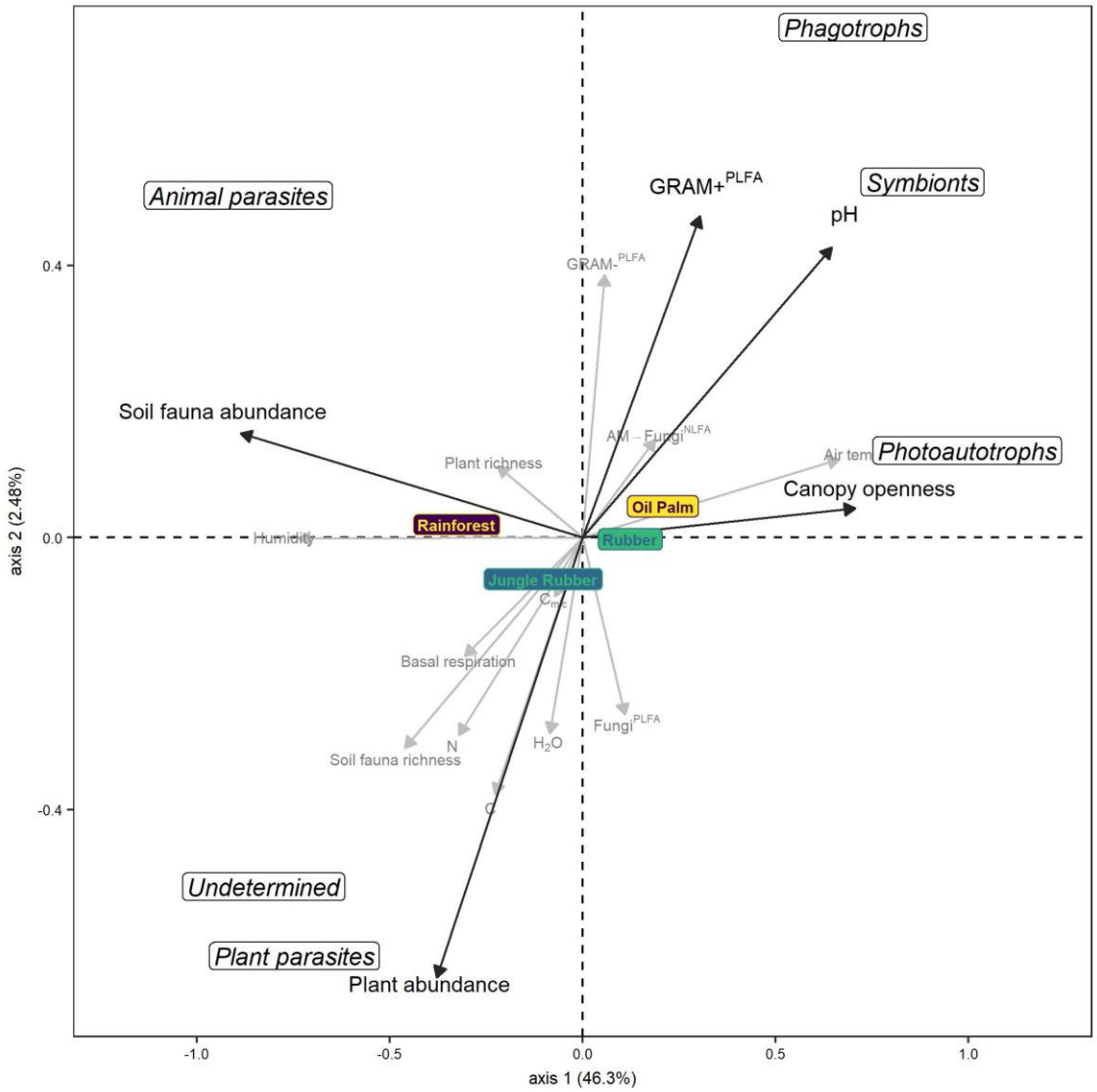

Figure 5: Distance-based RDA of protist OTUs in soil of four land-use systems (rainforest, jungle rubber, rubber and oil palm plantation). Factors in black and bold are significant in the forward selection procedure and are used as constraining factors (soil pH, canopy openness, plant abundance, soil fauna abundance, the sum of Gram-positive bacterial phospholipid fatty acids = GRAM+^{PLFA}), factors in grey are not significant (water content = H_2O, microbial basal respiration = Basal respiration, microbial biomass = C_{mic}, C concentration = C, N concentration = N, air temperature, humidity, plant richness, soil fauna richness, the sum of Gram-negative bacterial phospholipid fatty acids = GRAM-^{PLFA}, the sum of fungal phospholipid fatty acids = Fungi^{PLFA} and relative marker of arbuscular mycorrhizal fungal neutral lipid acid = AM-Fungi^{NLFA}). The position of trophic groups represents their centroid.

Discussion

Effects of land-use change on diversity and community composition of bacteria, fungi and various invertebrate groups have been investigated in soils of lowland Sumatra (Barnes et al., 2017; Clough et al., 2016; Drescher et al., 2016; Klarner et al., 2017; Schneider et al., 2015). However, information on protists, an abundant and diverse group of soil microbial eukaryotes, is lacking. Protists comprise a wide range of phylogenetic and trophic groups and are important determinants and indicators of ecosystem functioning. This study presents the first attempt towards understanding effects of land-use change on protist community structure and trophic assembly in tropical lowlands using high-throughput sequencing of environmental DNA.

General response

We hypothesised that protists are less diverse in more intensively managed land-use systems. Contrasting this hypothesis, the species richness of protists was only significantly reduced in rubber plantations, but similar in rainforest, jungle rubber and oil palm plantations. Seppey et al. (Seppey et al., 2017) also found the species richness of protists to differ little between forest, meadow and arable systems. In our study the Simpson index was high in each of the studied systems (mean 0.96 ± 0.04) indicating that protist diversity is high in each of the land-use systems investigated and little affected by conversion of rainforest into plantations. Contrasting these overall community characteristics, the community compositions of protists differed strongly between the four land-use systems, as indicated by DFA. The overall high species richness but different community composition in the studied ecosystems reflects that protists comprise phylogenetically diverse groups of single cell eukaryotes with very different ecological traits and functions. Thus, to uncover changes in the functioning of protist communities

associated with changes in land-use the community structure of individual trophic groups need to be considered.

Trophic groups

Contrasting protists in general, but conform to our hypothesis, the relative abundance and relative species richness of individual trophic groups of protists differed significantly between land-use systems. In addition, community composition of individual trophic groups of protists differed between land-use systems, suggesting that the community of each land-use system comprises specific protist taxa. For each trophic group of protists the community in rainforest was most distinct from that in the other land-use systems. The shift in community composition from rainforest to intensively managed land-use systems was represented by the first DFA axis, while the second axis separated protist communities of rubber and oil palm plantations. This pattern even applied to protists of undetermined trophic function. Separation of land-use systems, however, was less pronounced in symbionts, which likely is due to the low recovery of symbionts by the method used. Overall, the results support the notion of Grossman et al. (2016) “that protistan community patterns are highly consistent with habitat types”.

Phagotrophic protists predominantly function as bacterial grazers (Clarholm, 1981, 2005), however, in part also as fungivores (Foissner, 1999a; Geisen et al., 2015a) or predators of other protists (Hess and Melkonian, 2014; Seppey et al., 2017) and even microfauna (Geisen et al., 2015b; Gilbert et al., 2000; Yeates and Foissner, 1995). Notably, species richness and abundance of phagotrophic protists increased in intensively managed land-use systems as compared to rainforest. By grazing on bacteria phagotrophic protists, in particular amoebae, increase the mobilization of bacterial nitrogen and thereby improve plant nutrition ('microbial loop in soil'; Bonkowski, 2004;

Clarholm, 1994; Koller et al., 2013b; Rosenberg et al., 2009). However, due to fertilisation plants are likely to invest less in supporting the microbial loop in plantations via root exudates, thereby detrimentally affecting the abundance of phagotrophic protists contrasting our observation. However, in addition to fertilizer input, plantations are limed and thereby soil pH is increased as compared to rainforest (Krashevska et al., 2015; Schneider et al., 2015). It often has been shown that pH is one of the main factors driving the structure of protist communities (Dupont et al., 2016; Lara et al., 2016; Mitchell et al., 2013), and this notion is supported by the increase in phagotrophs with increasing pH in plantations in the present study. Changes in protist communities with soil pH, however, are likely to be indirect via soil pH changing the structure of bacterial communities (Nicol et al., 2008; Schneider et al., 2015). In fact, phagotrophs also correlated with increased abundance of Gram-positive bacteria comprising potential prey, suggesting that phagotrophs function as bacterial grazers and this is particularly pronounced in plantations. This is consistent with findings of Schneider et al. (2015) documenting that bacteria and archaea thrive in rubber and oil palm plantations. Further, phagotrophs correlated closely with photoautotrophs, potentially reflecting that phagotrophs increasingly feed on algae in rubber and oil palm plantations, which is in line with recent findings (Seppey et al., 2017).

Representatives of phagotrophic protists, including bacterial grazers, algivores and predators, formed part of the top ten most abundant OTUs in the studied rainforest and plantation systems. In each of the studies ecosystems the slow moving reticulose amoeboid grazer *Ischnamoeba* sp. was present, which is assumed to exclusively feed on bacteria (Berney et al., 2015). Further, *Telonema* spp. comprising algivore species was common in plantations. The two described members of this genus (*T. antarcticum* and *T.*

subtilis) are common in marine and brackish waters (Klaveness et al., 2005; Vørs, 1992). They are apparently a diverse, deep branching member of chromistan lineage (Shalchian-Tabrizi et al., 2006, 2007). To the best of our knowledge, we report *Telonema* sp. for the first time in soils. In rainforest an example of a predator of other protists is *Lacrymaria* sp. This genus paralyses its prey with toxicysts prior to consuming it (Rosati et al., 2008). The genus *Platyophyra* is known to prey on bacteria and diatoms but additionally harbours symbiotic algae (Foissner and Kreutz, 1996), highlighting that classification of protists in trophic groups is not straightforward. This genus occurred in high abundance in jungle rubber. Further, *Vermamoeba* sp. and *Acanthamoeba* sp. frequently occurred in jungle rubber; both function as bacterial grazers with *Acanthamoeba* sp. also feeding on algae (Marciano-Cabral and Cabral, 2003). Although not directly related to their ecological function, both protists are medically relevant to humans; *Vermamoeba* sp. is associated with different pathogens, i.e. *Stenotrophomonas maltophilia* (Cateau et al., 2014) or *Legionella taurinensis* (Pagnier et al., 2015) and *Acanthamoeba* species are the causative agents of granulomatous amoebic encephalitis and amoebic keratitis (for details, see Marciano-Cabral and Cabral (2003).

Photoautotrophic protists, traditionally termed algae and occurring in the sunlit uppermost soil layers, increased in richness and abundance in plantation systems benefitting from the more open canopy. In addition to increased canopy openness photoautotrophic protists may benefit from weed control in plantations contributing to increased sunlight reaching the soil surface. Further, the application of fertilisers is likely to favour the growth of photoautotrophs and to alter their community structure (Gilbert et al., 1998). Notably, Chlorophyta, i.e. green algae, are well adapted to harsh environmental conditions and disturbances as indicated by their frequent occurrence in

deserts (Lewis et al., 2005). Thereby, Chlorophyta are well adapted to thrive in plantations with Chlorophyceae and Trebouxiophyceae dominating the photoautotrophs in plantations. As representatives of these groups, *Chrysochromulina* sp. and *Prymnesium* sp. were among the top ten most abundant OTUs in rainforest and jungle rubber, respectively. Species of these genera in marine systems are known to produce toxins with haemolytic, ichthyotoxic and cytotoxic properties, affecting other algae and protists (Fistarol et al., 2003; Nielsen et al., 1990; Schmidt and Hansen, 2001). By producing toxins *Prymnesium parvum* may even kill its own predator, *Oxyrrhis marina*, and by consuming it switching to a phagotrophic lifestyle (Tillmann, 2003). This again highlights that the positioning of protists into trophic groups is not straightforward and this also applies to photoautotrophs.

Symbionts typically are tightly linked to host species, although the linkage may vary in space and time (Martin and Schwab, 2012). Although less than 1 % of the total OTUs in our study were classified as symbionts only one of the OTUs could be ascribed to genus level, i.e. *Saccinobaculus* sp., an endosymbiont living in the hindgut of cockroaches (Heiss and Keeling, 2006). This genus only occurred in rainforest and jungle rubber, where cockroaches reach a high species richness (Mumme et al., 2015). The other OTUs were identified as Syndiniales, endosymbionts of ciliates, algae and other protists (Hoek et al., 1995). Indeed, the species richness of Syndinales increased with the species richness of phagotrophs and photoautotrophs, which dominate in plantations, likely reflecting increased host availability in plantations. However, certain Syndiniales in marine systems are known to be parasitic (Guillou et al., 2008), calling for careful interpretation of these findings.

Parasites are closely linked to their host species resembling symbionts, however, they detrimentally affect them (Martin and Schwab, 2012). Parasitic protists reach high abundance and species richness, and may strongly influence animals, fungi and plants as well as other protists, although this is mainly documented for marine systems (de Vargas et al., 2015; Skovgaard, 2014) including deep-sea hydrothermal vents (Moreira and López-García, 2003). However, recent studies suggest that this also applies to soils (Dupont et al., 2016; Geisen, 2016; Mahé et al., 2017). Notably, both groups of parasites identified, i.e. animal and plant parasites, reached higher abundance and species richness in rainforest as compared to plantation systems, matching the higher abundance and species richness of soil invertebrates and plants in rainforest as indicated by RDA. However, in addition to lower host availability, adverse environmental conditions in plantations may contribute to lower abundance and species richness of parasites in plantations, e.g. increased light intensity in plantations may detrimentally affect parasites, as exposure to UV may kill cysts of Eimeriidae (Thomas et al., 1995) causing coccidiosis in animals. Despite that, OTUs belonging to Eimeriidae were the most abundant animal parasites in rubber and oil palm plantations. By contrast, the most abundant animal parasite protists in the more natural land-use systems included *Gregarina* sp., parasites of cockroaches (Clopton and Gold, 1996) and earwigs (Clopton et al., 2008).

The ever present *Eurychasma* sp., an oomycote with broad host range (Muller et al., 1999), was the most abundant plant parasite in each of the land-use systems. Peronosporomycetes, causative agents of downy mildew (Palti and Cohen, 1980), were less abundant in the more intensively managed land-use systems. This might be linked to the increased light intensity as red light inhibits the sporulation of *Peronospora* spp. (Cohen et al., 2013). *Polymyxa* sp., known to infect wheat and other crop species (Ketta et

al., 2012; Thompson et al., 2011; Xu et al., 2018), formed part of the more abundant plant parasites in oil palm plantations. However, although *Polymyxa* sp. may infect a wide range of host species (Legrève et al., 2000), its effect on oil palms is unknown.

Conclusions

Applying amplicon sequencing of the 18S rRNA of environmental DNA this study for the first time provided insight into the relative abundance and diversity of protists in rainforest and tropical agro-ecosystems. The results suggest that overall protist species richness is reduced in rubber plantations. By contrast, however, the community structure of protists is strongly affected by the conversion of rainforest into plantation systems with the relative abundance and relative species richness of the individual trophic groups responding differently. The abundance and in part also the species richness of phagotrophs, photoautotrophs and symbionts increased due to conversion of rainforest into plantation systems, whereas both abundance and species richness of parasites declined. Symbionts generally contributed little to protist abundance and species richness. Notably, within trophic groups individual taxa generally responded in a similar way, suggesting that trophic groups of protists reflect general patterns in changes in the structure of the micro-decomposer food web with conversion of rainforest into plantation systems.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Appendix Chapter 2

Supplementary Table 1: Categorisation of protist taxa into five trophic groups.

