Functional Roles of Cercozoa in the Rhizosphere and Phyllosphere of Plants

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Summary

Protists are a group of highly diverse unicellular eukaryotic organisms. They are globally distributed and inhabit all types of terrestrial and aquatic habitats. Protists represent an enormous functional diversity and occupy various ecological niches as bacterivores, fungivores, algivores, predators, primary producers, saprotrophs or parasites of plants and animals. Among protists, the Cercozoa are one of the most diverse, speciose and ecologically important of all protist phyla. Protists in terrestrial systems often show an association to plants and they are well-known predators on plant surfaces. However, a comprehensive understanding about the distribution and functions of plant-associated protists in the rhizosphere (i.e. belowground compartments of plants) and the phyllosphere (i.e. aboveground compartments of plants, mainly leaves) is lacking. Therefore this thesis aims to increase the knowledge on the diversity and functional roles of plant-associated Cercozoa.

In the first chapter several cercomonad Cercozoa strains were isolated from the phyllosphere and rhizosphere of plants from three functional groups. Their potential phyllosphere and rhizosphere as well as plant specificity was investigated and revealed that cercomonad communities show a deterministic assembly in the above- and belowground compartments of plants. During the course of this study, three novel cercomonad species were described and ten new cercomonad genotypes reported. This indicates that cercozoan taxa preferentially associated with the phyllosphere exist and that Cercozoa diversity is far from being completely revealed. The second chapter aims at deciphering the feeding preferences of leaf-associated cercomonads and their predation effects on the composition, function and interaction of phyllosphere bacterial communities. Predation-induced shifts in bacterial community composition could be linked to phenotypic protist traits and we showed that leaf-associated cercomonads significantly structured bacterial community composition which led to an altered interaction pattern among bacterial taxa. This study further demonstrated that cercomonad predation can have significant impact on the physiological function of bacterial communities. The third chapter of this thesis aimed to reveal the spatial and temporal dynamics between leaf-associated cercomonad Cercozoa and phyllosphere bacteria on leaves. We could prove that leaf-associated cercomonads are active and feeding on bacterial cells on the leaf surface and confirm the activity of protists on plant surfaces is closely connected to moisture on leaves. The last chapter aimed to increase the knowledge on the ecology and function of plant-associated cercozoan testate amoebae. Four different strains of *Rhogostoma* spp. were isolated from *Arabidopsis* leaves, agricultural soil and rhizosphere soil of *Ocimum basilicum* and *Nicotiana* sp. Detailed morphological description

for two novel *Rhogostoma* species isolated from the phyllosphere and rhizosphere is provided. The potential ingestion of bacteria, algae and fungi was investigated, providing indications on how the Rhogostomidae also prey on other (co-isolated) members of the phyllosphere microbiome.

Zusammenfassung

Protisten sind eine Gruppe von höchst diversen einzelligen eukaryotischen Organismen. Sie sind weltweit verbreitet und bevölkern alle Arten von terrestrischen und aquatischen Habitaten. Protisten zeigen eine enorme funktionelle Vielfalt und besetzen unzählige ökologische Nischen als Bakterivoren, Fungivoren, Algivoren, Prädatoren, Primärproduzenten, Saprobionten oder Parasiten von Pflanzen und Tieren. Unter den Protisten sind die Cercozoa eine der vielfältigsten, spezifischsten und ökologisch wichtigsten aller Protisten Phyla. Protisten in terrestrischen Systemen zeigen häufig eine Assoziation zu Pflanzen und sind bekannte Prädatoren auf Pflanzenoberflächen. Es fehlt jedoch ein umfassendes Verständnis über die Verteilung und die Funktionen von pflanzenassoziierten Protisten in der Rhizosphäre (d. h. die unterirdischen Kompartimente von Pflanzen) und der Phyllosphäre (d. h. die oberirdischen Kompartimente von Pflanzen, hauptsächlich Blätter). Diese Arbeit zielt daher darauf ab, das Wissen über die Diversität und funktionellen Rollen von pflanzenassoziierten Cercozoa zu erweitern.

Im ersten Kapitel wurden mehrere Cercomonaden aus der Phyllosphäre und Rhizosphäre von Pflanzen aus drei funktionellen Gruppen isoliert. Die potentielle Phyllosphären-, Rhizosphären- und Pflanzenspezifität wurde untersucht und zeigte, dass Cercomonadengemeinschaften eine deterministische Zusammensetzung in den ober- und unterirdischen Kompartimenten von Pflanzen aufweisen. Im Verlauf dieser Studie wurden drei neue Cercomonaden-Arten beschrieben und zehn neue Cercomonaden-Genotypen berichtet, was darauf hinweist, dass Cercozoen die bevorzugt mit der Phyllosphäre assoziiert sind existieren und dass die Diversität der Cercozoa bei weitem nicht vollständig offenbart ist. Das zweite Kapitel zielt darauf ab, die Ernährungspräferenzen von blattassoziierten Cercomonaden und deren Prädationseffekte auf die Zusammensetzung, Funktion und Interaktion von Phyllosphären-Bakteriengemeinschaften zu entschlüsseln. Räuberinduzierte Veränderungen in der Zusammensetzung bakterieller Gemeinschaften konnten mit phänotypischen Protistenmerkmalen in Verbindung gebracht werden und wir zeigten, dass blattassoziierte Cercomonaden die bakterielle Gemeinschaftszusammensetzung signifikant strukturiert , was zu einem veränderten Interaktionsmuster zwischen bakteriellen Taxa führte. Diese Studie zeigte weiterhin, dass die Cercomonaden Prädation erhebliche Auswirkungen auf die physiologische Funktion von Bakteriengemeinschaften haben kann. Das dritte Kapitel dieser Arbeit zielte darauf ab, die räumliche und zeitliche Dynamik zwischen blattassoziierten Cercomonaden und Phyllosphärenbakterien auf Blättern aufzuzeigen. Wir konnten nachweisen, dass blattassoziierte Cercomonaden aktiv auf der Blattoberfläche bakterielle Zellen fressen und bestätigen, dass die Aktivität von Protisten auf Pflanzenoberflächen eng