Trophic group	**Taxon**				
Phagotrophs	Amoebozoa	Dictyamoeba			
Phagotrophs	Amoebozoa	Dictyostelia			
Phagotrophs	Amoebozoa	Discosea	Flabellinia		
Phagotrophs	Amoebozoa	Discosea	Longamoebia	Centramoebida	Acanthamoeba
Phagotrophs	Amoebozoa	Discosea	Dactylopodia	Mayorella	
Phagotrophs	Amoebozoa	Gracilipodida			
Phagotrophs	Amoebozoa	Ischnamoeba			
Phagotrophs	Amoebozoa	Myxogastria			
Phagotrophs	Amoebozoa	Protosteliales			
Phagotrophs	Amoebozoa	Tubulinea			
Phagotrophs	Amoebozoa	Variosea			
Phagotrophs	Amoebozoa	Cavosteliida			
Phagotrophs	Amoebozoa	Protosporangiida			
Phagotrophs	Amoebozoa	Schizo-plasmodiida			
Photoautotrophs	Archaeplastida	Chloroplastida			
Photoautotrophs	Archaeplastida	Rhodophyceae			
Phagotrophs	Excavata	Discoba	Discicristata	Euglenozoa	Kinetoplastea
Phagotrophs	Excavata	Discoba	Discicristata	Heterolobosea	Tetramitia
Phagotrophs	Excavata	Discoba	Jakobida	Jakobida	Jakobida
Phagotrophs	Excavata	Malawimonas			
Symbionts	Excavata	Metamonada	Parabasalia		
Symbionts	Excavata	Metamonada	Preaxostyla	Oxymonadida	
Phagotrophs	Excavata	Metamonada	Preaxostyla	Paratrimastix	
Phagotrophs	Incertae sedis	Ancyromonadida			
Phagotrophs	Incertae sedis	Apusomonadida			
Phagotrophs	Incertae sedis	Breviatea			
Phagotrophs	Incertae sedis	Centrohelida			
Phagotrophs	Incertae sedis	Palpitia	Palpitomonas		
Phagotrophs	Incertae sedis	Telonemia			
Photoautotrophs	Incertae sedis	Cryptophyceae			
Photoautotrophs	Incertae sedis	Haptophyta			
Photoautotrophs	Incertae sedis	Picozoa			
Phagotrophs	Incertae sedis	Rigifilida			
Phagotrophs	Opisthokonta	Holozoa	Choanoflagellida		
Phagotrophs	Opisthokonta	Holozoa	Filasterea		
Animal parasites	Opisthokonta	Holozoa	Ichthyosporea		
Phagotrophs	Opisthokonta	Nucletmycea	Discicristoidea		
Animal parasites	SAR	Alveolata	Apicomplexa		
Phagotrophs	SAR	Alveolata	Ciliophora		
Phagotrophs	SAR	Alveolata	Colponema		

Phagotrophs/ Photoautotrophs	SAR	Alveolata	Dinoflagellata		
Photoautotrophs	SAR	Alveolata	Protalveolata	Chromerida	
Phagotrophs	SAR	Alveolata	Protalveolata	Colpodellida	
Animal parasites	SAR	Alveolata	Protalveolata	Perkinsidae	
Symbionts	SAR	Alveolata	Protalveolata	Syndiniales	
Phagotrophs	SAR	Rhizaria	Cercozoa	Cercomonadida	
Phagotrophs	SAR	Rhizaria	Cercozoa	Cholamonas	
Phagotrophs	SAR	Rhizaria	Cercozoa	Glissomonadida	
Phagotrophs	SAR	Rhizaria	Cercozoa	Granofilosea	Massisteria
Phagotrophs	SAR	Rhizaria	Cercozoa	Gromia	
Phagotrophs	SAR	Rhizaria	Cercozoa	Gymnophrys	
Phagotrophs	SAR	Rhizaria	Cercozoa	Imbricatea	Marimonadida
Phagotrophs	SAR	Rhizaria	Cercozoa	Imbricatea	Nudifila
Phagotrophs	SAR	Rhizaria	Cercozoa	Imbricatea	Silicofilosea
Phagotrophs	SAR	Rhizaria	Cercozoa	Imbricatea	Spongomonadida
Phagotrophs	SAR	Rhizaria	Cercozoa	Metromonadea	
Phagotrophs	SAR	Rhizaria	Cercozoa	Pansomonadida	Aurigamonas
Plant parasites	SAR	Rhizaria	Cercozoa	Phytomyxea	
Phagotrophs	SAR	Rhizaria	Cercozoa	Sainouron	
Phagotrophs	SAR	Rhizaria	Cercozoa	Thecofilosea	
Phagotrophs	SAR	Rhizaria	Cercozoa	Vampyrellidae	
Phagotrophs	SAR	Rhizaria	Retaria		
Phagotrophs	SAR	Stramenopiles	Bicosoecida		
Phagotrophs	SAR	Stramenopiles	Cantina		
Phagotrophs	SAR	Stramenopiles	Hyphochytriomycetes		
Phagotrophs/ Plant parasites	SAR	Stramenopiles	Labyrinthulomycetes		
Photoautotrophs	SAR	Stramenopiles	Ochrophyta	Bolidomonas	
Phagotrophs/ Photoautotrophs	SAR	Stramenopiles	Ochrophyta	Chrysophyceae	
Photoautotrophs	SAR	Stramenopiles	Ochrophyta	Diatomea	
Photoautotrophs	SAR	Stramenopiles	Ochrophyta	Eustigmatophyceae	
Photoautotrophs	SAR	Stramenopiles	Ochrophyta	Phaeophyceae	
Plant parasites	SAR	Stramenopiles	Peronosporomycetes		
Phagotrophs	SAR	Stramenopiles	Placididea		
Photoautotrophs	SAR	Stramenopiles	Xanthophyceae		

Supplementary Table 2: Environmental factors used for db-RDA

Sample name	Land-use system	H_2O [%]	C_{mic} [mg C_{mic} g^{-1} dw]	Basal Respiration [µg $O_2 g^{-1}$ dw h^{-1}]	pH	C concentration	N concentration	NLFA of arbuscular mycorrhizal fungi (16:1ω5c) [nmol g^{-1} l dw]	sum of relative PLFAs for gram-positive bacteria (i15:0, a15:0, i16:0, i17:0) [% nmol g-1 l dw]	sum of relative PLFAs of gram-negative bacteria (16:1ω7, cy17:0, cy19:0) [% nmol g-1 l dw]	sum of relative PLFAs of fungi (18:2ω6,9, 18:3ω6, 18:3ω3) [% nmol g-1 l dw]	Air temperatur [°C]	Humidity [%]	Canopy openness [%]	Plant abundance [Individals]	Plant richness [Number of species]	Soil fauna abundance [Individuals]	Soil fauna richness [Number of species]
BF1a	F	72,12	317,34	4,86	3,93	4,20	0,34	7,64	64,06	41,51	20,63	24,50	97,25	2,36	278	119	3760	26
BF1b	F	90,00	450,04	5,29	3,81	3,94	0,33	7,72	66,07	39,44	23,83	24,50	97,25	2,36	278	119	3760	26
BF1c	F	61,22	438,93	4,52	3,85	3,48	0,29	7,68	65,06	40,48	22,23	24,50	97,25	2,36	278	119	3760	26
BF2a	F	140,02	496,66	8,00	3,96	5,77	0,61	5,74	45,19	45,12	23,45	24,48	98,62	3,35	279	96	5453	28
BF2b	F	210,78	720,91	6,71	4,07	4,88	0,51	4,92	44,12	45,07	22,37	24,48	98,62	3,35	279	96	5453	28
BF2c	F	222,42	926,30	12,53	3,89	10,39	0,89	5,33	44,66	45,10	22,91	24,48	98,62	3,35	279	96	5453	28
BF3a	F	147,19	303,64	6,53	3,01	23,45	0,60	4,48	55,21	19,46	27,49	24,42	96,01	2,04	193	109	7777	26
BF3b	F	56,75	430,87	9,72	3,34	6,32	0,36	3,66	54,17	16,86	30,92	24,42	96,01	2,04	193	109	7777	26
BF3c	F	209,12	332,77	3,13	3,16	14,88	0,48	4,07	54,69	18,16	29,20	24,42	96,01	2,04	193	109	7777	26
BF4a	F	38,64	540,33	8,90	3,32	6,86	0,35	7,68	24,49	13,91	33,21	24,85	94,57	2,22	203	136	7697	25
BF4b	F	43,07	741,13	10,63	3,34	5,41	0,30	8,58	53,96	50,26	30,28	24,85	94,57	2,22	203	136	7697	25
BF4c	F	40,21	544,65	4,44	3,60	4,29	0,26	8,13	39,23	32,08	31,74	24,85	94,57	2,22	203	136	7697	25
BJ1a	J	123,37	726,16	7,40	4,22	5,97	0,39	7,88	44,27	39,62	28,76	25,00	94,94	5,75	181	30	2161	26
BJ1b	J	137,63	855,86	9,52	4,50	15,54	0,49	20,16	56,53	34,10	31,55	25,00	94,94	5,75	181	30	2161	26
BJ1c	J	227,18	1118,49	9,70	4,40	8,12	0,56	14,02	50,40	36,86	30,15	25,00	94,94	5,75	181	30	2161	26
BJ2a	J	90,75	477,07	6,91	4,26	4,67	0,31	10,92	61,96	29,73	24,29	25,42	91,38	6,39	169	36	3007	25
BJ2b	J	86,95	400,44	4,71	4,40	5,79	0,41	24,10	55,70	20,03	40,47	25,42	91,38	6,39	169	36	3007	25
BJ2c	J	113,29	713,80	8,02	4,66	6,60	0,48	17,51	58,83	24,88	32,38	25,42	91,38	6,39	169	36	3007	25
BJ3a	J	117,75	705,58	7,08	4,02	5,00	0,41	10,68	48,12	19,50	29,35	24,92	94,56	5,00	205	36	3186	19
BJ3b	J	185,87	672,59	9,24	4,58	8,04	0,68	7,56	32,80	19,70	33,92	24,92	94,56	5,00	205	36	3186	19
BJ3c	J	191,43	575,23	7,41	4,68	7,26	0,57	9,12	40,46	19,60	31,63	24,92	94,56	5,00	205	36	3186	19
BJ4a	J	126,98	721,82	8,04	4,24	7,73	0,54	9,82	68,58	14,47	21,83	25,25	93,32	5,85	168	33	2104	24
BJ4b	J	98,36	598,14	6,51	4,18	6,89	0,46	20,50	44,19	18,70	40,71	25,25	93,32	5,85	168	33	2104	24
BJ4c	J	107,42	432,32	4,69	4,09	9,40	0,53	15,16	56,39	16,59	31,27	25,25	93,32	5,85	168	33	2104	24
BO1a	O	104,55	563,79	8,14	4,22	3,85	0,28	7,10	75,41	41,91	26,53	25,45	91,29	14,49	58	126	1347	21
BO1b	O	135,53	284,09	3,81	4,47	4,64	0,31	8,12	69,57	20,35	24,00	25,45	91,29	14,49	58	126	1347	21
BO1c	O	114,45	324,72	4,70	4,37	3,07	0,24	7,61	72,49	31,13	25,26	25,45	91,29	14,49	58	126	1347	21
BO2a	O	129,56	237,15	3,14	4,27	3,07	0,29	4,60	74,29	18,84	21,74	25,53	89,46	12,68	52	134	3315	22

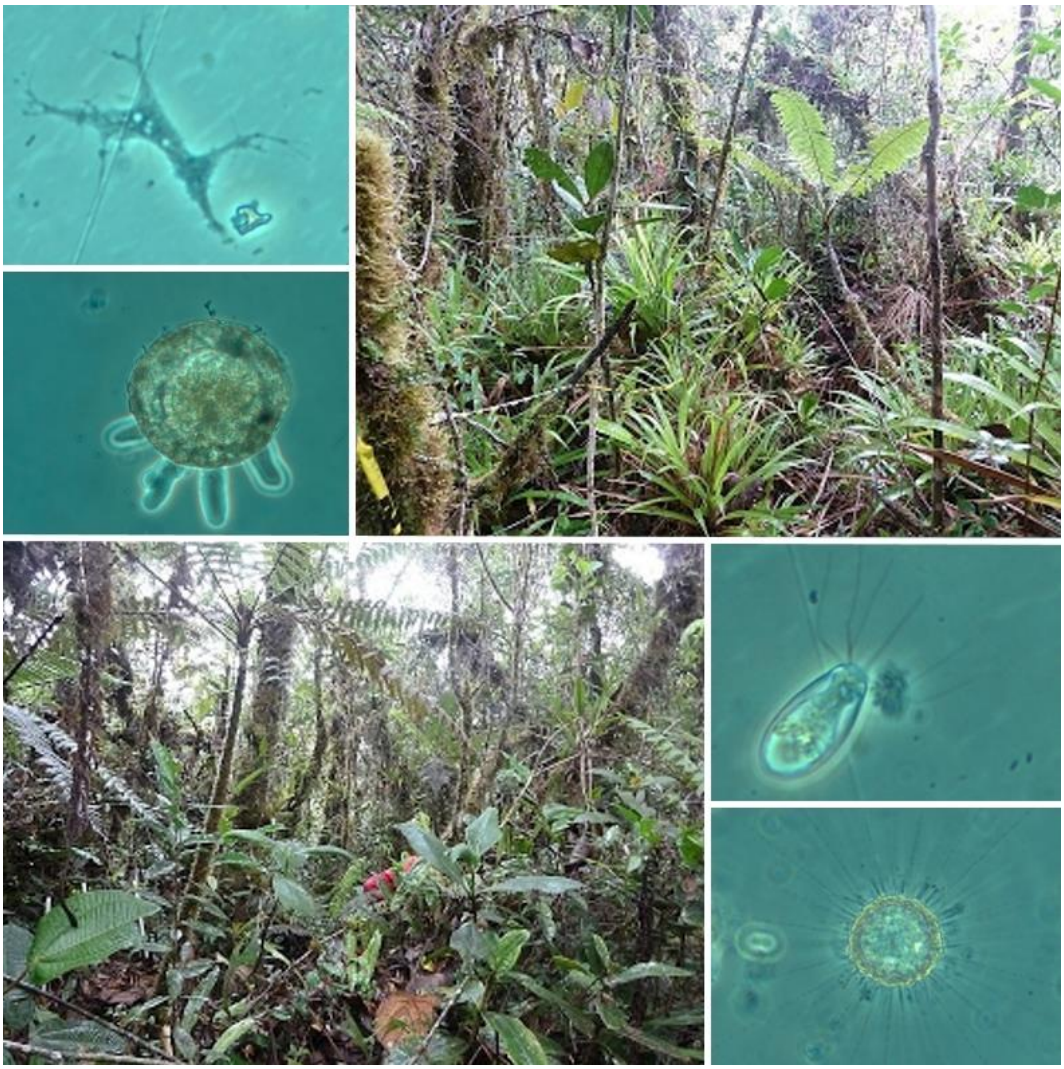
BO2b	O	171,19	323,75	4,01	4,35	4,08	0,32	9,72	73,75	29,80	22,28	25,53	89,46	12,68	52	134	3315	22
BO2c	O	69,81	459,96	3,97	4,42	3,58	0,30	7,16	74,02	24,32	22,01	25,53	89,46	12,68	52	134	3315	22
BO3a	O	65,96	911,94	8,57	4,41	5,99	0,48	15,32	74,92	41,79	27,70	25,78	86,90	10,90	44	116	836	20
BO3b	O	90,13	679,92	6,48	4,59	5,55	0,44	6,96	64,39	35,81	26,48	25,78	86,90	10,90	44	116	836	20
BO3c	O	97,97	745,11	9,39	4,48	4,31	0,40	11,14	69,65	38,80	27,09	25,78	86,90	10,90	44	116	836	20
BO4a	O	47,59	637,81	7,24	4,59	3,57	0,29	11,08	67,39	37,40	25,56	25,64	89,76	19,43	58	121	4636	19
BO4b	O	60,49	489,60	5,82	4,42	5,57	0,34	4,08	49,42	22,06	28,65	25,64	89,76	19,43	58	121	4636	19
BO4c	O	47,07	391,98	4,41	4,57	3,85	0,29	7,58	58,40	29,73	27,10	25,64	89,76	19,43	58	121	4636	19
BR1a	R	90,14	453,52	5,94	4,17	5,04	0,33	9,72	54,07	12,23	26,97	25,95	88,76	23,41	65	207	3327	22
BR1b	R	81,43	586,66	6,53	4,23	3,14	0,28	11,24	62,25	33,51	26,07	25,95	88,76	23,41	65	207	3327	22
BR1c	R	103,98	408,14	4,09	4,28	2,55	0,23	10,48	58,16	22,87	26,52	25,95	88,76	23,41	65	207	3327	22
BR2a	R	119,09	592,10	5,43	4,22	3,52	0,27	10,62	49,19	27,96	27,25	25,12	92,92	13,29	73	175	946	16
BR2b	R	66,92	472,11	3,58	4,30	2,48	0,20	11,64	62,35	33,77	25,56	25,12	92,92	13,29	73	175	946	16
BR2c	R	109,31	467,38	4,42	4,40	3,26	0,26	11,13	55,77	30,86	26,41	25,12	92,92	13,29	73	175	946	16
BR3a	R	105,20	574,36	5,39	4,49	6,63	0,23	0,00	63,46	33,85	35,62	25,45	91,42	12,97	75	158	1213	21
BR3b	R	71,15	516,19	4,32	4,39	1,84	0,14	8,14	48,58	19,08	27,55	25,45	91,42	12,97	75	158	1213	21
BR3c	R	139,26	632,53	6,45	4,58	11,42	0,31	4,07	56,02	26,46	31,58	25,45	91,42	12,97	75	158	1213	21
BR4a	R	117,29	450,10	4,44	4,40	4,77	0,41	17,92	62,26	33,99	25,49	25,67	90,86	20,61	79	132	1187	22
BR4b	R	100,65	417,05	3,29	4,34	3,75	0,35	14,92	46,70	25,49	19,12	25,67	90,86	20,61	79	132	1187	22
BR4c	R	141,04	448,16	4,41	4,43	6,50	0,44	16,42	31,13	17,00	12,74	25,67	90,86	20,61	79	132	1187	22
HF1a	F	27,03	546,67	5,88	3,97	2,59	0,19	14,00	52,74	31,23	24,42	24,88	96,39	2,37	315	161	3232	22
HF1b	F	37,45	479,96	5,98	3,86	3,05	0,23	3,83	65,32	38,17	15,34	24,88	96,39	2,37	315	161	3232	22
HF1c	F	35,06	283,62	5,05	3,96	3,67	0,24	8,92	59,03	34,70	19,88	24,88	96,39	2,37	315	161	3232	22
HF2a	F	38,42	310,87	5,70	3,94	5,16	0,34	23,86	64,11	36,64	22,12	24,91	95,11	2,56	294	182	958	20
HF2b	F	51,04	460,40	7,89	3,70	7,36	0,42	15,00	61,98	38,32	24,70	24,91	95,11	2,56	294	182	958	20
HF2c	F	33,96	373,22	4,46	3,80	3,79	0,25	19,43	63,04	37,48	23,41	24,91	95,11	2,56	294	182	958	20
HF3a	F	42,02	570,11	5,72	4,05	4,46	0,30	4,46	54,20	30,68	26,16	24,67	98,10	2,22	333	155	3432	23
HF3b	F	51,01	347,55	4,19	3,77	4,90	0,28	5,96	60,38	37,07	25,76	24,67	98,10	2,22	333	155	3432	23
HF3c	F	47,84	529,37	5,27	4,27	3,48	0,28	5,21	57,29	33,88	25,96	24,67	98,10	2,22	333	155	3432	23
HF4a	F	44,39	481,90	6,89	3,93	3,96	0,27	4,43	54,80	33,23	29,52	24,92	95,47	3,19	301	154	3389	23
HF4b	F	33,62	409,05	4,71	4,05	5,11	0,32	9,47	61,47	37,38	21,83	24,92	95,47	3,19	301	154	3389	23
HF4c	F	37,51	394,99	3,71	4,19	3,58	0,27	6,95	58,13	35,31	25,67	24,92	95,47	3,19	301	154	3389	23
HJ1a	J	42,99	1038,48	5,50	4,27	3,64	0,28	13,60	62,87	37,40	24,33	25,37	91,91	11,39	128	84	2889	25
HJ1b	J	33,49	464,84	4,54	4,30	2,74	0,24	7,83	66,28	37,70	16,00	25,37	91,91	11,39	128	84	2889	25
HJ1c	J	39,29	498,52	3,44	4,02	3,94	0,29	10,72	64,57	37,55	20,17	25,37	91,91	11,39	128	84	2889	25
HJ2a	J	42,63	372,62	4,12	4,12	2,86	0,19	9,38	64,15	30,60	29,45	25,06	93,75	6,85	130	129	2521	25
HJ2b	J	34,92	468,38	4,05	4,42	3,15	0,23	9,80	47,00	21,47	33,35	25,06	93,75	6,85	130	129	2521	25
HJ2c	J	27,35	370,17	4,41	4,04	3,98	0,28	9,59	55,57	26,04	31,40	25,06	93,75	6,85	130	129	2521	25
HJ3a	J	32,24	541,58	4,35	4,31	2,73	0,22	16,90	63,12	37,95	21,71	25,13	91,50	6,76	131	130	1063	18
HJ3b	J	84,87	347,78	2,91	4,28	4,27	0,32	10,92	46,63	20,24	30,82	25,13	91,50	6,76	131	130	1063	18
HJ3c	J	31,59	335,36	4,42	4,38	4,40	0,29	13,91	54,87	29,09	26,26	25,13	91,50	6,76	131	130	1063	18
HJ4a	J	35,87	353,62	4,83	4,55	4,66	0,31	13,56	62,20	38,31	24,42	25,13	93,61	7,48	179	145	1620	20
HJ4b	J	41,94	247,78	4,81	4,45	5,99	0,28	14,36	49,29	19,54	31,12	25,13	93,61	7,48	179	145	1620	20
HJ4c	J	32,65	375,45	6,02	4,71	3,42	0,23	13,96	55,75	28,92	27,77	25,13	93,61	7,48	179	145	1620	20