mit der Feuchtigkeit auf den Pflanzenblättern verbunden ist. Das letzte Kapitel zielte darauf ab, das Wissen über die Ökologie und Funktion pflanzenassoziierter Schalenamöben der Cercozoa zu erweitern. Vier verschiedene Stämme von *Rhogostoma*-Arten wurden von *Arabidopsis*-Blättern, landwirtschaftlichem Boden und der Rhizosphäre von Basilikum (*Ocimum basilicum*) und Tabak (*Nicotiana* sp.) isoliert. Zwei neue *Rhogostoma*-Arten isoliert aus der Phyllosphäre und der Rhizosphäre wurden morphologisch detailliert beschrieben. Die potenzielle Nahrungsaufnahme von Bakterien, Algen und Pilzen wurde untersucht und lieferte Hinweise darauf, wie die Rhogostomidae auch auf andere Mitglieder des Phyllosphären-Mikrobioms Einfluss nehmen.

General Introduction

Protists

Protists or Protozoa terms a highly diverse group of unicellular eukaryotic organisms. These microscopic organisms (2-200 µm) reproduce primarily through asexual mechanisms, are ubiquitous and globally distributed (Bates et al. 2013). Protists represent the vast majority of eukaryotic diversity and inhabit all types of terrestrial and aquatic environments (Adl et al. 2012; Ekelund and Ronn 1994; Sherr and Sherr 2002). Their ability to form resting cysts make them highly adaptive to environmental changes, allowing them to survive harsh conditions (Mueller and Mueller 1970; Rivera et al. 1992). In terrestrial systems protists occur in high abundances, with up to 10^4 - 10^7 active individuals per gram dry soil (Adl and Gupta 2006) and recent estimates indicate a global existence of 70.000 - 150.000 species of which only a small fraction is yet known (De Vargas et al. 2015; Grossmann et al. 2016; Mahé et al. 2017).

For centuries, protists were classified based on morphologic similarities. Traditionally, they were divided into the four most abundant morphogroups, i.e. naked and testate amoebae, flagellates and ciliates. These morphogroups comprise organisms of fundamental different phylogeny and lifestyles (Hausmann et al. 2003) and were assigned next to plants, animals and fungi to the eukaryotic kingdom Protista (Haeckel 1866; Whittaker 1969). However, this system did not reflect the evolutionary relationships between protists, since even morphological highly similar taxa comprise heterotrophic protists and photosynthetic algae.

In the last decade molecular phylogenetics fundamentally changed protist taxonomy. By comparing the 18S small subunit of the ribosomal DNA, or “SSU rDNA”, scientists for the first time were able to reveal the true phylogenetic and evolutionary relationships among protist taxa. Based on analyses of genetic markers for phylogeny (e.g. SSU rDNA and LSU rDNA), protists are now organized in a manner that better represents their evolutionary relationships and meanwhile a general consensus in protist taxonomy has been reached (Adl et al. 2012; Adl et al. 2005; Baldauf 2008; Burki 2014; Cavalier-Smith 1998a; 1993; Levine et al. 1980).

Instead of being a separated kingdom, protists turned out to be highly para- and polyphyletic which evolved all over the eukaryotic tree of life, as illustrated in Figure 1 according to Burki (2014).