HO1a	O	31,06	601,21	4,32	4,76	2,76	0,18	7,27	64,23	49,96	39,93	25,37	92,25	8,18	73	35	1725	18
HO1b	O	36,18	493,82	4,48	4,65	3,05	0,24	23,26	33,81	25,38	32,57	25,37	92,25	8,18	73	35	1725	18
HO1c	O	43,18	428,04	4,69	4,79	2,55	0,22	15,27	49,02	37,67	36,25	25,37	92,25	8,18	73	35	1725	18
HO2a	O	30,48	428,97	3,17	5,02	1,65	0,14	4,23	55,50	38,08	27,32	25,62	90,01	14,76	72	38	678	19
HO2b	O	26,10	317,43	2,73	5,68	1,62	0,15	33,44	30,47	42,13	24,18	25,62	90,01	14,76	72	38	678	19
HO2c	O	38,05	305,93	2,60	5,56	5,27	0,19	18,84	42,98	40,10	25,75	25,62	90,01	14,76	72	38	678	19
HO3a	O	27,21	225,25	1,96	4,66	1,72	0,14	9,91	70,42	48,82	22,72	25,40	91,16	15,72	44	35	749	14
HO3b	O	22,46	381,67	7,07	4,96	2,07	0,15	45,08	64,59	38,60	22,01	25,40	91,16	15,72	44	35	749	14
HO3c	O	20,80	339,01	2,16	4,77	1,62	0,13	27,50	67,50	43,71	22,37	25,40	91,16	15,72	44	35	749	14
HO4a	O	32,31	522,69	2,14	5,15	3,35	0,23	21,46	66,14	48,35	36,19	25,59	90,40	11,52	92	32	1585	16
HO4b	O	26,27	267,10	1,67	4,91	2,29	0,15	16,12	63,29	33,25	19,91	25,59	90,40	11,52	92	32	1585	16
HO4c	O	29,49	249,59	1,64	4,76	2,93	0,23	18,79	64,71	40,80	28,05	25,59	90,40	11,52	92	32	1585	16
HR1a	R	33,60	193,24	2,24	4,42	1,34	0,15	5,82	78,31	40,71	24,31	25,35	91,12	15,20	96	85	2236	20
HR1b	R	42,35	514,60	2,98	4,35	3,01	0,26	24,00	32,22	22,32	32,74	25,35	91,12	15,20	96	85	2236	20
HR1c	R	39,16	287,56	3,47	4,57	2,56	0,23	14,91	55,27	31,51	28,53	25,35	91,12	15,20	96	85	2236	20
HR2a	R	43,92	423,12	3,43	4,21	2,72	0,24	8,95	56,04	29,97	26,32	25,49	91,28	11,80	45	73	2245	26
HR2b	R	43,25	165,63	3,31	4,32	1,96	0,17	9,44	57,87	20,08	24,41	25,49	91,28	11,80	45	73	2245	26
HR2c	R	41,27	287,64	2,89	4,49	2,34	0,21	9,20	56,96	25,02	25,37	25,49	91,28	11,80	45	73	2245	26
HR3a	R	34,50	354,97	3,52	4,54	1,67	0,14	16,94	64,74	38,03	28,15	25,18	91,24	11,95	51	111	1320	20
HR3b	R	26,96	228,46	2,96	4,53	1,83	0,15	19,34	36,07	25,82	26,93	25,18	91,24	11,95	51	111	1320	20
HR3c	R	30,95	205,89	2,80	4,50	1,60	0,14	18,14	50,40	31,92	27,54	25,18	91,24	11,95	51	111	1320	20
HR4a	R	44,47	461,77	4,09	4,42	3,62	0,27	17,54	33,87	20,52	26,65	25,39	90,94	15,91	85	167	944	17
HR4b	R	40,09	481,55	3,43	4,46	3,18	0,24	12,76	64,87	19,34	25,54	25,39	90,94	15,91	85	167	944	17
HR4c	R	42,28	471,66	3,76	4,44	3,40	0,25	15,15	49,37	19,93	26,09	25,39	90,94	15,91	85	167	944	17

Chapter 3

Response of protists to nitrogen addition, AMF manipulation and mesofauna reduction in a tropical montane rainforest in Southern Ecuador

Garvin Schulz, Tessa Camenzind, Laura M. Sánchez-Galindo, Dominik Schneider, Stefan Scheu, Valentyna Krashevska



Abstract

The tropical Andes, a species-rich and nutrient-limited system, might be sensitive to increased nitrogen (N) inputs from the atmosphere. However, the effects of elevated N are still poorly understood, especially the effects on functionally important groups of the belowground system driving nutrient cycling such as protists. We used high throughput sequencing (HTS) of environmental DNA of two different litter layers from field installed microcosms to explore how increased N affects protists in a tropical montane rainforest in southern Ecuador. We also manipulated and studied arbuscular mycorrhizal fungi (AMF) and mesofauna involved in the N cycle, as possible competitors for and sources of nutrients for protist communities. We obtained 2,503,427 reads of protists accounting for 44.2 % of the total reads and ascribed them to 4,369 ASVs. Alveolata, Sarcomastigota, and Archaeplastida were more abundant in the upper L layer, while Rhizaria, Excavata, and Hacrobia were more abundant in the lower F/H layer. The protist community was affected strongly by added N in both layers, while reduction of mesofauna had a stronger effect in the lower F/H layer compared to the L layer. Changes in AMF concentration had the lowest impact on protists. Photoautotrophs and animal parasites showed a higher relative abundance in the L layer, while phagotrophs and plant parasites showed a higher relative abundance in the F/H layer. In both layers added N increased the relative abundance of phagotrophs and animal parasites and decreased that of plant parasites, while the relative abundance of mixotrophs decreased in the L layer but increased in the F/H layer. In the L layer, with higher AMF concentration, the relative abundance of mixotrophs decreased, while in the F/H layer the relative abundance of photoautotrophs increased and that of plant parasites decreased. With reduced mesofauna abundance the relative abundance of phagotrophs increased and that of animal parasites decreased in both layers, while the relative abundance of plant parasites increased only in the L layer. Overall, the results

demonstrate that analysing protist communities of multiple layers is needed to acquire insight into the response of protist communities to environmental changes.

Introduction

The tropical Andes are a biodiversity 'hotspot' with a high number of endemic animal and microbial taxa (Myers et al., 2000; Bax and Francesconi, 2019) involved in complex interactions with plants (Hagedorn et al., 2019; de la Cruz-Amo et al., 2020). These species-rich but nutrient-poor systems are likely to be sensitive to changes in nutrient inputs, such as nitrogen (N) and phosphorus (P), especially at high elevation (Graefe et al., 2010; Dalling et al., 2016; Camenzind et al., 2018). Changes in nutrient input occur even in pristine tropical forests; since the 1960s the global atmospheric amount of N has increased mainly due to increased combustion of fossil fuels (Galloway et al., 2008). Atmospheric N input into distant non-agricultural ecosystems has been increased and will increase further in the future (Mahowald et al., 2005). Even minor input of nutrients affects not only plants, but also alters soil microbial communities and threatens the biodiversity of these hotspots (Homeier et al., 2012; Krashevska et al., 2014). Cycling of nutrients in large is mediated in the belowground system via decomposition processes, performed by interactions of a diverse community of soil- and litter-dwelling organisms (Bardgett, 2005; Scheu et al., 2005).

One of the most important players in the complex belowground system are protists, single-celled organisms comprising the majority of all eukaryotic life forms (Adl et al., 2019). Protists may reach high densities and diversity in soils (Foissner, 1999; Geisen and Bonkowski, 2017), and include virtually all trophic levels in ecosystems (Geisen et al., 2018). Therefore, they are ideal model organisms for studying interactions in the belowground system and factors impacting these interactions. For example, phagotrophs

are grazers and predators of microorganisms and smaller soil animals (Clarholm, 2005; Geisen et al., 2015a; Mitchell, 2015; Adam et al., 2017). Photoautotrophs are primary producers and contribute to the organic input into soils (Metting, 1981; Bamforth, 2008; Seppey et al., 2017). Mixotrophs use photosynthesis for nutrition, but simultaneously employ a phagotrophic lifestyle or switch between both modes (Unrein et al., 2014; Jassey et al., 2015). Further, parasites of plants and animals constitute a large faction in the belowground system, rivalling the diversity of arthropods (Kinne and Lauckner, 1980; Foissner, 1987; Strona, 2015; Mahé et al., 2017; Geisen et al., 2018). While it is known that N affects protist communities (Wang et al., 2014; Pan et al., 2020), it is not clear which trophic groups are most vulnerable to increased atmospheric N input. Although the importance of protists in the belowground system has been stressed (Bonkowski, 2004; Bonkowski and Clarholm, 2012; Geisen and Quist, 2021), experiments revealing interactions between protists and other players in soil, such as arbuscular mycorrhizal fungi (AMF) (Koller et al., 2013; Henkes et al., 2018) and mesofauna (Erktan et al., 2020), are limited. This is due, at least in part, to the fact that extraction and cultivation of the majority of protist taxa is difficult or impossible (Geisen et al., 2015b). However, high throughput sequencing (HTS) of environmental DNA offers the possibility to analyse whole protist communities (Mahé et al., 2017; Heger et al., 2018; Schulz et al., 2019; Oliverio et al., 2020). Most of the existing data is based on samples from the top layers of the mineral soil, although protists also colonize the litter layer, which is of eminent importance for element cycling. In the tropical montane rainforest of Southern Ecuador, our study region, testate amoebae are the only group of protists which have been investigated in litter and soil so far (Krashevska et al., 2007, 2010, 2014). These mostly microbial grazers showed high abundance and diversity at 2,000 m a.s.l., with both being higher in the upper litter than the deeper fermentation layer (Krashevska et al., 2007).

Testate amoebae benefit from the addition of high and moderate amounts of N, but likely compete with saprotrophic fungi and AMF for nutrients (Krashevska et al., 2010, 2014). However, it is unknown how other groups of protists respond to increased input of N, and variations in AMF or mesofauna abundance, both potential competitors for resources of protists (Treseder and Cross, 2006; Camenzind and Rillig, 2013).

In this study we use HTS of environmental DNA targeting the 18S gen to investigate protist community structure in an experimental setup. We manipulated N, AMF and mesofauna abundance to investigate their impact on the diversity and community composition of protists, as well as on protist functioning in a tropical montane rainforest in southern Ecuador. We hypothesised that diversity, community composition and relative abundance of trophic groups of protists will be differentially affected (1) by the addition of N, (2) the reduction of AMF, and (3) the reduction of mesofauna, with the effect of N input and AMF reduction being strongest in autotrophic protists essentially relying on mineral N, whereas the effect of reduced mesofauna to be strongest in phagotrophic protists relying on organic matter resources and microorganisms as food as most detritivore soil mesofauna species. Further, we expected mesofauna to acts as hosts for parasitic protists thereby favouring their diversity. Generally, we assume these effects to be more pronounced on the functional than on the taxonomic level.

Material & Methods

Study site

The study site is located near the Estación Científica San Francisco at 2,000 m a.s.l. (3°59'S, 79°05'W) in the northern part of the Podocarpus National Park on the eastern slope of the Andes in southern Ecuador. The forest is classified as lower montane rainforest (Homeier et al., 2008) and part of the private reserve Reserve Biologica San Francisco. It is in close

to pristine condition and well-studied in regard to vegetation and above- and belowground arthropods, with extraordinary richness in tree species as well as other plant and animal species (Beck et al., 2008; Homeier et al., 2010). The climate is warm (mean annual temperature of ~15 °C) and humid (mean annual precipitation ~2,200 mm). The soil is Cambisol with a thick organic layer but nutrient-poor (Wilcke et al., 2002). The organic layer had a thickness of 11 cm and was separated into litter and fermentation layer (Krashevska et al., 2007).

Experimental setup

The experiment was setup as described in Sánches-Galindo et al. (2019). Briefly, microcosms consisted of plastic tubes of 15 cm diameter and 20 cm length. Two rectangular holes (10 x 15 cm) were cut opposite to each other into the tube and covered with 45 µm nylon mesh. Further, the tubes were equipped with two sheets of 45 µm nylon mesh at 2 cm and 7 cm from the bottom to allow drainage of water but prevent ingrowth of roots and mycorrhizal hyphae. The microcosms were closed with a lid of 4 mm nylon mesh to allow colonization by mesofauna (Figure 1A).

The effect of the three factors N addition, rotation (reduction of AMF), and defaunation (reduction of mesofauna; each with two levels) on protist communities in the L and F/H layer were investigated in the framework of the nutrition manipulation experiment (NUMEX) (Homeier et al., 2012). Briefly, NUMEX is a fertilisation experiment, set up in 2007 in a complete randomised block design with four blocks. For our experiment four soil samples of approximately 3,000 cm3 were taken randomly with a stainless-steel corer of 14.5 cm inner diameter from two subplots (2 x 2 m) of the N addition and control plots of the four blocks of the NUMEX experiment at 2,000 m a.s.l. The N addition plots received 50 kg N ha-1 y-1 applied as urea twice per year. The samples comprising the L and F/H

layer were placed into the microcosm tubes fitting on top of the upper 45 µm mesh sheet. For defaunation half of the cores were frozen at -20 °C for one week. Microcosms were placed into the holes from which the samples were excavated. To manipulate AMF abundance half of the microcosms were rotated 45° every two days preventing ingrowth of AMF hyphae (Figure 1B). Microcosms were installed in June 2015 and harvested after 5 months in November 2015. At harvest, the soil cores were taken out of the tubes and vertically split into half and then separated into L and F/H layer, resulting in 128 samples in total (2 x fertilisation, 2 x rotation, 2 x defaunation, 4 x blocks, 2 x layers, 2 x subsamples). Half of each sample was frozen at 20 °C at the day of sampling and transported to Germany for analysis of fatty acids, soil properties and extraction of environmental DNA. The other half of each sample was used for extraction and determination of soil mesofauna (Sánchez-Galindo et al., 2019).

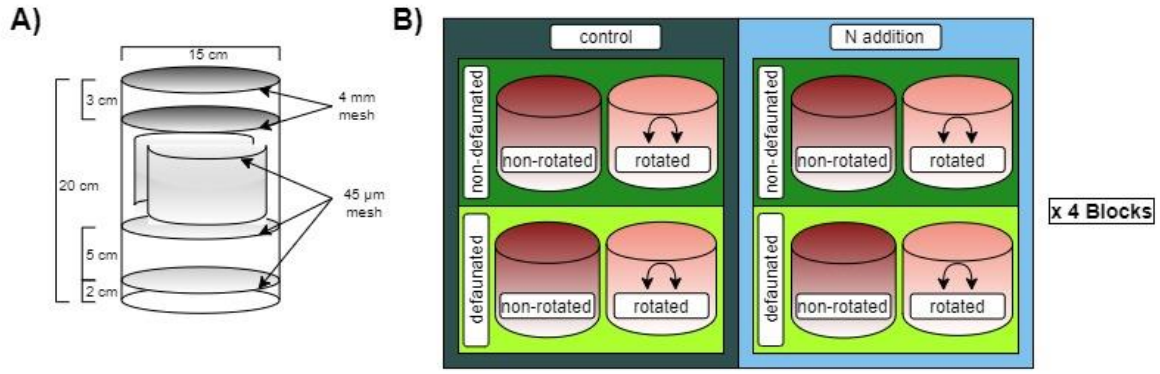


Figure 1: Scheme of the microcosms (A) and study design (B).