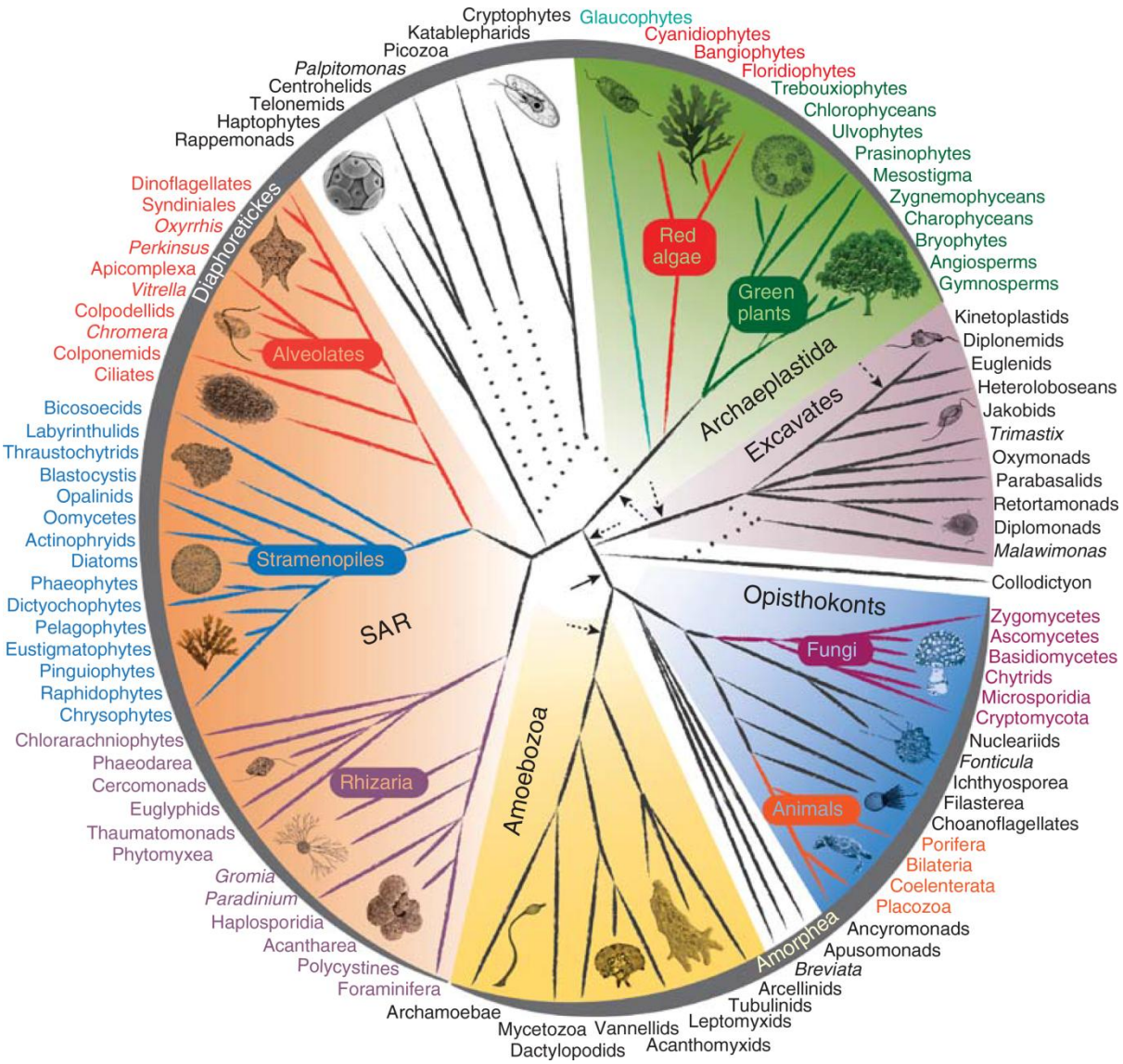

Figure 1 - Protists constitute the majority of lineages across the eukaryotic tree of life. This schematic represents a synthesis of information on morphology, phylogenetic analyses as well as phylogenomic analyses. Five “supergroups” are indicated by colored boxes, all of which contain multiple protistan lineages. Cartoons illustrate the diversity constituting the largest assemblages. The branching pattern does not necessarily represent the inferred relationships between the lineages. Dotted lines denote uncertain relationships, including conflicting positions. The arrows point to possible positions for the eukaryotic root; the solid arrow corresponds to the most popular hypothesis (Amorphea-bikont rooting), the broken arrows represent alternative hypotheses. (Extracted from Burki (2014)).

For instance, flagellates are members of the supergroups Excavata, Ophistokonta, Amoebozoa and the huge supergroup SAR (=Stramenopiles, Alveolates and Rhizaria) (Adl et al. 2012; Burki et al. 2007; Domonell et al. 2013; Ekelund et al. 2001; Finlay et al. 2000; Kang et al. 2017). However, during eukaryotic evolution several lineages evolved locomotion by amoeboid movement and lost their flagella and/ or gained or secondarily lost autotrophy, leading to the intermingled physiology and ecology of protists (Nowack 2014; Rogers et al. 2007; Stechmann and Cavalier-Smith 2002). The majority of naked amoebae belong to Amoebozoa and the remaining are members of the supergroups Excavata, Stramenopiles and Rhizaria (Adl et al. 2012; Adl et al. 2005). Testate amoebae evolved independently in at

least three different lineages, the Amoebozoa, Rhizaria and Stramenopiles (Kosakyan et al. 2016; Nikolaev et al. 2005; Wylezich et al. 2002), whereas only ciliates form a monophyletic group within the Alveolata (Lynn and Sogin 1988; Sogin et al. 1986).

Functional roles of protists in soil

Protists in soils are extremely diverse and considered to represent the major consumers of bacterial production, forming the basis of the heterotrophic eukaryotic food web that channels the energy flow via bacteria to higher trophic levels (bacterial energy channel) (Bonkowski et al. 2009; Crotty et al. 2011; De Ruiter et al. 1995; Hunt et al. 1987; Moore and Hunt 1988). However, soil protists play various roles in the soil ecosystem and represent an enormous functional versatility.

The majority of protists in soil are bacterivorous and it has been shown that predation by protists is the main source of mortality for soil bacteria, leading to the liberation of nutrients from consumed bacteria (the microbial loop in soil (Clarholm 1985)). Predation of bacterivore protists further stimulates plant productivity, by the release of nutrients from bacteria and by changes in bacterial community composition (Bonkowski and Clarholm 2012; Bonkowski 2004). Nonetheless, bacteria are not the only prey of phagotrophic soil protists. A wide range of soil protist taxa are fungal feeders and obligate and facultative mycophagous protists are common soil inhabitants (Chakraborty and Old 1982; Ekelund 1998; Geisen 2016a; Geisen et al. 2016; Petz et al. 1986). Hence protist functioning in soils is not restricted to bacterivory and soil protists also possess important functional roles in the fungal energy channel (Geisen and Bonkowski 2018; Geisen 2016a; Geisen et al. 2016). Other phagotrophic soil protists are top predators and feed on other protists and/ or algae (Dumack et al. 2016b; Jassey et al. 2012; Page 1977; Seppey et al. 2017; Smirnov et al. 2011). These top predators are not limited to single celled prey and some protists even prey on metazoan invertebrates like nematodes (Bjornlund and Ronn 2008; Geisen et al. 2015c; Ronn et al. 2012). For example the tiny but common soil protist *Cryptodifflugia operculata* is able to kill and feed on nematodes by the practice of pack hunting to slay their much larger victims (Geisen et al. 2015c).