DNA extraction and amplification of 18S rRNA genes

DNA was extracted from 200 mg of lyophilised sample material using the MoBio PowerSoil isolation kit (Dianova, Hamburg, Germany) following the manufacturer's instructions. The variable region V4 of the 18S rRNA gene was amplified using the general eukaryotic primers TA-Reuk454FWD1 (5'-CCAGCASCYGCGGTAATTCC-3') and TA-ReukREV3 (5'-ACTTTCGTTCTTGATYRA-3') (Stoeck et al. 2010) paired with the MiSeq-

Adapters Forward overhang (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and Reverse overhang (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). For amplification, the Phusion High Fidelity DNA Polymerase kit (ThermoFisher Scientific, Germany) was used. The PCR reaction mixture contained 10 µl of 5-fold Phusion GC Buffer, 1 µl of the forward and reverse primers (1 µM), 1 µl MgCl2 (50 mM), 1 µl dNTPs (50 mM), 2.5 µl DMSO, 0.5 µl Phusion Polymerase (1 U) and 1 µl template DNA. The following thermocycling scheme was used for amplification: initial denaturation at 98 °C for 1 min, 35 cycles of denaturation at 98 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min, followed by a final extension period at 72 °C for 5 min. Amplicon length was ~400 bp. All amplicon PCRs were performed three times and pooled in equal amounts for sequencing. PCR products were purified using the QIAquick Purification Kit (Qiagen, Germany) following the manufactures protocol. Göttingen Genomic Laboratory determined the sequences of the 18S rRNA amplicons using MiSeq.

Bioinformatic analysis of 18S rRNA gene sequences

Paired-end sequencing data from the Illumina MiSeq were quality-filtered with fastp (version 0.19.4) (Chen et al., 2018) using default settings with the addition of an increased per base Phred score of 20, base pair corrections by overlap (-c), as well as 5′- and 3′-end read trimming with a sliding window of 4, a mean quality of 20 and minimum sequence size of 50 bp. After quality control, the paired-end reads were merged using PEAR (version 0.9.11) (Zhang et al., 2014) and primers clipped using cutadapt (version 1.16) (Martin, 2011) with default settings. Sequences were then processed using VSEARCH (version 2.8.4) (Rognes et al., 2016). This included sorting and size-filtering of the paired reads to ≥250 bp (--sortbylength --minseqlength 250), dereplication (--derep_fulllength). Dereplicated amplicon sequence variants (ASVs) were denoised with UNOISE3 using

default settings (--cluster_unoise –minsize 8) and chimeras were removed (--uchime3_denovo). An additional reference-based chimera removal was performed (--uchime_ref) against the SILVA SSU NR database (version 132). Raw reads were mapped to ASVs (--usearch_global–id 0.97). The taxonomy was assigned using BLAST 2.7.1+ (Altschul et al., 1990) against the PR2 database (version 4.10) resulting in a total of 10,368 ASVs. The taxonomy was checked manually and curated following Ruggiero et al. (2015), Cavalier-Smith (2017) and Adam et al. (2017).

Data analysis

All statistical analysis, data handling and graphical processing was done in R 3.6.3 (R Core Team, 2020) using the packages ampvis2 (Andersen et al., 2018), car (Fox and Weisberg, 2019), compositions (van den Boogaart and Tolosana-Delgado, 2008), ggpubr (Kassambara, 2020), gridExtra (Auguie, 2017), kableExtra (Zhu, 2019), MOTE (Buchanan et al., 2019), nlme (Pinheiro et al., 2017), tidyverse (Wickham and Henry, 2017), vegan (Oksanen et al., 2017) and zCompositions (Palarea-Albaladejo and Martín-Fernández, 2015).

Metagenomic data are compositional by nature (Pawlowsky-Glahn and Egozcue, 2006). To account for that, we used the centred log-ratio transformation (clr) (Aitchison, 1982) in contrast to the popular rarefaction approach, which drops a lot of information (McMurdie and Holmes, 2014). In order to apply this transformation, we imputed zeros in the read counts using the count zero multiplicative (czm) replacement. The clr-transformed data was used for all further analyses, except for the assessment of alpha diversity, i.e. richness and evenness. Due to the clr-transformation we could use Euclidean distances for all PERMANOVAs (Pawlowsky-Glahn and Egozcue, 2006).

Differences of the protist communities, based on the clr-transformed read counts, between the L and F/H layer were assessed using principal component analysis (PCA) and tested with a PERMANOVA with 10,000 replicates. Additionally, the major protist groups Euglenozoa, Excavata, Sarcomastigota, Stramenopiles, Alveolata, Rhizaria, Hacrobia and Archaeplastida, based on their clr-transformed read counts, were tested for differences between layers with a PERMANOVA with 10,000 replicates. In addition to report p-values we calculated the effect size ω2 of all applicable analyses (Olejnik and Algina, 2003; Lakens, 2013).

We used exploratory data analysis to test for successful treatment procedures. The treatment was considered successful if in the rotation treatments the concentration of the AMF marker NLFA 16:1ω5 in rotated the microcosms was reduced, if in the defaunation treatments the abundance of mesofauna was reduced and if in the N addition treatments the concentration of N was increased. This was tested by individual ANOVAs for each of the three treatments. The rotation treatment did not significantly affect the AMF marker concentration, but the concentration of the AMF marker varied across the samples and showed a normal distribution; in addition to using the rotation treatment as factor we therefore used the AMF marker concentration as covariate in all analyses (see 'Efficacy of experimental setup' in Results and Discussion for details).

Alpha diversity was analysed by comparing the observed ASV richness based on untransformed read counts, usually underestimating real richness (Calle, 2019). Therefore, we used the Chao1 index, which adjusts for missed species, in addition, resulting in elevated numbers compared to the observed richness (Calle, 2019). Evenness within samples was assessed by using the Shannon index as well as the inverse Simpson

index. All four diversity measures were used in individual ANOVAs as independent variables to test for significant treatment effects.

Community composition was assessed by principal component analysis (PCA) of the clr-transformed read counts, looking into each treatment individually. To test for treatment effects on the community, the scores of PCA axis 1 and 2 were used as variables in a MANOVA for each layer.

To gain further insight into the functional variation of the protist communities, protists were assigned to five trophic groups: phagotrophs, photoautotrophs, mixotrophs, animal and plant parasites, based on recent studies (Adam et al., 2017; Adl et al., 2019; Schulz et al., 2019). We used the most inclusive taxonomic ranks, e.g. class, of the respective protists for assignment whenever possible. Higher taxonomic ranks, e.g. family or genus, were used to prevent assignment of protists to multiple trophic groups (Appendix 1).

PERMANOVAs (with 10,000 replicates) using the clr-transformed read counts for all five trophic groups for each layer revealed significant effects of the experimental treatments N addition and defaunation in both layers and of the AMF marker concentration in the F/H layer on the clr-transformed read counts (Appendix 2 and 3). Therefore, the effects of the experimental treatments, variation in the concentration of the AMF marker concentration and possible interactions on each trophic group were analysed individually with further PERMANOVAs with the same number of replicates. Lastly, we compared the ranked clr-transformed read counts of the respective trophic groups to assess effects of the experimental treatments on the most abundant protists.

Results

Data quality and general community overview

Using general eukaryotic primers to amplify the variable V4 region of the 18S rRNA gene (Stoeck et al. 2010), we obtained 5,021,942 curated sequence reads from 128 samples, which could be ascribed to 10,368 ASVs. After filtering out reads of Fungi, Metazoa and Streptophyta (37.0 %) as well as all unidentified reads (18.8 %), the remaining reads of protists targetable with this PCR-based approach accounted for 44.2 % of the total reads comprising 2,503,427 reads, ascribed to 4,369 ASVs (Table 1). Rhizaria and Alveolata of the SAR supergroup dominated the protist community, followed by Sarcomastigota, Stramenopiles, Archaeplastida, Hacrobia, Excavata and Euglenozoa in both, ASV richness and read counts (Table 1). The clr-transformed read counts of all protists in the L and F/H layers differed significantly ($F_{1,126} = 14.61$, $p < 0.001$, $\omega^2 = 0.10$, Figure 2A). The major protist groups in the L and F/H layers differed significantly as well ($F_{1,126} = 12.44$, $p < 0.001$, $\omega^2 = 0.08$, Figure 2B). Alveolata, Sarcomastigota, and Archaeplastida were more abundant in the L layer (Appendix 4), while Rhizaria, Excavata, and Hacrobia were more abundant in the F/H layer (Appendix 5).

Table 1: Reads and ASVs of all eukaryotic (all eukaryotes) and protist phyla (protists) amplified by PCR of 128 samples from Southern Ecuador. For protists unidentified reads, Fungi, Metazoa and Streptophyta were filtered out. Percentages based on total numbers of combined samples of unfiltered eukaryote data and filtered protist data, respectively.

			all eukaryotes		protists	
Phylum	**# of reads**	**# of ASVs**	**read %**	**ASV %**	**read %**	**ASV %**
Unikonts						
Euglenozoa						
Euglenozoa	1,031	13	0.02	0.13	0.04	0.30
Excavata						
Loukozoa	48	1	< 0.01	0.01	< 0.01	0.02
Metamonada	78	2	< 0.01	0.02	< 0.01	0.05
Percolozoa	2,112	13	0.04	0.13	0.08	0.30
Sarcomastigota						
Amoebozoa	212,636	530	4.23	5.11	8.49	12.13
Choanozoa	24,166	129	0.48	1.24	0.97	2.95
Sulcozoa	1,786	10	0.04	0.10	0.07	0.23
Opisthokonta						
Metazoa	1,176,044	1,837	23.42	17.72		
Fungi	727,698	2,004	14.49	19.33		
Bikonts						
Harosa						
Incertae Sedis - Harosa	368	9	0.01	0.09	0.01	0.21
Stramenopiles						
Bigyra	69,013	456	1.37	4.40	2.76	10.44
Ochrophyta	4,719	38	0.09	0.37	0.19	0.87
Alveolata						
Pseudofungi	3,508	23	0.07	0.22	0.14	0.53
Ciliophora	119,160	354	2.37	3.41	4.76	8.10
Miozoa	760,123	723	15.14	6.97	30.36	16.55
Rhizaria						
Cercozoa	1,242,675	1,625	24.74	15.67	49.64	37.19
Hacrobia						
Cryptista	281	5	0.01	0.05	0.01	0.11
Haptophyta	927	14	0.02	0.14	0.04	0.32
Heliozoa	4,130	33	0.08	0.32	0.16	0.76
Picozoa	5,504	56	0.11	0.54	0.22	1.28
Archaeplastida						
Viridiplantae						
Chlorophyta	46,632	306	0.93	2.95	1.86	7.00
Streptophyta	116,976	214	2.33	2.06		
Biliphyta						
Rhodophyta	4,530	29	0.09	0.28	0.18	0.66
Unidentified						
No blast hit	497,797	1,944	9.91	18.75		

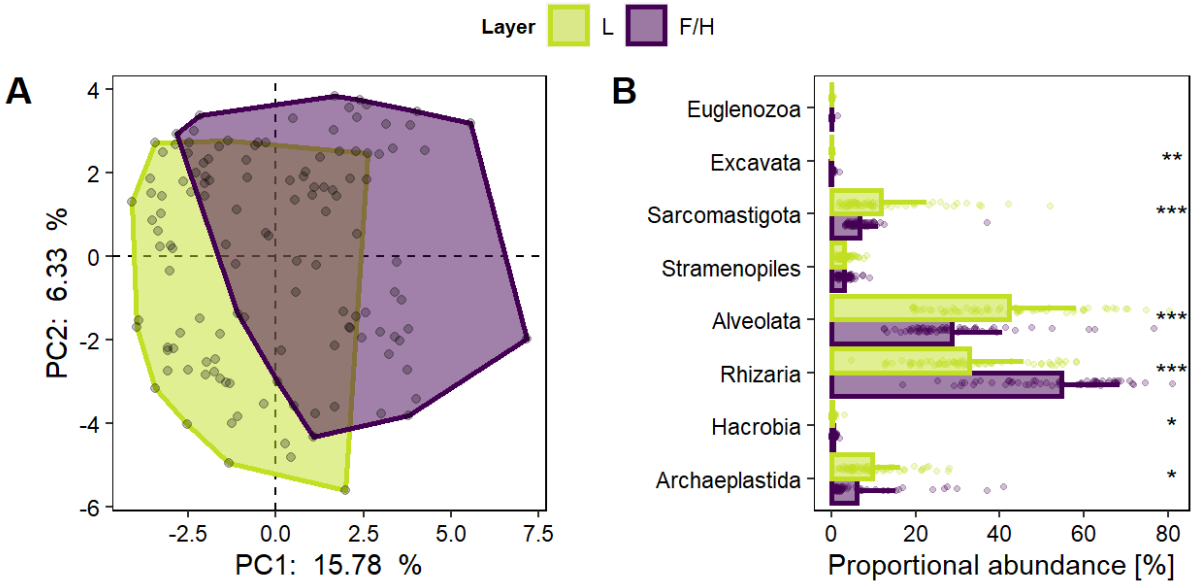


Figure 2: Differences between the L and F/H layer of (A) read abundances of all protists visualised as PCA and (B) proportional abundances of major protist groups. Asterisks mark significant differences in proportional abundances between layers (t-test with $p \leq 0.01$ = **, $p \leq 0.001$ = ***).

Efficacy of experimental treatments

Data exploration of the three experimental treatments - N addition, rotation and defaunation - revealed that only N addition and defaunation were successful (Figure 3A and 3C). N addition increased N concentration in both layers (L layer: $F_{1,62} = 13.53$, $p < 0.001$, $\omega^2 = 0.16$; F/H layer: $F_{1,62} = 8.08$, $p = 0.006$, $\omega^2 = 0.1$). Defaunation reduced mesofauna abundance in both layers (L layer: $F_{1,62} = 27.17$, $p < 0.001$, $\omega^2 = 0.28$; F/H layer: $F_{1,62} = 17.86$, $p < 0.001$, $\omega^2 = 0.21$). However, there was no effect of rotation in either layer as measured by the concentration of the AMF marker (Figure 3B). Rather, the AMF marker concentration showed a roughly bimodal distribution in both layers, suggesting an uneven distribution of AMF, not influenced by the rotation of the microcosms. Therefore, we retained the rotation treatment as an independent variable but included the concentration of the AMF marker as covariate in the following statistical analyses to test for possible effects on protists.

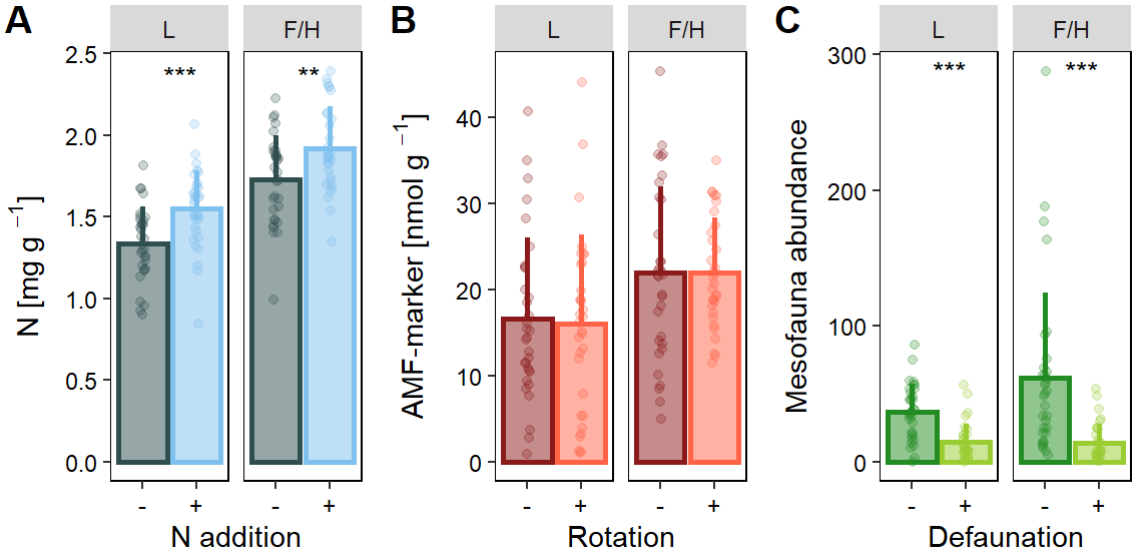


Figure 3: Effects of (A) N addition, (B) rotation and (C) defaunation measured as N concentration, concentration of the AMF marker NLFA 16:1ω5 and mesofauna abundance, respectively; - control, + treatment. Asterisks mark significant differences within layers (t-test; $p \leq 0.01$ = **, $p \leq 0.001$ = ***).

Variation in community composition between layers

In the L layer, neither ASV richness nor any of the diversity indices varied significantly within treatments (Appendices 2 and 3). In the F/H layer, ASV richness increased by N addition ($F_{1,56} = 19.90$, $p \leq 0.001$, $\omega^2 = 0.23$) and defaunation ($F_{1,56} = 4.17$, $p = 0.046$, $\omega^2 = 0.05$). The Chao1 index increased by N addition ($F_{1,56} = 21.67$, $p \leq 0.001$, $\omega^2 = 0.24$) and defaunation ($F_{1,56} = 4.68$, $p = 0.035$, $\omega^2 = 0.05$) as well. In the L layer, the protist community was significantly affected by N addition and defaunation, while in the F/H layer the AMF marker concentration also affected the protist community, whereas rotation was neither significant in the L nor in the F/H layer (Figure 4). In both layers, N addition explained most of the variation in the relative community abundance data (78 % and 72 % for the L and F/H layer, respectively) and had overall a large effect size ($\omega^2 = 0.76$ and $\omega^2 = 0.70$, respectively). Defaunation explained 14 % and 61 % of the variation in the respective layer but had a rather low effect size ($\omega^2 = 0.10$) in the L layer compared

to the F/H layer (ω2 = 0.58). The concentration of the AMF marker showed a significant effect in the F/H layer, explaining 11 % of the variation, but with a small effect size (ω2 = 0.08), and was non-significant in the L layer.