Apart from the majority of protists being phagotrophic with a variety of prey organisms, several soil protist lineages gather energy via phototrophy, saprotrophy or parasitism. It might seem contradictory to obtain energy by phototrophy in the opaque soil environment, however, phototrophic protists (i.e. algae) in soils are numerous and diverse although they are limited to the upper part of sunlit soil (Bates et al. 2013; Geisen et al. 2015a; Grossmann et al. 2016). Phototrophic (or mixotrophic) protists are mainly associated with soil crusts and

might represent an important carbon input into soil systems (Geisen and Bonkowski 2018; Jassey et al. 2015; Schmidt et al. 2016; Seppey et al. 2017). Furthermore, together with cyanobacteria, they contribute to soil crust formation as some form mucilage (e.g. Zygnematophyceae) and thereby perform an important function for soil stabilization against erosion in drylands (Weber et al. 2016).

Recently, highthroughput sequencing (HTS) approaches performed on terrestrial habitats, revealed that saprotrophs and parasites are also common and abundant members of soil protist communities (Dupont et al. 2016; Geisen 2016b; Geisen et al. 2015a; Geisen et al. 2015b; Grossmann et al. 2016; Mahé et al. 2017; Venter et al. 2017). Among those are plant infecting protists like pathogenic oomycetes (e.g. *Phytophthora*) or plasmodiophorids in the Endomyxa. The latter group includes the causative agent of clubroot in *Brassica* species and powdery potato scab (Neuhauser et al. 2014). In addition, animal infecting taxa such as apicomplexans form a considerable proportion of protists in soil. Apicomplexa is a phylum that includes a huge diversity of obligate parasites comprising vertebrate parasites such as *Cryptosporidium*, *Plasmodium* and *Toxoplasma* as well as invertebrate parasites like Gregarines that infect predominantly Arthropods.

Altogether, functional diversity of protists in soil is enormous. Their ecological roles in the transfer of nutrients in the soil food web is likely very important. However, detailed knowledge on a wide range of species, the community compositions and taxonspecific ecological function of soil protists is largely missing.

Protists in the rhizosphere of plants

Plants are “meta-organisms” or “holobionts”, since they live in close association with a diversity of microorganisms. Host-associated microbiomes contain microorganisms that are essential for plant health and nutrition and these microbiomes maintain a continuous relationship over the lifetime of their host-plant (Berg et al. 2014).

The rhizosphere of plants is the narrow zone of soil that is influenced by root secretions and is a habitat of high microbial activities and abundances, with up to 10^{11} microorganisms per gram root (Berg et al. 2005; Egamberdieva et al. 2008; Herron et al. 2013). Furthermore, it has been demonstrated that plants are able to determine their species-specific rhizosphere microbiome (Berg et al. 2006; Garbeva et al. 2008; Micallef et al. 2009; Miethling et al. 2000). By the secretion of root exudates containing carbohydrates, amino acids and specific secondary metabolites, plants attract or repel microorganisms and select for specific microbial communities in the rhizosphere (Bais et al. 2002; Doornbos et al. 2012; Moe 2013;

Weston and Mathesius 2013; Zhang et al. 2011). Members of the root-microbiome can provide a number of beneficial services to the host plant including the delivery of nutrients, protection against diseases, stimulation of growth and tolerance to abiotic stress (Bulgarelli et al. 2013; Lugtenberg and Kamilova 2009; Yang et al. 2009). Additionally, many soil-borne microorganisms have been found to support also the defense in aboveground compartments of the plant (Berendsen et al. 2012; Zamioudis and Pieterse 2012). Nonetheless specific microorganisms are able to protect and support the plant, their efficacy is influenced by a multitude of interactions among the microbial community.

In the rhizosphere numbers of bacterivorous protists increase up to 30-fold compared to bulk soil (Turner et al. 2013; Zwart et al. 1994) and there is evidence that plant diversity influence protist richness in soil (Tedersoo et al. 2016). However, the contributions of protists to the microbiomes of plants are not well known, although they play an important role in the rhizosphere of plants. It has been shown that protist in the rhizosphere affect plant health and productivity by grazing on bacterial communities. Protist grazing shifts the bacterial community composition (Bonkowski 2004; Rosenberg et al. 2009) resulting in changed root architecture (Kreuzer et al. 2006), altered hormone balance (Krome et al. 2010) and increased plant biomass and reproduction (Alphei et al. 1996; Krome et al. 2009).

Investigations started to unveil plant-associated protists (Agler et al. 2016; Arcate et al. 2006; Hulvey et al. 2010; Ploch et al. 2016; Sapp et al. 2018), however detailed knowledge on the major protist players in root-microbiomes is still missing. Since the diversity of protists in morphology and phylogeny is enormous (Adl et al. 2012; Cavalier-Smith 1998a; 1993) it seems inevitable that at least certain protist species represent an essential fraction of the plant microbiome and conduct important ecological functions.

Leaf-associated protists in the phyllosphere of plants

In contrast to the rhizosphere, the phyllosphere comprises the aboveground compartments of plants and is dominated by leaves (Vorholt 2012). Plant leaves are forming the largest biological surface on Earth with 10^8 km² globally (Penuelas and Terradas 2014), an area approximately twice as large as the global land surface. Similarly to the rhizosphere, plant leaves harbor microbiomes although these epiphytic microbial communities experience tremendous influence of environmental fluctuations. Leaf colonizers are influenced by physical and chemical extremes during diurnal cycles in moisture availability, temperature and UV-radiation (Leveau 2006; Lindow 2006). Bacteria are by far the most numerous colonizers of leaves (Lindow and Brandl 2003) and research on bacterial assemblages in the phyllosphere has gained much interest in recent years (Müller et al. 2016). Studies have

shown that assemblage of microbial communities are highly influenced by the plant genotype (Dees et al. 2015; Horton et al. 2014; Kim et al. 2012; Redford et al. 2010; Wagner et al. 2016). Furthermore plant traits such as anatomy, secondary metabolites, nutrient availability (Bodenhausen et al. 2014; Kembel et al. 2014; Ritpitakphong et al. 2016; Ryffel et al. 2016; Vorholt 2012) and environmental features such as geography, climate, and season (Copeland et al. 2015; Finkel et al. 2011; Jackson and Denney 2011; Rastogi et al. 2012; Redford and Fierer 2009) also play an important role in community structuration. Similarly to the rhizosphere, the leaf-microbiome exhibit important functions as some microorganisms interact with the plant to stimulate growth and inhibit or promote pathogen infection of tissues (Lindow and Brandl 2003). However, microbial communities on leaves are taxonomically more diverse including also fungi, yeasts, algae and protists (Lindow and Brandl 2003) and complex interactions are expected to occur (Vorholt 2012).

Among leaf-associated protists, plant parasitic oomycetes have been extensively studied due to their importance for plant health (Fawke et al. 2015; Gerbore et al. 2014; Jiang and Tyler 2012; Larousse and Galiana 2017). Well known pathogenic oomycetes are among the Albuginaceae which are dominant in the phyllosphere of Brassicaceae (Ploch and Thines 2011; Thines et al. 2009). Recently it has been revealed that the genus *Albugo* represents important hub taxa as they act as mediators between the plant and its microbiome (Agler et al. 2016).

Despite that, very little is known on interactions of phagotrophic protozoa and their prey within microbial communities on leaf surfaces. Although the ability of phagotrophic protists to colonize and reproduce in new habitats is mainly defined by the given conditions, like moisture, temperature and food abundance (Adl and Coleman 2005), studies confirm the regular presence of diverse ciliate, amoebae and flagellate taxa on plant leaves (Bamforth 1973; Mueller and Mueller 1970; Ploch et al. 2016; Sapp et al. 2018; Vaerewijck et al. 2014; Vaerewijck et al. 2011). However, systematic reports on non-pathogenic protists in the phyllosphere are scarce and phagotrophic protists have been mainly studied in terms of their potential as human pathogens on vegetables (Ciurea-Van Saanen 1981; Gourabathini et al. 2008; Napolitano and Collettieggolt 1984; Napolitano 1982; Rude et al. 1983; Vaerewijck et al. 2014; Vaerewijck et al. 2011). Similar to the rhizosphere, predation by protozoa may strongly contribute to the spatio-temporal structure of phyllosphere communities, but has been largely ignored in phyllosphere studies.

Among flagellates, cercozoan taxa appear to be the dominant colonizers of the phyllosphere (Amaral Zettler et al. 2005; Ploch et al. 2016; Sapp et al. 2018). Cercozoa are well adapted to life in the phyllosphere, because they withstand environmental extremes and are

especially adapted, and quickly respond to fluctuating environmental conditions (Ekelund et al. 2003; Holtze et al. 2003). The ability of Cercozoa to rapidly excyst, feed and multiply with generation times of 5-10 h (Ekelund 1996), is a perfect adaptation to highly fluctuating and extreme environmental conditions found in the plant phyllosphere.

Although non-pathogenic protists appear to be common phyllosphere colonizers, little is known about their diversity and impact on the microbial food webs in the phyllosphere. We have only a vague idea of these complex interactions, with respect to fundamental questions such as which microorganisms are present and what they do there. One first prerequisite to increase our knowledge on these interactions is to unravel the identity and feeding habits of leaf-associated protists and to determine their potential prey spectra. Further, phyllosphere protists as predators of bacteria on plant leaves have been rather neglected. Since many bacteria in the phyllosphere have the ability to influence plant growth (Lindow 2006), these interactions deserve further attention.

Cercozoa

Among protists, the phylum Cercozoa CAVALIER-SMITH 1998 accommodates very divergent organisms. Cercozoa is highly diverse in morphology and ecology, comprising amoeboflagellates, flagellates, filose testate amoebae, endophytic biotrophs and parasites (Bass et al. 2009a; Bass et al. 2009b; Cavalier-Smith and Chao 2003; Dumack et al. 2016b; Hibberd and Norris 1984; Howe et al. 2011; Howe et al. 2009; Neuhauser et al. 2014). Together with the Radiolaria and Foraminifera, both with a more conserved morphology, they constitute the eukaryotic supergroup Rhizaria (Adl et al. 2012; Cavalier-Smith 1998a; 1998b). Only two decades ago, Cercozoa were assigned as a monophyletic group (Cavalier-Smith 1997). Cercozoa are mostly heterotrophic protists, dwelling abundantly in soil and in all freshwater and marine habitats, feeding on bacteria, fungi and/or algae (Bass and Cavalier-Smith 2004; Dumack et al. 2016b; Geisen et al. 2015a; Urich et al. 2008).