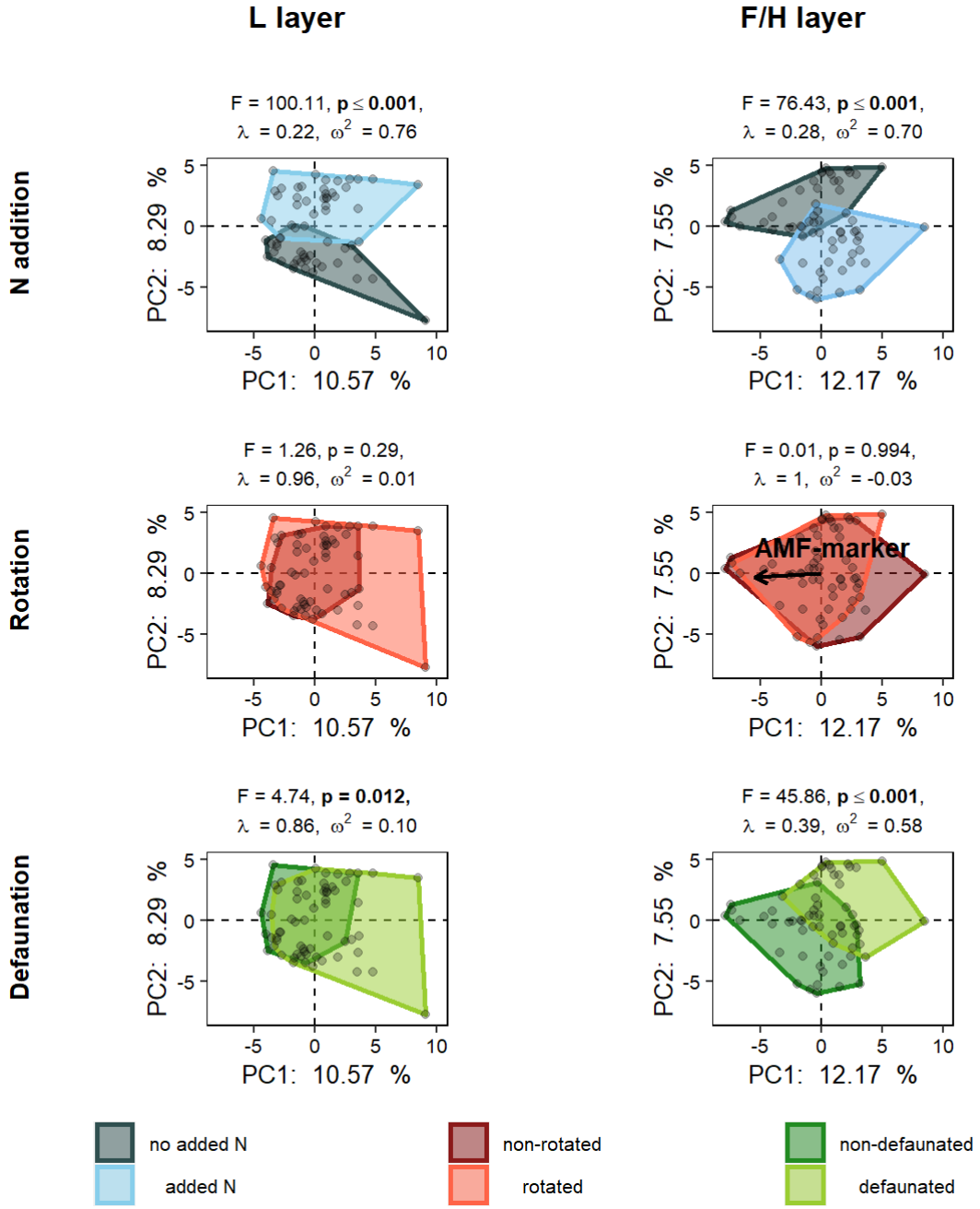


Figure 4: Principle component analysis (PCA) on the effects of the treatments N addition (blue), rotation (green) and defaunation (red) on protist communities in the L layer (left) and F/H layer (right). The significant effect (λ = 0.89, $F_{2,58}$ = 3.67, p = 0.032, ω2 = 0.08) of the covariate AMF marker concentration is indicated as black arrow. Dark colour indicates control, light colour indicates treatment.

Distribution of trophic groups between layers

Three of the five trophic groups of protists differed in their clr-transformed relative abundances between the L and F/H layer ($F_{1,127} = 23.77$, $p < 0.001$, $\omega^2 = 0.15$; Figure 5).

Phagotrophs showed a higher proportional abundance in the F/H layer (79 % compared to 63 % in the L layer). The top ten ASVs in this layer could be ascribed exclusively to Glissomonadida, while in the L layer few Cercomonadida ASVs appeared in the middle and upper ranks. The majority of Glissomonadida belonged to Allapsidae and Sandonidae, bacterivorous groups.

Photoautotrophic protists showed a higher relative abundance in the L layer (5 % compared to 2 % in the F/H layer). The top ten ASVs in the L layer were ascribed to green algae (Chlorophyta) and comprised eight Trebouxiophyceae, one Chlorophyceae and one Ulvophyceae. The following algae could be further identified to genus level: *Elliptochloris*, *Coccomyxa*, *Pseudococcomyxa*, *Viridirella*, *Printzina*, *Lobosphaeropsis* and *Leptosira*. In the F/H layer more Chlorophyceae ASVs (*Chlorosarcinopsis*, *Coccomyxa*) as well as a brown algae ASV (Phaeophyceae) appeared in the middle ranks.

Animal parasites showed a higher relative abundance in the L Layer (31 % compared to 18 % in the F/H layer). The top ten animal parasitic ASVs belonged exclusively to Gregarines. In the L layer *Gregarina* and *Ascogregarina* could be further identified, and in the F/H layer additionally *Paraschneideria*.

Neither mixotrophs nor plant parasites showed differences in their relative abundances between layers (both 2 %). The mixotrophs comprised Ochrophyta (Stramenopiles), Haptophyta and Cryptista (Hacrobia), and Miozoa (Alveolata). The plant parasites comprised mostly Plasmodiophorida (Endomyxea) and fewer numbers of Peronosporales (Oomycota).

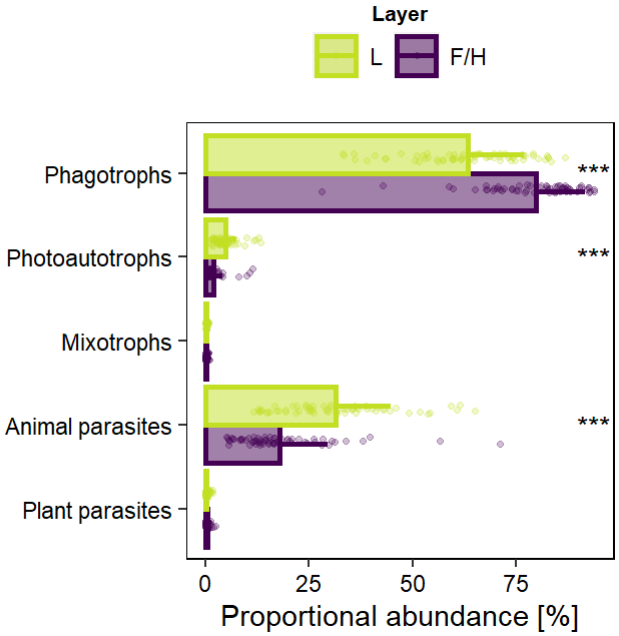


Figure 5: Distribution of trophic groups of protists in the L and F/H layer based on clr-transformed read counts, shown as proportional abundances. Asterisks mark significant differences between layers (t-test with $p \leq 0.001$ = ***).

Treatment effects on trophic groups

N addition: In the L layer N addition affected phagotrophs, mixotrophs, animal parasites and plant parasites. The relative abundance of phagotrophs and animal parasites increased by 3.0 % and 0.1 %, the relative abundance of mixotrophs decreased by 4.2 % (Figure 6A-C), whereas that of plant parasites generally decreased 61.1 %. However, the strong decrease of the relative abundance of plant parasite varied with defaunation (N addition × defaunation interaction; $F_{1,58} = 5.27$, $p = 0.024$) and was mainly due to the very high relative abundance of plant parasites in the defaunated treatment without N addition (Figure 6D). In the F/H layer N addition affected phagotrophs, mixotrophs and plant parasites. The proportional abundance of phagotrophs and mixotrophs increased by 8.2 % and 40.0 %, plant parasites decreased by 46.7 % (Figure 6E-G).

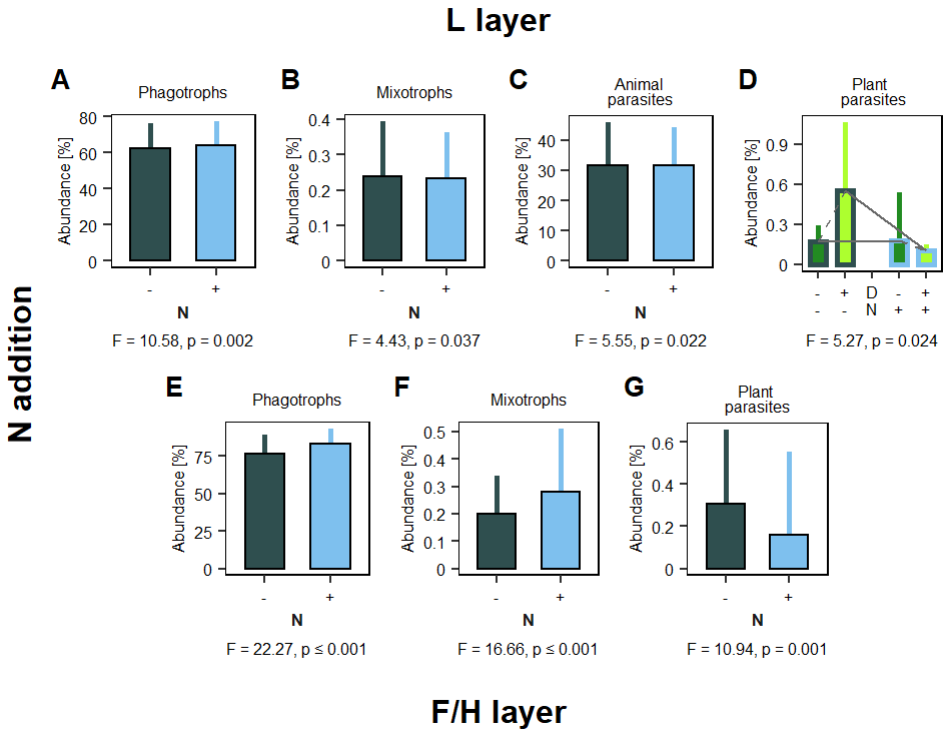


Figure 6. Effects of N addition (control = -, N addition = +) on the proportional abundance of trophic groups of protists in the L (top) and F/H layer (bottom). Panel D shows the interactive effects of N addition (N; control = -, N addition = +) and defaunation (D; non-defaunated = -, defaunated = +) on the relative abundance of plant parasitic protists in the L layer.

Based on the ranked clr-transformed read counts, the top ten phagotrophic ASVs in the L layer without N addition could be ascribed to ASVs of the families Glissomonadida and Cercomonadida, both Cercozoa, but were mixed through the ranks. Additionally, a Colpodellida ASV (Miozoa) occurred in the middle and lower ranks. In the L layer with N addition Glissomonadida also dominated the upper ranks while the Cercomonadida filled the lower ranks. A Spongomonadida ASV occurred in the lower ranks as well, while the Colpodellida disappeared.

In the F/H layer the phagotrophs in microcosms with and without N addition were dominated exclusively by Glissomonadida of the families Allapsidae, Cercomonadidae and

Sandonidae. Although their proportional abundances differed between treatments, the order of their ranks did not.

The top ten mixotrophic ASVs in the L layer without N addition could be ascribed to members of three superphyla, i.e. Alveolata, Hacrobia and Stramenopiles. In the L layer without N addition members of Chrysophyceae Clade C dominated the top and middle ranks, with *Rufusiella* (Dinophyceae) second and *Isochrysis* (Prymnesiophyceae) third, while a further *Rufusiella* ASV and Prymnesiphyceae (Clade C1 and *Algiosphaera*) filled the lower ranks. In the L layer with N addition, no Hacrobia were in the top ten ranks. ASVs ascribed to members of Chrysophyceae Clade C and *Paraphysomonas* Clade F dominated virtually all upper and middle ranks. A *Rufusiella* ASV ranked first while a different *Rufusiella* together with other Dinophyceae filled the lower ranks.

Of the mixotrophs in the F/H layer without N addition a member of Clade C of the Chrysophyceae ranked highest, followed by Cryptomonodales (Cryptophyceae) and *Rufusiella* (Dinophyaceae). The middle ranks were filled by *Ochromonas* (Chrysophyseae) and Cryptomonodales ASVs (Cryptophyceae), while the lower ranks were filled by members of the Prymnesiophyceae (*Isochrysis*, *Algiosphare* and an ASV of Clade C1). With N addition, Cryptophyceae were reduced in diversity and filled the lower ranks. By contrast, Chrysophyceae increased in diversity with more members of Clade C dominating the top ranks, but also the middle and lower ranks were filled by the genus *Paraphysomonas* Clade F. The Dinophyceae filled the upper ranks as well.

The top ten animal parasitic ASVs in the L layer without N addition could be exclusively ascribed to Gregarines. In the L layer with N addition, an ASV ascribed to the phylum Perkinsozoa entered the lower ranks but could not be further identified.

The top ten plant parasitic ASVs in both layers could be ascribed to mostly Phytomyxea and few Oomycota. Phytomyxea comprised *Spongospora*, *Woronia* and *Plasmodiophora* with other not further identified Plasmodiophorida. The Oomycota comprised the two Peronosporales *Phytium* and *Phytophthora*.

In the microcosms of the L layer without N addition *Spongospora* dominated the top ranks, followed by *Plasmodiophora* and further unidentified Plasmodiophorida ASVs, with *Pythium, Woronia* and *Phytophthora* in the lower ranks. With fertilisation *Woronia* dominated the top rank in the L layer. *Woronia* actually increased in proportional abundance, while the other Phytomyxea where reduced but kept the same order in their ranks as in the non-fertilised ingrowth cores. The Oomycota increased in their proportional abundance with N addition as well.

In the plant parasites of the F/H layer Plasmodiophora dominated the top ten ranks as in the L layer. However, with N addition *Woronia* appeared in the middle and lower ranks. Only one ASV of the Oomycota and *Phytophthora* (Peronosporales), occurred in the middle ranks without N addition and in the lower ranks with N addition.

Rotation and AMF marker concentration: Although the rotation did not affect the relative abundance of protists, the concentration of the AMF marker did. In the L layer, with increasing AMF marker concentration, the relative abundance of mixotrophs decreased, whereas in the F/H layer the relative abundance of photoautotrophs increased and the relative abundance of plant parasites decreased (Figure 7A-C).

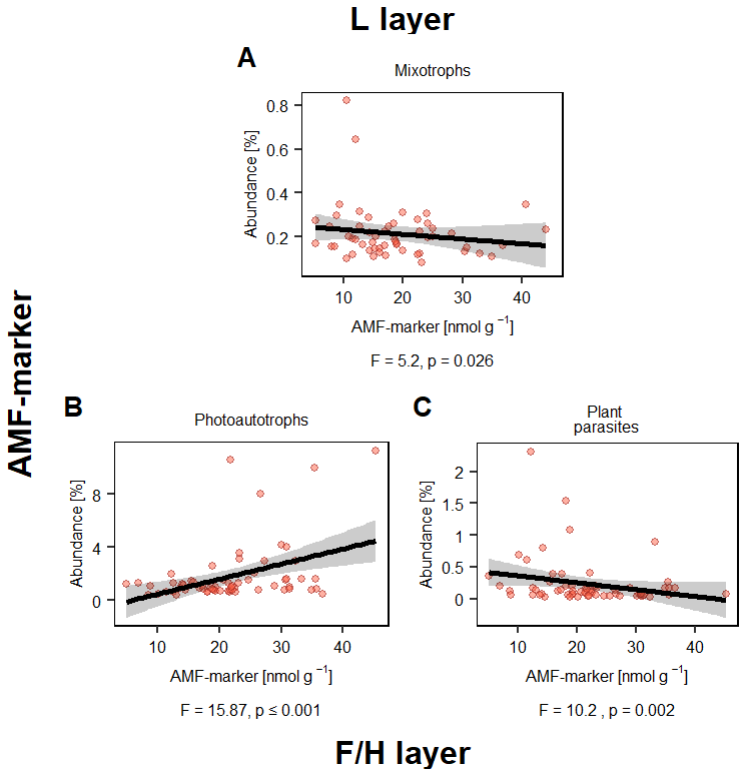


Figure 7: Significant correlations of the concentration of the AMF marker with the relative abundance of trophic groups of protists in the L (top) and F/H layer (bottom).

Defaunation: In the L layer, with decreasing mesofauna abundance, i.e. in the defaunated microcosms, the relative abundance of phagotrophs and plant parasites increased by 21.9 % and 94.1 %, while the relative abundance of animal parasites decreased by 32.0 % (Figure 8A, B). However, the increase of plant parasites varied with N addition (N addition × defaunation interaction; $F_{1,58} = 5.27$, $p = 0.024$; Figure 6D). In the F/H layer defaunation increased the relative abundance of phagotrophs by 18.5 %, while the animal parasites decreased by 49.8 % (Figure 8C, D).