Today Cercozoa are known to be one of the most diverse, species-rich and ecologically important of all protozoan phyla and include the majority of eukaryotes with filose pseudopods or cilia that glide on surfaces instead of swimming (Cavalier-Smith and Chao 2003). Studies have shown, only a small portion of cercozoan taxa has been described and many cercozoan species are still to be discovered (Bass et al. 2009a; Bass and Cavalier-Smith 2004; Ploch et al. 2016).

Cercomonadida - the dominant terrestrial amoeboflagellates

Within the Cercozoa the Cercomonadida are bacterivorous amoeboflagellates, known to be most abundant and widespread in soils (Bates et al. 2013; Mylnikov and Karpov 2004). In particular, the taxon *Cercomonas* has been reported to outnumber all other soil protozoan taxa in grassland and forest soils (Domonell et al. 2013). Cercomonads are small biflagellate heterotrophic protozoa which can change to a more pronounced amoeboid movement upon encounter of bacterial biofilms. Cercomonads were found to be functionally important during the breakdown of dead organic matter (Griffiths et al. 1993). Especially in the rhizosphere of plants, compared to bulk soil, the cercomonad taxa *Cercomonas* and *Heteromita* have been reported to be enriched and to outnumber all other soil protist taxa (Darbyshire and Greaves 1967; Holtze et al. 2003; Lara et al. 2007; Turner et al. 2013). In agreement with this, Murase et al. (2006) reported cercomonads as being the dominant protist group in rice field soils; and stable isotope probing in the rice rhizosphere confirmed cercomonads as being dominant feeders on rhizosphere bacteria (Lueders et al. 2004). Furthermore, Tedersoo et al. (2016) reported an influence of plant diversity on cercozoan richness, giving evidence for the affinity of cercomonads to plants.

Recently it was shown that cercozoan predators also exhibit a high diversity in the plant phyllosphere (Ploch et al. 2016) and that they form an integral part of the *Arabidopsis thaliana* microbiome (Sapp et al. 2018). In particular, Ploch et al. (2016) reported that species of six major orders of the Cercozoa could be found to be associated to leaves of Brassicaceae whereof the majority of detected taxa belonged to bacterivore, amoeboflagellates such as *Cercomonas* and *Eocercomonas* (Cercomonadida) (Bass et al. 2009b; Karpov et al. 2006) and small, gliding flagellates in the Glissomonadida (Howe et al. 2009). Despite these advances, we have still only a vague idea on the diversity and specific functional roles of plant-associated cercomonads in rhizosphere and phyllosphere of plants.

Testacea in the Cercozoa

The phylum Cercozoa is highly speciose and consists mainly of flagellates, amoeboflagellates and naked amoebae (Bass et al. 2009a; Dumack et al. 2016a; Hess and Melkonian 2013; Howe et al. 2011). Nestling between those are several distinct polyphyletic testate amoebae lineages. The order Euglyphida in the class Imbricatea with tests made of siliceous plates (Cavalier-Smith 1998a; 1998b; Wylezich et al. 2002), the family Rhogostomidae in the order Cryomonadida with organic thecae (Dumack et al. 2017c; Howe et al. 2011) and the families Chlamydophryidae, Rhizaspididae and Pseudodifflugiidae in the order Tectofilosida with thin organic hyaline tests and tests composed of agglutinated foreign material, respectively (Dumack et al. 2017c; Dumack et al. 2016c; Dumack et al. 2016b;

Wylezich et al. 2002). To complete, the latter orders branching in the class Thecofilosea (Cavalier-Smith and Chao 2003).

The Rhogostomidae accommodate thecate amoebae with a cleft-like opening which have been isolated from soils, sediments and freshwaters, or were detected on plant leaves (Belar 1921; Howe et al. 2011; Ploch et al. 2016). Although testate amoebae in general have been of considerable interest to protistologists and ecologists, only little is known about their diversity and ecology. Recently it has been shown that Rhogostomidae are predominantly bacterivorous, secondly algivorous with no evidence for fungal ingestion (Dumack et al. 2017b; Dumack et al. 2017c; Dumack et al. 2016c; Seppey et al. 2017; Wylezich et al. 2002). However, very little is known on the ecology and function of Rhogostomidae, especially in terms of their different feeding habits and predation pressure on bacteria, fungi and algae in terrestrial systems. Since small, testate amoebae such as cercozoan *Rhogostoma* spp. have also been detected to occur regularly in the phyllosphere (Ploch et al. 2016), leaf-associated bacterivorous and algivorous Rhogostomidae deserve further attention.

Aims

A comprehensive understanding about the distribution and functions of plant-associated protists in the rhizosphere and phyllosphere is lacking. The main objectives of this thesis were to investigate the diversity and functional roles of plant-associated cercomonads and cercozoan testate amoebae. This PhD thesis had four major goals:

1.) Comparative analysis of the diversity of plant-associated cercomonad taxa from the rhizosphere and phyllosphere of different plant species.

2.) Isolation and characterization of phenotypic traits of leaf-associated cercomonad Cercozoa and comparison of the protist cultures in respect to their direct and indirect effects on the diversity and functional traits of leaf-associated bacterial communities.

3.) To reveal patterns in the spatial and temporal dynamics between leaf-associated cercomonad Cercozoa and phyllosphere bacteria on plant leaves in experiments with labelled bacteria using epifluorescence microscopy.

4.) Isolation and characterization of plant-associated cercozoan testate amoebae and comparison of prey spectra of isolates from the phyllosphere, rhizosphere and soil.