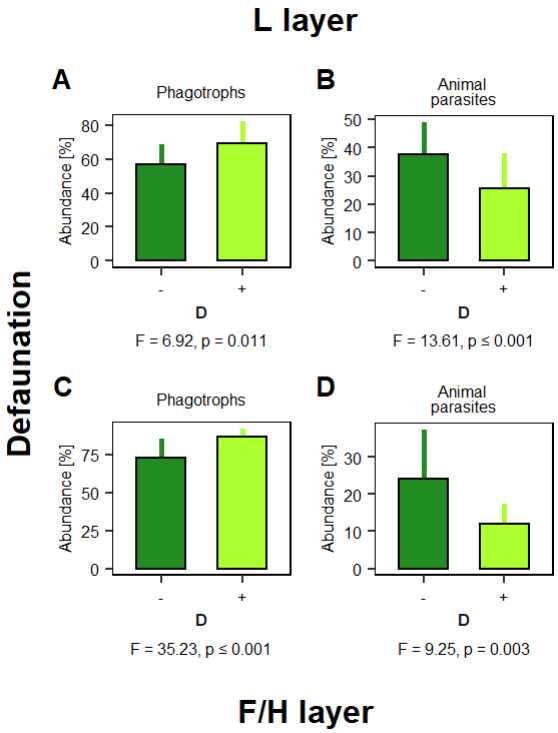


Figure 8: Significant effects of defaunation (control = -, defaunated = +), i.e. reduction of mesofauna, on the relative abundance of trophic groups of protists in the L (top) and F/H layer (bottom).

Based on the ranked clr-transformed read counts, the top ten phagotrophic ASVs in the L layer without defaunation, i.e. with mesofauna, could be ascribed virtually exclusively to members of the Cercozoa. Several Glissomonadida of the family Allapsidae filled the top ranks, while not further identifiable Glissomonadida, but also ASVs of the genus *Cercomonas* (Cercomonadida) as well as two Colpodellida (Miozoa) filled the middle and lower ranks. With defaunation, i.e. reduced mesofauna, another Glissomonadida family, Sandonidae including the genus *Sandona*, occupied the top ranks. Allapsidae and Cercomonadidae mixed in the middle and lower ranks, while contrasting microcosms without defaunation both Colpodellida were not in the top ranks.

The top ten phagotrophic ASVs in the F/H layer without defaunation could be ascribed to a mix of Glissomonadida (Sandonidae, Allapsidae as well as not further identifiable

Glissomonadida) with the addition of an ASV ascribed to Variosea (Amoebozoa) and an apicomplexan Colpodellida (Miozoa). With defaunation Sandonidae dominated the top ranks but also appeared in the lower ranks. Allapsidae filled the middle ranks, while the ASVs ascribed to Variosea and Colpodellida disappeared from the top ranks.

The top ten ASVs of animal parasites in the L layer could be ascribed exclusively to Gregarines. Some ASVs could be further identified to the genera *Gregarina* and *Ascogregarina*. In the non-defaunated microcosms, i.e. with mesofauna, the *Gregarina* dominated the upper ranks, while *Ascogregarina* appeared in the lower ranks. With defaunation the animal parasites kept their order of ranks, i.e. no changes in dominance, but changed in the relative abundance of the individual ASVs.

The animal parasites in the F/H layer kept their order of ranks but changed in their individual proportional abundance, as in the L layer. However, an ASV could be ascribed to the genus *Paraschneideria* and appeared in the lower ranks, while the *Ascogregarina* appeared in the middle ranks.

The top ten plant parasitic ASVs in the L layer without defaunation could be ascribed to a mixture of Phytomyxea (Cercozoa) and Oomycota (Pseudofungi), but exclusively to Phytomyxea in microcosms with defaunation. All Phytomyxea could further be identified as Plasmodiophorida, including the genera *Spongospora* and *Woronia*. The Oomycota could further be identified as *Pythium* and *Phytophthora* of the Peronosporales. All families were well mixed in the non-defaunated microcosms, while in the defaunated microcosms no Oomycota were present in the top ranks. Instead, additional ASVs of the genus *Woronia* appeared in the upper and middle ranks. However, a single *Spongospora* dominated clearly the top ranks.

Table 4: Effects of N addition, rotation and defaunation as independent factors as well as the concentration of the AMF marker as covariate on the abundance of trophic groups of protists in the L and F/H layer (PERMANOVAs, 10,000 replicates). Note that only the interaction between defaunation and N addition was included as interactions with rotation (as well as rotation as main factor) were generally not significant.

		L layer			F/H layer		
Trophic group	**Treatment**	**$F_{(1,58)}$**	**p**	**ω^2**	**$F_{(1,58)}$**	**p**	**ω^2**
Phagotrophs	N addition	10.58	**0.002**	0.13	22.27	**< 0.001**	0.25
	Rotation	0.04	0.843	-0.02	2.11	0.149	0.02
	Defaunation	6.92	**0.011**	0.08	35.23	**< 0.001**	0.35
	AMF-marker	0.02	0.9	-0.02	2.93	0.097	0.03
	Defaunation x N addition	0.27	0.595	-0.01	< 0.01	0.998	-0.02
Photoautotrophs	N addition	1.58	0.209	0.01	1.1	0.3	0.01
	Rotation	< 0.01	0.995	-0.02	0.75	0.388	0.01
	Defaunation	0.67	0.413	-0.01	0.8	0.379	0.01
	AMF-marker	0.18	0.67	-0.01	15.87	**< 0.001**	0.19
	Defaunation x N addition	1.39	0.242	0.01	3.78	0.058	0.04
Mixotrophs	N addition	4.43	**0.037**	0.05	16.66	**< 0.001**	0.2
	Rotation	0.42	0.527	-0.01	0.72	0.393	0.01
	Defaunation	3.92	0.056	0.04	1.53	0.225	0.01
	AMF-marker	5.2	**0.026**	0.06	0.95	0.336	0.01
	Defaunation x N addition	0.61	0.431	-0.01	0.7	0.397	0.01
Animal parasites	N addition	5.55	**0.022**	0.07	0.19	0.664	-0.01
	Rotation	0.48	0.498	-0.01	0.69	0.417	0.01
	Defaunation	13.61	**< 0.001**	0.16	9.25	**0.003**	0.11
	AMF-marker	2.06	0.153	0.02	0.26	0.618	-0.01
	Defaunation x N addition	1.47	0.238	0.01	0.02	0.889	-0.02
Plant parasites	N addition	12.36	**< 0.001**	0.15	10.94	**0.001**	0.13
	Rotation	0.84	0.366	0.01	< 0.01	0.954	-0.02
	Defaunation	7.87	**0.005**	0.1	1.66	0.208	0.01
	AMF-marker	0.28	0.599	-0.01	10.2	**0.002**	0.13
	Defaunation x N addition	5.27	**0.024**	0.06	0.59	0.445	-0.01

Discussion

We investigated the response of protist communities to increased N deposition, a limiting macroelement in montane ecosystems (Camenzind et al., 2018), similar to that forecasted by anthropogenic change scenarios (Fabian et al., 2005; Homeier et al., 2012). We also investigated the role of concentrations of arbuscular mycorrhizal fungi (AMF), common fungi associated with plants in tropical montane rainforests (Treseder and Cross, 2006), on protist communities. Furthermore, we investigated the possible effects of the reduction of mesofauna, important soil arthropods (Maraun et al., 2008), on protist communities. To our knowledge this study is the first employing HTS for investigating protist communities in the tropical montane rainforests of Southern Ecuador. In general, 44.2 % of the total number of 5,021,942 reads could be ascribed to 4.369 protist ASVs, resembling figures from studies on other tropical regions (Mahé et al., 2017; de Araujo et al., 2018), but being higher than those from studies of temperate regions (Venter et al., 2017). Most studies on protists focus on the mineral soil ignoring organic layers despite that the density and diversity of microorganisms and animals in organic layers typically exceeds that in the mineral soil (Krashevska et al., 2008; Maraun et al., 2008). To account for the vertical distribution of protist communities, we analysed protist communities of the upper and lower part of the organic layer.

Community composition

Of the three treatments, N addition had the strongest impact, e.g. large effect size and high explained variance, on the protist communities in both layers. The low amount of N added (50 kg N ha^{-1} y^{-1} applied twice per year) resembling the atmospheric anthropogenic input at our study region highlights the sensitivity of microorganisms to nutrient inputs at this nitrogen-limited ecosystem (Homeier et al., 2012; Camenzind et al., 2018). Both, ASV richness and Chao1 index increased with N addition, reflecting an overall increase in

protist diversity, but this was restricted to the F/H layer. This lack of effect in the L layer might have been due to the accumulation of the added N in the F/H layer 7 years after the N addition treatment in the NUMEX experiment started. The Shannon index and the inverse Simpson index was high in both layers irrespective of the addition of N, indicating species-rich protist communities that differ in abundance but not in species diversity.

Although the rotation of the microcosms did not reduce the concentration of the AMF marker NLFA 16:1ω5 in the microcosms, we detected a weak but significant effect of the concentration of the AMF marker on the protist communities in the F/H layer, where it explained 11 % of the variance in the relative abundance data. This is in line with recent studies showing direct or indirect effects of AMF on protists (Koller et al., 2013; Krashevska et al., 2014; Bukovská et al., 2018; Henkes et al., 2018). AMF mostly suppress protists and other microorganisms, however, in a context dependant way. To facilitate N capture AMF depend on the microbial loop, in which protists as grazers of bacteria play a crucial role (Bonkowski, 2004; Jansa et al., 2019). Presumably, AMF suppress only selected groups of protists excluding e.g., bacterial grazers, to improve N capture.

The reduction of mesofauna abundance in the defaunation treatment affected protist communities in both layers but the effect in the F/H layer was less pronounced than that of N addition. Generally, the effect was weak in the L layer with a small effect size and only 14 % of explained variance in the relative abundance data, it was much stronger in the F/H layer with a large effect size and 61 % of explained variance. In the F/H layer the reduced mesofauna abundance was associated with an increased ASV richness and Chao1 index of protist communities, while the Shannon index and the inverse Simpson index were not affected. This mirrored the effect of N addition, i.e. increased abundance of protists but no effect on the community composition. Lower abundance in the non-

defaunated treatments with higher numbers of mesofauna might be due to mesofauna preying on protists, but also an indirect effect via reduction of mesofauna as consumers of nematodes (de Ruiter et al., 1995; Rusek, 1998; Crotty et al., 2012). Alphei et al. (1996) showed experimentally interactions between nematodes, earthworms and protists, whereas nematodes increased the abundance of flagellates but decreased the abundance of amoebae. In our study the mesofauna comprised Collembola and Acari (Sánchez-Galindo et al., 2019), including groups preying on nematodes, such as Uropodina (Koehler, 1997) but also Oribatida (Heidemann et al., 2011). Reduction of mesofauna likely increased nematode abundances, which in this case was advantageous for protists and increased generally their abundance. While defaunation reduced the mesofauna in both layers, it was stronger in the F/H layer (Sánchez-Galindo et al., 2019), suggesting a more pronounced influence of mesofauna on protists in the deeper F/H layer compared to the upper L layer.

Trophic groups

To explore possible effects of the treatments on protists in terms of their function, we assigned ASVs to trophic groups including phagotrophs, photoautotrophs, mixotrophs, and animal and plant parasites. Although the general distribution of the trophic groups of protists showed differences in the relative abundance between the L and F/H layers for three of the five groups, i.e. phagotrophs, photoautotrophs and animal parasites, the treatment effects are discussed in detail for each affected trophic group of each layer separately.

N addition: In both layers, N addition affected phagotrophs, mixotrophs, and plant parasites, while in the L layer animal parasites were also affected. The relative abundance of phagotrophs and animal parasites increased by N addition, while the relative

abundance of plant parasites decreased. The relative abundance of mixotrophs decreased slightly in the L layer, but increased in the F/H layer by N addition.

The addition of N likely benefitted bacteria, increasing the food resource of grazing protists and thereby their relative abundance. This has been shown for phagotrophic protists in a greenhouse experiment (Xiong et al., 2018) as well as in agricultural fields (Schulz et al., 2019). While the addition of N may benefit phagotrophic protists in general, Zhao et al. (2019) showed that only certain taxa may indeed benefit, while others might be detrimentally affected. Typically, in N addition experiments higher amounts of N have been applied than in our study, highlighting the sensitivity of microbial communities of montane rainforests to changes in nutrient inputs. Interestingly, the relative abundance of phagotrophs was higher in the F/H than in the L layer, which contrasts bacteria as indicated by phospholipid fatty acid markers (Sánchez-Galindo et al., 2019). As the most abundant ASVs in both layers were ascribed to bacterivorous groups, such as the glissomonads Allapsidae and Sandonidae (Howe et al., 2011), this suggests that phagotrophs actually exert a stronger top-down pressure on bacteria in the F/H than in the L layer. This might be a species specific effect of this glissomonads (Glücksman et al., 2010). However, not all phagotrophs benefitted from the addition of N addition, which might be related to the fact that they not only include bacterivores but also predators. While an ASV of the Colpodellida, predatory flagellates preying on other protists (Simpson and Patterson, 1996), was among the most abundant phagotrophs in the L layer without N addition, it disappeared from the predominant phagotrophs in the treatments with N addition. This suggests that either their food source, mostly algae, benefited from the N addition and were thus better able to defend themselves against predation by

Colpodellida, or the N addition had directly a negative effect on this predatory phagotrophs.

Mixotrophs can switch trophic strategies depending on the availability of nutrients, i.e. bacteria and other protists as well as the availability of light (Liu et al., 2016). As discussed above, N addition likely increased the abundance of bacteria also serving as prey for mixotrophs. However, the addition of N only increased the relative abundance of mixotrophs in the F/H layer, whereas it was reduced in the L layer. In the F/H layer mixotrophs necessarily rely little on photosynthesis but need to predominantly live as phagotrophs. Therefore, similar arguments for the beneficial effects of N addition as for phagotrophs may apply, but again, their response likely varies among taxa. Different species of *Ochromonas* thrive in dark environments if bacterial prey is present (Sanders et al., 2001), while other species like *Isochrysis* employs phagotrophy to survive in the absence of light, but cannot grow properly relying on bacteria alone (Anderson et al., 2018). Some Chrysophyceae like *Paraphysomonas* grow better if, in addition to prey, nutrients are added (Sin et al., 1998). This suggests that the addition of N had not only increased bacterial prey and thereby indirectly influenced mixotrophic protists but in addition beneficially affected the growth of individual mixotrophs directly by increasing nutrient availability.

Animal parasites in the L layer increased slightly in relative abundance with N addition. Virtually all dominant ASVs were gregarines, one member of Perkinsozoa occurred in samples with N addition. Gregarines are parasites of virtually all major arthropod lineages including mites and collembolans (Chen, 1999; Clopton, 2009), while Perkinsozoa infect marine molluscs but also amphibians in freshwater environments (Mangot et al., 2011; Gleason et al., 2014). As the animal parasites could not further be identified than phylum

or class level, interpretation is rather limited. Information on wet environments such as rainforests are scarce too.

Plant parasites in both layers decreased in relative abundance with N addition, with a stronger effect in the L than the F/H layer. They comprised mostly Plasmodiophorida (Cercozoa, Endomyxea) and low numbers of Peronosporales (Pseudofungi, Oomycota). Plasmodiophorida have a wide variety of hosts, such as vascular plants, green and brown algae, diatoms, but also other plant parasites, e.g. Oomycota (Bulman and Braselton, 2014). Peronosporales are a diverse group of mostly plant parasites with narrow to wide host spectra (Beakes et al., 2014; Thines, 2014). Both groups are well known from arable systems, where they play a significant role as pathogens (Adam et al., 2017). In such systems fertilisation with N can reduce their abundance (Zhao et al., 2019). This is likely due to the positive effects of N on their hosts improving defence (Mur et al., 2016). Mittelstraß et al. (2006) showed that low rather than high amounts of N fertiliser improved plant resistance to *Phytophthora* (Peronosporales), resembling the effect of low N addition in our study. Overall, the effect of the N addition was strongest in plant parasites among all the trophic groups of protists, underscoring that the impact of indirect effects on microbial communities in low nutrient ecosystem.

However, in the litter layer N addition and defaunation had an interactive effect on the plant parasites. Mesofauna can influence nitrogen availability and mobility in litter (Kandeler et al., 1999; Scheu et al., 2005). This might indicate that the amount of nitrogen released by the mesofauna had a positive effect on the hosts of the plant parasites, comparable to the positive effect of N addition on plant defence. Conversely, the nitrogen released by the mesofauna was missing in the defaunated microcosms and the plant parasites could more easily infest their hosts, resulting in increased relative abundance.

N addition overpowered any positive effects of defaunation for plant parasites, and reduced their relative abundance below the initial level in the control treatments.

Rotation: By rotating of the microcosms every second day we expected to strongly reduce colonization of the microcosms by AMF hyphae. Contrasting this assumption, rotation did not reduce AMF. Presumably, this is due to a different growth form of AMF in tropical forests compared to temperate systems where AMF form extensive exploitative mycelia extending away from roots to capture nutrients in soil (Phillips et al., 2013). In montane rainforests AMF may predominantly stay closely attached to roots (Camenzind and Rillig, 2013), which in lieu develop an extensive network in the litter layer (Kottke et al., 2004). Despite the failure to manipulate AMF via rotation of the microcosms the amount of AMF, as measured by the concentration of the AMF marker NLFA 16:1ω5 in the microcosms, varied widely allowing to explore associated variations in protist communities by using the concentration of the AMF marker as covariate in our statistical models. Doing that we assumed that AMF hyphae were still active within the microcosms potentially in a saprotrophic stage (Azcón-Aguilar and Barea, 1995). The significant correlation with the overall abundance of protists in the F/H layer, as well as with mixotrophs in the L layer and photoautotrophs and plant parasites in the F/H layer suggest that protist community composition in fact varies with AMF abundance. We expected, that reduced AMF abundance would be beneficially affect protists in general. Contrasting this assumption, the effect on the relative abundance of individual trophic groups of protists differed and varied between layers. In the L layer the relative abundance of mixotrophs increased with reduced concentration of the AMF marker and the same was true for plant parasites in the F/H layer. By contrast, the abundance of photoautotrophs decreased with reduced concentration of the AMF marker. AMF are important for P and N acquisition of plants,

which is mediated by interactions of AMF with bacteria and protists (Bonkowski et al., 2019; Jansa et al., 2019). It has been shown that AMF can suppress not only specific bacteria but also protists such as *Acanthamoeba* (Bukovská et al., 2018; Henkes et al., 2018). However, *Acanthamoeba* as bacterivorous phagothrophs were not affected by changes in the concentration of the AMF marker in our experiment, but the negative effect of AMF on mixotrophs in the L layer may have been linked to the phagotrophic phase of these protists. Overall, the results based on correlations between protists and concentrations of the AMF marker fatty acid need to be interpreted with caution as not only hyphae but also spores of AMF contain high amounts of the marker fatty acid (Olsson, 1999) complicating their interpretation.