Following hypotheses were proposed:

H1: Cercomonads can be found ubiquitously in the rhizosphere and phyllosphere of plants. However, the taxonomic composition is only partly known and the rhizosphere and phyllosphere are colonized by distinct cercozoan taxa.

H2: Phyllosphere cercomonad taxa affect the community composition and function of leaf-associated bacterial communities.

H3: Predation by cercomonads in the phyllosphere determines the spatial occurrence and dynamics of phyllosphere bacteria.

H4: Cercozoan testate amoebae from the phyllosphere, rhizosphere and soils differ in their prey spectra.

Chapter 1

Diversity of Cercomonad Species in the Phyllosphere and Rhizosphere of Different Plant Species with a Description of *Neocercomonas epiphylla* (Cercozoa, Rhizaria) a Leaf-Associated Protist

This chapter aimed to better characterize the diversity of plant-associated cercomonads and to contribute to a better resolution of the systematics of cercomonads and their association with plants. 75 cercomonad strains were isolated from the phyllosphere and rhizosphere of plants from three functional groups: grasses (*Poa* sp.), legumes (*Trifolium* sp.) and forbs (*Plantago* sp.). The potential phyllosphere and rhizosphere as well as plant specificity of the cercozoan genera *Cercomonas*, *Neocercomonas* and *Paracercomonas* was investigated by a comparative analysis. Three novel cercomonad species were described, including *Neocercomonas epiphylla* that was consistently and exclusively isolated from the phyllosphere.

Chapter 2

Grazing of Leaf-Associated Cercomonads (Protists: Rhizaria: Cercozoa) Structures Bacterial Community Composition and Function

This chapter investigates how grazing of leaf-associated cercomonads modified the composition and function of leaf-associated bacterial communities. The taxonomic and functional changes of the whole bacterial community due to predation effects of leaf-associated cercomonad Cercozoa were explored using a shotgun metagenomics

approach. Phenotypic protists traits could be linked to predation-induced shifts in bacterial community composition and altered bacterial community interactions were investigated.

Chapter 3

Spatial and temporal dynamics between leaf-associated cercomonad Cercozoa and phyllosphere bacteria on bean leaves (*Phaseolus vulgaris*)

This chapter aimed to reveal the spatial and temporal dynamics between leaf-associated cercomonad Cercozoa and bacterial strains of *Pantoea eucalypti* on leaves. We studied if cercomonads graze and reproduce on *P. eucalypti* and investigated their spatial and temporal interactions on bean leaves (*Phaseolus vulgaris*) by direct examination using epifluorescence microscopy.

Chapter 4

Rhogostomidae (Cercozoa) from soils, roots and plant leaves (*Arabidopsis thaliana*): Description of *Rhogostoma epiphylla* sp. nov. and *R. cylindrica* sp. nov.

The last chapter investigates the ecology and function of plant-associated cercozoan testate amoebae. Four different strains of *Rhogostoma* spp. were isolated from *Arabidopsis* leaves, agricultural soil and rhizosphere soil of *Ocimum basilicum* and *Nicotiana* sp. Detailed morphological description for two novel *Rhogostoma* species isolated from the phyllosphere and rhizosphere is provided. The potential ingestion of bacteria, algae and fungi was investigated, providing indications on how the Rhogostomidae also prey on other (co-isolated) members of the phyllosphere microbiome.

Chapter 1

Diversity of Cercomonad Species in the Phyllosphere and Rhizosphere of Different Plant Species with a Description of *Neocercomonas epiphylla* (Cercozoa, Rhizaria) a Leaf-Associated Protist

Chapter 1 - Diversity of Cercomonad Species in the Phyllosphere and Rhizosphere of Different Plant Species with a Description of *Neocercomonas epiphylla* (Cercozoa, Rhizaria) a Leaf-Associated Protist

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Abstract

Cercomonads are among the most abundant and diverse groups of heterotrophic flagellates in terrestrial systems and show an affinity to plants. However, we still lack basic knowledge of plant-associated protists. We isolated 75 Cercomonadida strains from the phyllosphere and rhizosphere of plants from three functional groups: grasses (Poa sp.), legumes (Trifolium sp.) and forbs (Plantago sp.), representing 28 OTUs from the genera *Cercomonas*, *Neocercomonas* and *Paracercomonas*. The community composition differed clearly between phyllosphere and rhizosphere, but was not influenced by plant species identity. From these isolates we describe three novel cercomonad species including *Neocercomonas epiphylla* that was consistently and exclusively isolated from the phyllosphere. For each new species we provide a detailed morphological description as well as an 18S rDNA gene sequence as a distinct marker of species identity. Our data contribute to a better resolution of the systematics of cercomonads and their association with plants, by describing three novel species and adding gene sequences of ten new cercomonad genotypes and of nine previously described species. In view of the functional importance of cercozoan communities in the phyllosphere and rhizosphere of plants, a more detailed understanding of their composition, function and predator-prey interactions are clearly required.

Introduction

The phylum Cercozoa (Cavalier-Smith and Chao 2003) is one of the dominant protist groups in terrestrial systems and highly diverse in both morphology and ecology (Bass and Cavalier-Smith 2004; Geisen et al. 2015a; Urich et al. 2008). Cercozoa harbor plant-infecting taxa like root-endophytic lineages and plant pathogens in the Endomyxa, but also a vast diversity of non-parasitic heterotrophic flagellates, amoeboflagellates and amoebae that feed on bacteria, algae and fungi (Bass et al. 2009a; Bass et al. 2009b; Dumack et al. 2016b; Flues et al. 2017; Glücksman et al. 2010; Hess and Melkonian 2013; Neuhauser et al. 2014).