Defaunation: Reduction of mesofauna affected phagotrophs and animal parasites in both layers, as well as plant parasites in the L layer. The relative abundance of phagotrophs and plant parasites increased with reduction of mesofauna, while the relative abundance of animal parasites was reduced. This suggests, that phagotrophic protists thrive in the absence of mesofauna, such as Oribatida and Collembola, potentially functioning as competitors for food in particular by feeding on bacteria (Ngosong et al., 2009). Although defaunation resulted in a more pronounced reduction in the mesofauna in the F/H than in the L layer, the effect on phagotrophs was somewhat less pronounced. This may have been due to the fact that there were more predatory protists, e.g. Colpodellidae, in the F/H than in the L layer, which likely had not benefited from the reduction in bacterivorous mesofauna.

The reduction of the relative abundance of animal parasites is most likely linked to the reduction of host organisms. The higher relative abundance of animal parasites in the L than the F/H layer suggests that the majority of hosts did not belong to mesofauna, which

was more abundant in the F/H than the L layer (Sánchez-Galindo et al., 2019). This suggests, that rather than mites and collembolans but other invertebrates comprised the majority of hosts for the animal parasites, which mainly comprised gregarines.

Unexpectedly, the relative abundance of plant parasites increased with the reduction of mesofauna abundance in the L layer. Presumably, mesofauna also consumed hosts of plant parasitic protists such as algae thereby reducing of algal parasites. However, mesofauna may also directly feed on plant parasitic protists.

Conclusions

We showed that the protist communities in tropical montane rainforests of Southern Ecuador are taxonomically but also trophically complex responding sensitively to an even moderate increase in N input as well as variations in mesofauna abundance and concentrations of the AMF marker fatty acid. N addition strongly affected virtually all trophic groups of protists, highlighting the susceptibility of microbial food webs to human disturbances. Reduced mesofauna abundance resulted in increased relative abundance of phagotrophs, presumably competing with mites and collembola for bacterial food, as well as reduced relative abundance of animal parasites. Although our treatment of reducing AMF abundance by rotating the microcosms was not successful, significant correlations between the AMF marker fatty acid and photoautotrophic, mixotrophic and plant parasitic protists suggest that at a wide range of protist trophic groups closely interact with AMF. While the experimental treatments typically affected trophic groups of protists in the L and F/H layer in a similar way, some trophic groups differentially responded in the two layers suggesting that protist communities needs to be studied across layers to fully understand their role in ecosystem functioning as well as their response to environmental changes.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Appendix Chapter 3

Appendix 1: Assignment of protists to trophic groups.

Infrakingdom	Phylum	Subphylum	Class	Trophic Group
Euglenozoa	Euglenozoa	Euglenoida	Euglenida	Photoautotroph
		Kinetoplastea	Kinetoplastida	
Excavata	Loukozoa	Malawimonadea	Malawimonadida	
	Metamonada	Fornicata		
	Percolozoa	Heterolobosea		
Sarcomastigota	Amoebozoa	Amoebozoa		
		Conosa	Mycetozoa_Dictyostelea	
			Mycetozoa_Myxogastrea	
			Variosea	
		Lobosa	Lobosa_G1	Phagotroph
			Lobosa	
			Tubulinea	
	Choanozoa	Choanoflagellatea	Acanthoecida	
			Choanoflagellida	
			Craspedida	
		Cristidiscoidea	Nucleariida	
		Filasterea	Capsasporida	
		Ichthyosporea	Ichthyosphonida	Animal parasite
			Rhynosporida	
	Sulcozoa	Glissodicea	Planomonadida	Phagotroph
		Thecomonadea	Apusomonadida	
Harosa	Incertae Sedis - Harosa	Telonemea	Telonemia	
Stramenopiles	Bigyra	Bicoecea	Anoecales	Phagotroph
			Bicoecales	
			Borokales	
			Pseudodendromonadales	
		Bicosoecea	Rictida	
		Bigyromonadea		
		Labyrinthulea	Labyrinthulales	Animal parasite
			Thraustochytriales	
		MAST	MAST_1	Phagotroph
			MAST_11	
			MAST_12	
			MAST_14	
			MAST_22	
			MAST_3	Animal parasite
			MAST_6	Phagotroph
			MAST_7	
			MAST_8	

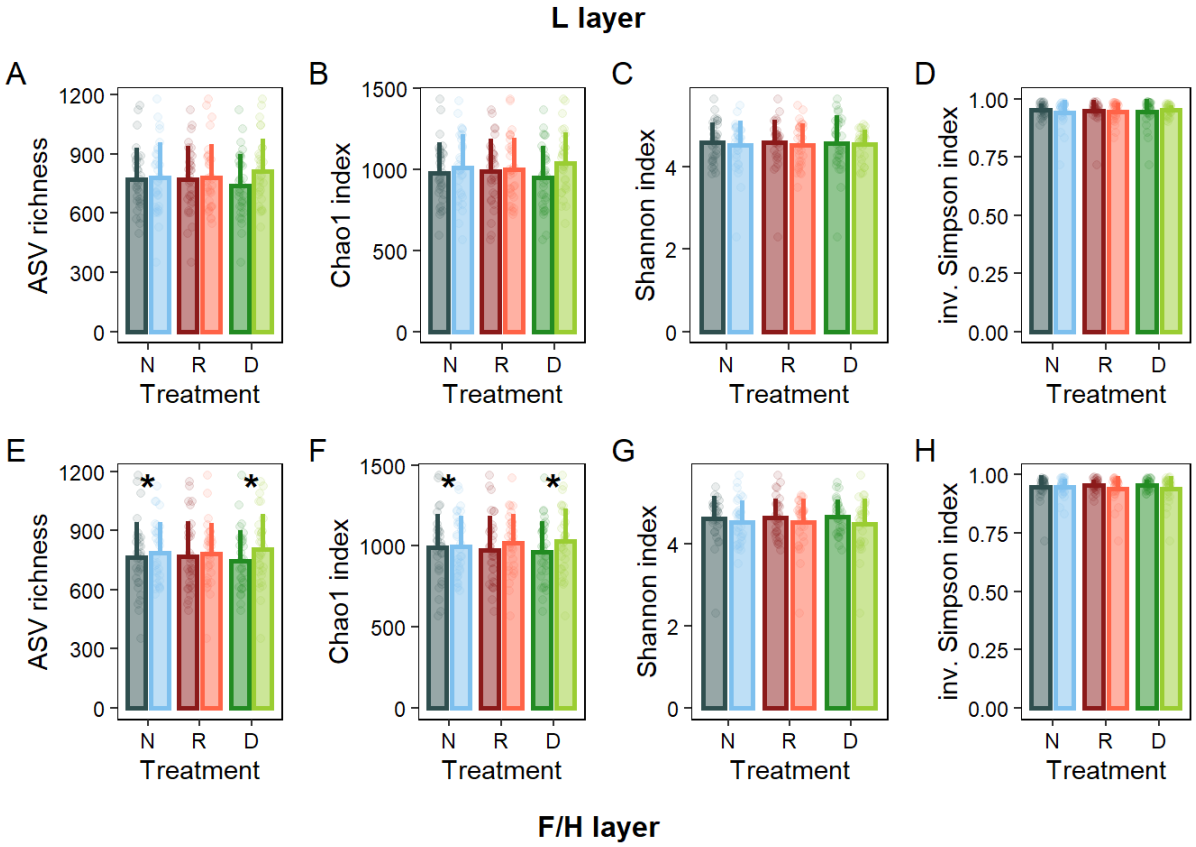
	Ochrophyta	Bacillariophyta		Photoautotroph
		Chrysophyceae		
		Eustigmatophyceae		
		Phaeophyceae		
		Stramenopiles_X_Group_6		
		Synurophyceae	Synurales	
	Pseudofungi	Oomycota		Plant parasite
Alveolata	Ciliophora	Colpodea		Phagotroph
		CONThreeP		
		Heterotrichea		
		Karyorelictea		
		Litostomatea	Haptoria_6	
			Haptoria_7	
			Rhynchostomatia	
			Trichostomatia	
		Nassophorea		
		Oligohymenophorea	Astomatia	Animal parasite
			Hymenostomatia	
			Peritrichia_2	Phagotroph
			Scuticociliatia_1	Animal parasite
			Scuticociliatia_2	
		Phyllopharyngea	Cyrtophoria_4	Phagotroph
			Suctoria	
		Plagiopylea		
		Spirotrichea	Euplotia	
			Hypotrichia	
			Strombidiida	
	Miozoa	Apicomplexa	Coccidia	Animal parasite
			Colpodellidae	Phagotroph
			Gregarines	Animal parasite
			Haemosporida	
			Piroplasmida	
		Colponemea	Colponemida	Phagotroph
		Dinophyceae	Gymnodiniales	Mixotroph
			Prorocentrales	
		Perkinsea	Perkinsida	Animal parasite
Rhizaria	Cercozoa	Endomyxa	Vampyrellida	Plant parasite
		Endomyxa_Phytomyxea	Plasmodiophorida	
		Filosa		Phagotroph
		Filosa_Granofilosea	Cryptofilida	
			Desmothoracida	
			Limnofilida	
		Filosa_Imbricatea	Euglyphida	
			Marimonadida	

			Spongomonadida	
			Thaumatomonadida	
		Filosa_Sarcomonadea	Cercomonadida	
			Glissomonadida	
			Pansomonadida	
		Filosa_Thecofilosea	Cryomonadida	
			Ebriida	
			Tectofilosida	
			Ventricleftida	
		Novel_clade_10_12	Novel_clade_10	
			Novel_clade_12	
			Tremulida	
		Phaeodarea	Phaeogromida	
Hacrobia	Cryptista	Cryptophyceae		Mixotroph
		Leucocryptea	Katablepharida	Phagotroph
	Haptophyta	Prymnesiophyceae	Calcihaptophycidae	Mixotroph
			Isochrysidales	
			Prymnesiales	
	Heliozoa	Centrohelea	Acanthocystida	Phagotroph
			Centroheliozoa	
			Pterocystida	
	Picozoa			
Archaeplastida	Chlorophyta	Chlorophyceae	Chlamydomonadales	Photoautotroph
			Sphaeropleales	
		Mamiellophyceae	Mamiellales	
		Trebouxiophyceae	Chlorellales	
			Ctenocladales	
			Microthamniales	
			Phyllosiphonales	
			Prasiolales	
			Watanabea_Clade	
		Ulvophyceae	Trentepohliales	
			Ulotrichales	
	Rhodophyta	Bangiophyceae	Bangiales	
		Compsopogonophyceae	Erythropeltidales	
		Florideophyceae	Batrachospermales	
			Ceramiales	
			Corallinales	
		Rhodellophyceae	Glaucosphaera_Clade	
			Rhodellales	

Appendix 2: ANOVA table of F-, p- and ω^2-values for the effects of N addition, rotation, defaunation and the concentration of the AMF marker (as covariate) on diversity indices (ASV richness, Chao1, Shannon, inverse Simpson) of protist communities in the L and F/H layer.

		L layer			F/H layer		
Index	**Treatment**	**$F_{(1,56)}$**	**p**	**ω^2**	**$F_{(1,56)}$**	**p**	**ω^2**
ASV richness	N addition	< 0.01	0.961	-0.02	19.90	**< 0.001**	0.23
	Rotation	0.03	0.869	-0.02	0.33	0.567	-0.01
	Defaunation	3.14	0.082	0.03	4.17	**0.046**	0.05
	AMF-marker	2.25	0.139	0.02	< 0.01	0.959	-0.02
Chao1	N addition	0.42	0.518	-0.01	21.67	**< 0.001**	0.24
	Rotation	0.06	0.802	-0.01	0.28	0.596	-0.01
	Defaunation	3.54	0.065	0.04	4.68	**0.035**	0.05
	AMF-marker	1.16	0.287	< 0.01	0.04	0.834	-0.02
Shannon	N addition	0.50	0.484	-0.01	1.31	0.257	< 0.01
	Rotation	0.16	0.695	-0.01	0.58	0.450	-0.01
	Defaunation	0.16	0.694	-0.01	0.62	0.435	-0.01
	AMF-marker	0.62	0.434	-0.01	0.03	0.863	-0.02
inverse Simpson	N addition	0.50	0.483	-0.01	0.24	0.627	-0.01
	Rotation	< 0.01	0.963	-0.02	< 0.01	0.979	-0.02
	Defaunation	3.07	0.085	0.03	0.14	0.709	-0.01
	AMF-marker	0.11	0.747	-0.01	0.21	0.647	-0.01

Appendix 3: Effects of N addition (N), rotation (R), and defaunation (D)on ASV richness (A, E), Chao1 index (B, F), Shannon index (C, G) and inverse Simpson index (D, H) of protist communities in the L layer (upper row) and F/H layer (lower row); dark shading = without treatment, light shading = with treatment; asterisks mark significant differences.



Appendix 4: Reads and ASVs of all eukaryotic (all eukaryotes) and protist phyla (protists) amplified by PCR of 64 samples of the L layer from Southern Ecuador. For protists unidentified reads, Fungi, Metazoa and Streptophyta were filtered out. Percentages based on total numbers of combined samples of unfiltered eukaryote data and filtered protist data, respectively.

			all eukaryotes		protists	
Phylum	**# of reads**	**# of ASVs**	**read %**	**ASV %**	**read %**	**ASV %**
Unikonts						
Euglenozoa						
Euglenozoa	362	6	0.01	0.06	0.04	0.15
Excavata						
Loukozoa	7	1	< 0.01	0.01	< 0.01	0.02
Metamonada	75	2	< 0.01	0.02	0.01	0.05
Percolozoa	315	12	0.01	0.13	0.03	0.30
Sarcomastigota						
Amoebozoa	124,086	490	4.98	5.30	12.71	12.24
Choanozoa	4,324	119	0.17	1.29	0.44	2.97
Sulcozoa	930	9	0.04	0.10	0.10	0.22
Opisthokonta						
Metazoa	888,139	1,698	35.62	18.36		
Fungi	326,086	1,837	13.08	19.87		
Bikonts						
Harosa						
Incertae Sedis - Harosa	156	7	0.01	0.08	0.02	0.17
Stramenopiles						
Bigyra	28,805	435	1.16	4.70	2.95	10.86
Ochrophyta	1,171	33	0.05	0.36	0.12	0.82
Alveolata						
Pseudofungi	749	18	0.03	0.19	0.08	0.45
Ciliophora	46,981	327	1.88	3.54	4.81	8.17
Miozoa	377,722	645	15.15	6.98	38.7	16.11
Rhizaria						
Cercozoa	347,807	1,483	13.95	16.04	35.63	37.04
Hacrobia						
Cryptista	77	3	< 0.01	0.03	0.01	0.07
Haptophyta	326	14	0.01	0.15	0.03	0.35
Heliozoa	2,036	29	0.08	0.31	0.21	0.72
Picozoa	843	54	0.03	0.58	0.09	1.35
Archaeplastida						
Viridiplantae						
Chlorophyta	35,891	290	1.44	3.14	3.68	7.24
Streptophyta	56,386	183	2.26	1.98		
Biliphyta						
Rhodophyta	3,402	27	0.14	0.29	0.35	0.67
Unidentified						
No blast hit	246,925	1,525	9.90	16.49		

Appendix 5: Reads and ASVs of all eukaryotic (all eukaryotes) and protist phyla (protists) amplified by PCR of 64 samples of the F/H layer from Southern Ecuador. For protists unidentified reads, Fungi, Metazoa and Streptophyta were filtered out. Percentages based on total numbers of combined samples of unfiltered eukaryote data and filtered protist data, respectively.

			all eukaryotes		protists	
Phylum	**# of reads**	**# of ASVs**	**read %**	**ASV %**	**read %**	**ASV %**
Unikonts						
Euglenozoa						
Euglenozoa	669	13	0.03	0.15	0.04	0.33
Excavata						
Loukozoa	41	1	< 0.01	0.01	< 0.01	0.03
Metamonada	3	1	< 0.01	0.01	< 0.01	0.03
Percolozoa	1,797	13	0.07	0.15	0.12	0.33
Sarcomastigota						
Amoebozoa	88,550	452	3.50	5.12	5.8	11.44
Choanozoa	19,842	120	0.78	1.36	1.30	3.04
Sulcozoa	856	9	0.03	0.10	0.06	0.23
Opisthokonta						
Metazoa	287,905	1,304	11.39	14.78		
Fungi	401,612	1,711	15.88	19.40		
Bikonts						
Harosa						
Incertae Sedis - Harosa	212	6	0.01	0.07	0.01	0.15
Stramenopiles						
Bigyra	40,208	427	1.59	4.84	2.63	10.8
Ochrophyta	3,548	35	0.14	0.40	0.23	0.89
Alveolata						
Pseudofungi	2,759	20	0.11	0.23	0.18	0.51
Ciliophora	72,179	308	2.85	3.49	4.73	7.79
Miozoa	382,401	656	15.12	7.44	25.04	16.6
Rhizaria						
Cercozoa	894,868	1,554	35.39	17.62	58.59	39.32
Hacrobia						
Cryptista	204	5	0.01	0.06	0.01	0.13
Haptophyta	601	12	0.02	0.14	0.04	0.30
Heliozoa	2,094	20	0.08	0.23	0.14	0.51
Picozoa	4,661	56	0.18	0.63	0.31	1.42
Archaeplastida						
Viridiplantae						
Chlorophyta	10,741	217	0.42	2.46	0.7	5.49
Streptophyta	60,590	171	2.40	1.94		
Biliphyta						
Rhodophyta	1,128	27	0.04	0.31	0.07	0.68
Unidentified						
No blast hit	250,872	1,682	9.92	19.07		

General Discussion

Protist, albeit accounting for the majority of all eukaryotic life, are generally mostly overlooked (Adam et al., 2017). This is especially true for the belowground system (Geisen et al., 2020; van Elsas et al., 2019). Due to their minuscule size and opaque environment, protists in soils are virtually invisible. Nevertheless, they participate, influence or control important functions of the soil, e.g. mineralisation, decomposition, and nutrient availability (Geisen et al., 2018). Furthermore, protists impact and interact with other organisms, such as bacteria, plants and animals, but also with other protists (Bonkowski et al., 2019; Gao et al., 2018).

The overall aim of this thesis was to assess diversity and function of protists in tropical soils, identifying environmental factors affecting them and to investigate possible interactions with other soil organisms. I approached this large group of organisms in two ways. In Chapter 1, I focused on the morphology of a single genus of testate amoebae in the Ecuadorian Andes, *Trigonopyxis.* I showed that the form of the pseudostome of its shell is useful for species delineation, whereas the size of the shell and the diameter of the pseudostome are variable, since the latter are both influenced by temperature and humidity, rather indicating changing environmental conditions. Although the detailed analysis of an individual taxon results in a plethora of important information about form, function and possible interactions with other soil organisms and environmental factors, it is far too time-consuming and unfeasible for analysing multiple sites and considering a wide range of protists. In Chapters 2 and 3, I used therefore high-throughput sequencing (HTS) of environmental DNA to assess whole protist communities. In Chapter 2, I showed that transformation of natural rainforest systems of the tropical lowlands in Sumatra, Indonesia, into rubber and palm oil plantations causes differential shifts in trophic groups

of protists, being beneficial for some groups but detrimental for others. Land-use transformation changed environmental conditions, affecting soil protists directly, e.g. increased light availability in plantations, or indirectly via detrimental effects on potential hosts or prey. The HTS approach was well suited for assessments and comparison of protists communities of different environments. In Chapter 3, returning to the Ecuadorian Andes, I used an experimental approach to look deeper into interactions of protists in the belowground system, exploring the importance of nutrients as well as possible competitors. Tropical soils show a stronger limitation in nutrient availability compared to soils of temperate regions, fostering competition and interactions in the soil system. I employed microcosms to manipulate nitrogen (N), arbuscular mycorrhizal fungi (AMF) and mesofauna across two soil horizons, showing how susceptible individual taxonomic and trophic groups of protists are with regard to disturbances and the complexity of the belowground system.

Morphological variation

The classical and for a long-time exclusive approach to assess nearly all protist taxa was first optical and later scanning electron microscopy (Honigberg et al., 1964; Levine et al., 1980). Morphological features are often the only characteristics suitable for identification of taxa in the group of testate amoebae (Clarke, 2003). In contrast to other species with stable morphology, the genus *Trigonopyxis* within the order Arcellinida with the type species *Trigonopyxis arcula* is commonly seen as morphologically highly variable (Bobrov & Mazei, 2004; Swindles et al., 2014). To identify reliable characteristics for morphology-based taxon delineation I investigated variations in shell size, pseudostome diameter and pseudostome form in *T. arcula* from three different sites of the Ecuadorian Andes, where these characters vary even more than previously described. The shell size, usually used

for taxon delineation (Mazei and Tsyganov 2006), is of limited value for characterizing taxa, if taken alone, as it varies markedly with environmental conditions such as temperature and humidity. However, shell size uniformly increases with decreasing temperature and might therefore be used as indicator for changes in environmental conditions in the recent past, but also in palaeoecological studies, given a solid sample record. I showed that, despite commonly assumed to be variable, the pseudostome form in the genus *Trigonopyxis* can be used to delineate five distinct morphotypes. However, I suggest not to separate different taxa of *Trigonopyxis* until molecular evidence is available, but rather to view them as different morphotypes of *T. arcula*, as suggested earlier (Bobrov et al. 1995). One potential exception arguably is *T. leidyi* with its unique pseudostome form. However, due to the variability in this character and the co-occurrence of a specific morphotype I refrained from considering *T. leidy* as a separate taxon but see it as an extreme variation of this morphotype. Again, molecular evidence is needed for clarification. Therefore, the genus *Trigonopyxis* may best be considered comprising a single polymorphic species named *T. arcula*. However, thorough morphological characterization allows distinguishing different morphotypes with varying response to changes in environmental conditions, e.g. along altitudinal gradients. To establish the validity of the five identified morphotypes of the genus *Trigonopyxis* molecular approaches are needed. This was not yet possible due to strong contamination of the available samples with fungi, preventing amplification of DNA sequences. Additionally, this is further limiting an incorporation of the detailed knowledge of this individual taxon into the larger scoped analyses of Chapters 2 and 3. Overall, this study not only provided an overview of the morphological variability of the genus *Trigonopyxis*, but serves as first step of a possible re-description and revision of the whole taxon.

Shifts in trophic groups

Instead of focusing on individual taxa, I employed high-throughput sequencing of environmental DNA to assess changes in relative abundance, species richness, and community structure of protist communities with conversion of tropical lowland rainforest into rubber agroforest (jungle rubber), and rubber and oil palm plantations; typical agricultural systems in Sumatra, Indonesia. A total of 4,219 operational taxonomic units (OTUs) were assigned to five trophic groups of protists: phagotrophs (52 %), animal parasites (29 %), photoautotrophs (12 %), plant parasites (1 %) and symbionts (<1 %). The results suggest that overall protist species richness is only reduced in rubber plantations compared to the other land-use systems. By contrast, the community structure of protists is strongly affected by the conversion of rainforest into plantation systems. The relative abundance and in part also the species richness of phagotrophs, photoautotrophs and symbionts increased due to conversion of rainforest into plantation systems, whereas both relative abundance and species richness of animal and plant parasites declined. I linked these changes to individual factors for every trophic group. Phagotrophs were presumably mainly driven by changes in soil pH and an increased abundance of Gram-positive bacteria, photoautotrophs by light availability, and both groups of parasites by the abundance and species richness of their hosts. Overall, the results show that species richness, relative abundance and community composition of individual trophic groups of protists in tropical lowland rainforest significantly differ from those in converted ecosystems, which likely is associated with changes in ecosystem functioning. The study provides novel insights into protist communities and their changes with land-use intensity in tropical lowland ecosystems. I showed that trophic groups of protists are powerful indicators reflecting changes in the functioning of converted ecosystems. Notably, within trophic groups individual taxa generally responded in a

similar way, suggesting that trophic groups of protists reflect general patterns in changes in the structure of the micro-decomposer food web with conversion of rainforest into plantation systems. Compared to the approach of Chapter 1, light and scanning electron microscopy, HTS was a well working tool in assessing protists on a community level and, indeed, made the study possible in the first place. However, without the knowledge of individual taxa and groups the identified protists could not be brought into an ecological context. Detailed analyses of single protists are probably needed more than before, due to improved access of their opaque environment on a community level.

Differential response to nutrient availability

The effects of elevated nitrogen levels on a functionally important group of the belowground system such as protists are still poorly understood, especially in nutrient-limited systems like the tropical Andes. As in Chapter 2, I used HTS of environmental DNA of the litter (L) and fermentation/humus (F/H) layers from field installed microcosms to explore how increased nitrogen (N) input affects protists in a tropical montane rainforest in Southern Ecuador. Arbuscular mycorrhizal fungi as well as mesofauna are possible competitors for, but also sources of, nutrients for protists and were manipulated in their abundance as well. A total of 4,369 amplicon sequence variants (ASVs) could be ascribed to six major protist groups. Alveolata, Sarcomastigota and Archaeplastida were more abundant in the upper L layer, while Rhizaria, Excavata and Hacrobia were more abundant in the lower F/H layer. Assignment to trophic groups showed a strong dominance of phagotrophs (L layer: 63 %, F/H layer: 79 %) and animal parasites (31 %, 18 %) over photoautotrophs (5 %, 2 %), plant parasites (both <2 %) and mixotrophs (both <2 %). The protist communities were taxonomically but also trophically complex and responded sensitively to an even moderate increase in N input as well as variations

in AMF concentration and mesofauna abundance. Nitrogen had the strongest effect in both layers, while reduced mesofauna abundance had a stronger effect in the lower F/H layer compared to the upper L layer. Changes in AMF concentration had the lowest impact on protists. In both layers, additional N increased the relative abundance of phagotrophs and animal parasites but decreased the relative abundance of plant parasites, whereas the relative abundance of mixotrophs decreased in the L layer but increased in the F/H layer, highlighting the susceptibility of microbial food webs to disturbances. While predatory phagotrophs were negatively affected either directly through N input or indirectly via positive effects of N on their prey, the majority of phagotrophs were bacterivores and benefitted strongly from an increased abundance of bacteria. Mixotrophic protists were likely outcompeted by their phagotrophic counterparts in the L layer, but responded positively to increased N amounts in the lower F/H layer, where mixotrophs cannot use photosynthesis but rely on nutrients as energy source. While the effects on animal parasites remained elusive due to broader identification to class level and limited data on animal parasites in this environment, plant parasites were negatively affected due to positive effects of N on their hosts, strengthening host defence against parasites. In the L layer, with higher AMF concentration, the relative abundance of mixotrophs decreased, while in the F/H layer the relative abundance of photoautotrophs increased and that of plant parasites decreased, suggesting that a wide range of protist trophic groups interacts with AMF. Generally, AMF are important for nutrient acquisition of plants, which is mediated by interactions of AMF with bacteria and protists. Although AMF can suppress not only specific bacteria but also protists, the positive effects on photoautotrophs remain elusive. However, these interpretations need to be taken with care as they are based on correlations of the AMF marker fatty acid NLFA 16:1ω5, which is not only present in active AMF hyphae but also in

spores. With reduced mesofauna abundance, the relative abundance of phagotrophs increased and that of animal parasites decreased in both layers, while the relative abundance of plant parasites increased only in the L layer. As phagotrophs thrive in the absence of certain mesofauna taxa, such as mites and collembolans, this suggests that they compete for the same microbial food. The reduction of animal parasites is most likely linked to a reduction of probable hosts. The unexpected effect on the relative abundance of plant parasites in the L layer suggests that the mesofauna either feeds on parasitized host plants, such as algae, or feeds directly on the zoospores of plant parasites. While the experimental treatments typically affected trophic groups of protists in the L and F/H layer in a similar way, some trophic groups differentially responded in the two layers, suggesting that protist communities need to be studied across layers to fully understand their role in ecosystem functioning as well as their response to environmental changes.

While Chapter 2 demonstrated that the soil protist community can be assessed altogether, the experimental approach of Chapter 3 highlighted the complexity of the soil system. Protists are a very diverse group of organisms and part of many different processes in the soil. Depending on the scope, general conclusions can be drawn from soil samples as in Chapter 2. A closer look, separating even physically close layers of the belowground system as in Chapter 3, revealed a more detailed but increasingly complex picture of the protist community and their interactions with other organisms in this systems.

Different methods for analysing protist communities

While I highlighted in Chapter 1 the importance of thorough morphological analysis of an individual taxon, Chapters 2 and 3 focused on the assessment of whole protist communities. Chapter 1 shows the importance of a time-consuming but thorough morphological analysis of individual protist taxa. Although current studies use mostly

molecular techniques for analysing protist communities, they need previously acquired knowledge, e.g. by morphological analysis, to link it to information based on DNA sequences and to achieve meaningful conclusions in a, for example, ecological context. Therefore, it is important to employ these time-consuming methods, as they form the basis for interpreting results based on state-of-the-art molecular methods. In the case of *T. arcula* single gene sequencing is needed to allow such a link. On the other hand, Chapters 2 and 3 resemble each other in using HTS to analyse whole protist communities in soils of tropical regions. However, methods in the field of HTS develop at a fast pace. Since the methods employed in Chapter 2 were used in our laboratory for the first time, I decided to use established methods for the analysis. For identification of sequences, we used the in 2007 implemented SILVA database, comprising bacteria, archaea and eukaryotes (Pruesse et al., 2007). However, as the primary focus of this database is bacteria, a proportion of protist sequences could not be identified. I used OTUs as the smallest unit, i.e. sequences with 97 % identity were considered as belonging to one species, probably lumping together different species (Blaxter et al., 2005). Rarefaction was used to cope with the strong imbalance in number of reads between the samples (Hughes & Hellmann, 2005). For this procedure, reads are randomly discarded until all samples have the same number of reads as in the sample with the lowest number of reads. In this study, the reads were reduced to 2,331 per sample, although the maximum number of reads per sample was 90,526 with a mean of 25,346 reads per sample. This is a high loss of information and is therefore the main critique of this method (McMurdie & Holmes, 2014), but is seen as necessary to prevent a skewed statistical analysis. All three mentioned points, selection of database for sequence identification, as well as the methods of species determination and normalization, were modified in Chapter 3. I chose the PR^2 database for sequence identification. Initiated in 2010, this database has a strong

focus on mostly single-celled eukaryotes and is curated and annotated by experts (Guillou et al., 2013). This reduces the amount of unidentified protists sequences. Instead of OTUs I used ASVs to increase the identification precision of protists, allowing a more detailed view on the protist communities (Callahan et al., 2017). An alternative to the high-loss normalization by rarefaction are transformations. To account for the compositional nature of the data, I chose the centred log-ratio transformation, where the geometric mean of the sample is used as the reference, to transform each sample into an unbound space, allowing any statistical analysis to be used (Aitchison, 1982; Pawlowsky-Glahn & Egozcue, 2006). However, neither of these improvements is invalidating the approach of Chapter 2 rather pointing out how fast this field of research is evolving. The current trend of differential abundance analysis will likely increase depth and accuracy of these analyses (Lin & Peddada, 2020), but is not without critique (Quinn et al., 2021).

Unambiguously, methods will further improve, allowing analyses with higher precision and increased accuracy. However, to employ them efficiently solid background information and curated databases are important to gain new knowledge of protists, linking them to functions and processes in soils.

Overall conclusions

In this thesis, I highlighted that the time-consuming process of analysing individual protist taxa using classical methods might be of even greater importance with the growing use of modern molecular methods, as molecular methods allow access to the heterogeneous soil system on a larger scale but are ill suited to explore other characteristics of protists, needed for ecological interpretations. In the particular case of *Trigonopyxis* I showed that the pseudostome form, rather than shell size and shell diameter commonly used for testate amoebae, are appropriate characteristics for taxa delineation. The latter

characteristics are affected by environmental conditions, e.g. humidity and temperature. Although indispensable, this approach is not feasible for ecological assessments at larger scales. In that regard, HTS of environmental DNA is a solid approach to analyse whole soil protist communities as shown in Chapters 2 and 3. I showed that changes in the environment, represented by increasing land-use, cause shifts in trophic groups of protists. Effects were direct, e.g. due to increased light availability in plantations, as well as indirect, e.g. due to reduced access of potential host organisms or increased bacterial food resources. As individual taxa within trophic groups responded similarly, trophic groups of protists reflect general patterns in changes in the structure of the belowground system. However, the details of these patterns, as shown in Chapter 3, are complex due to many interactions of protists with nutrients and other soil organisms such as fungi and mesofauna. As shown in Chapter 2, especially nutrients in the form of nitrogen addition affected the soil protist community strongly, but showed differential effects depending on trophic group and soil layer, highlighting the heterogeneity of the soil system. Mesofauna abundance affected soil protists depending on the layer as well, hinting at resource competition with some protists, while mesofauna species may serve as host for other protist groups. Although I could not manipulate AMF abundance as initially planned, correlations showed positive and negative effects of AMF on different soil protist groups, indicating specific interactions between fungi and certain protist taxa. Overall, I showed that soil protists are a very heterogeneous group of organisms, interacting with many other organisms in the belowground system. Although the knowledge on protists and their functions in soils is growing steadily, many topics await further exploration. In my thesis I linked individual taxa (*Trigonopyxis*), but also whole soil protist communities to changes in environmental conditions and showed the complexity of the belowground

system. Disentangling these functions and interactions, while strenuous and time-consuming, allows us to understand the soils we live on.

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List of Publications

Schulz, G., Maraun, M., Völcker, E., Scheu, S., & Krashevska, V. (2018). Evaluation of morphological characteristics to delineate taxa of the genus Trigonopyxis (Amoebozoa, Arcellinida). *Protist*, *169*(2), 190–205. https://doi.org/10.1016/j.protis.2018.02.005

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Thesis declarations

Declaration of the author's own contribution to manuscripts with multiple authors

Chapters 2 comprises a manuscript that has been published in a peer-reviewed journal; Chapter 3 and 4 comprise manuscripts that are currently in preparation to peer reviewed journals. In all manuscripts, I am the first author; I have collected and analyzed the data, written the manuscripts, developed the main ideas, created tables, figures and appendices and contributed significantly to the study design. All co-authors contributed to interpretation and finalizing the manuscripts.

Plagiarism declaration

I declare that I have written this doctoral dissertation independently. All persons contributing to the manuscripts have been named so. All sentences or passages quoted from other people's work have been specifically acknowledged by clear cross-referencing. I have not submitted this dissertation in any form for another degree at any university or institution. I bindingly confirm that the contents of the digital version are identical with the written version.

Garvin Schulz

Göttingen, February 2022