Within the Cercozoa the Cercomonadida are bacterivorous amoeboflagellates, known to be most abundant and widespread in soils (Bates et al. 2013; Mylnikov and Karpov 2004). In particular, the genus *Cercomonas* has been reported to outnumber all other soil protozoan taxa in grassland and forest soils (Geisen et al. 2015a). Ploch et al. (2016) reported a high diversity of cercozoan taxa in the phyllosphere of Brassicaceae. Cercomonadida appear to be also enriched in the rhizosphere of plants compared to bulk soil (Turner et al. 2013), suggesting that they could be general rhizosphere colonizers. In agreement with this, Tedersoo et al. (2016) reported an influence of plant diversity on cercozoan richness. Despite these reports on the affinity of cercomonads to plants, we have only a vague idea on the diversity and distribution of cercomonads in the rhizosphere and phyllosphere of plants.

In contrast to the rhizosphere, the phyllosphere comprises the aerial plant surface and is colonized by host-specialized microbial populations that are well adapted to the diurnal environmental fluctuations (Vorholt 2012). Plant leaves are forming the largest biological surface on Earth with 10^8 km² globally (Penuelas and Terradas 2014), an area approximately twice as large as the global land surface. Bacteria are by far the most numerous colonizers of leaves (Lindow and Brandl 2003) and research on bacterial assemblages in the phyllosphere has gained much interest in recent years (Müller et al. 2016). Nevertheless, microbial communities on leaves are taxonomically more diverse and include also fungi, yeasts, algae and protists (Lindow and Brandl 2003). Studies confirm the regular presence of ciliates, amoebae and flagellates on plant leaves (Bamforth 1973; Mueller and Mueller 1970; Ploch et al. 2016; Vaerewijck et al. 2014; Vaerewijck et al. 2011). However, systematic reports on protists in the phyllosphere are scarce and phyllosphere protists have been mainly studied in terms of their potential as human pathogens or vectors of bacterial pathogens on vegetables (Ciurea-Van Saanen 1981; Gourabathini et al. 2008; Napolitano and Collettieggolt 1984; Napolitano 1982; Rude et al. 1983; Vaerewijck et al. 2014; Vaerewijck et al. 2011). Only one molecular study on leaf-associated Cercozoa has been conducted to date (Ploch et al. 2016). Increasing evidence suggests that Cercozoa can have a considerable impact on the composition and function of bacterial communities (Flues et al.

2017; Glücksman et al. 2010), giving evidence that phyllosphere Cercozoa are considerably understudied. However, we virtually lack a basic understanding of whether, how, and which of the Cercozoa are associated with plants.

We still require fundamental knowledge of plant-associated protists, and therefore a comprehensive understanding about their distribution and ecological functions in different rhizosphere and phyllosphere systems has not been achieved. In this study we aim to better characterize the diversity of plant-associated cercomonads. 75 cercomonad strains were isolated from the phyllosphere and rhizosphere of three plant species belonging to three different functional groups (grasses, legumes and non-leguminous forbs) and identified by their 18S rDNA. We investigated the phyllosphere and rhizosphere as well as plant specificity of the cercozoan genera *Cercomonas*, *Neocercomonas* and *Paracercomonas* and provide a comparative analysis. Based on unambiguous differences in 18S rDNA and morphological characters, we further describe three novel cercomonad species including one species that was exclusively isolated from the phyllosphere. For each new species we provide a detailed morphological description as well as an 18S rDNA gene sequence as a distinct marker of species identity.

Material and Methods

Sampling and identification

Populations of three plant species (*Poa* sp., *Trifolium* sp., *Plantago* sp.) were sampled in spring 2014 at a grassland site on the campus of the University of Cologne, Germany (50°55´30.1"N 6°56´07.4"E). At sampling, three leaves of each plant were harvested. Subsequently the root system of the same plant was recovered with a soil corer (5 cm diameter, 20 cm length). Leaf and root samples were collected from 22 individual plants per species and stored in sterile plastic bags for further isolation of cercozoans. In the laboratory, leaves were cut into pieces, submerged in 1.5 ml Neff´s Modified Amoeba Saline (NMAS) (Page 1976) and incubated in 24-well plates (Sarstedt, Nümbrecht, Germany) for up to three days prior to analyses. The rhizosphere samples were prepared by diluting 2 g fresh weight of roots with adhering rhizosphere soil in 50 ml NMAS-medium, which was gently shaken (30 rpm, 20 min) to detach protists from soil particles. For incubation, the suspension was diluted by a factor of 4 and 20 µl of the suspension were incubated in 180 µl Wheat Grass (WG)-medium in a 96-well plate (Sarstedt, Nümbrecht, Germany). The WG was made by adding 0.15 g dried wheat grass powder (Sanatur GmbH, Singen, Germany) to Prescott and James (PJ) medium (Prescott and James 1955). The samples were incubated for up to three weeks and screened weekly for cercomonad-like cells with an inverted phase-contrast microscope (Nikon Eclipse TS100; Ph1; 40-400x magnification).

Isolation and cultivation

Cercomonad cells were picked with a glass micro-pipette and transferred to 60 mm Petri dishes with NMAS containing one sterile quinoa (*Chenopodium quinoa*) grain as carbon source for bacteria. Cercozoan strains were subcultured several times until free from other eukaryotes or co-cultured with accompanying protists. Cercomonad cells were subcultured approximately every two months.

Microscopical observations

Pictures and videos were taken with a Nikon digital sight DS-U2 camera (program: NIS-Elements V4.13.04) with a Nikon Eclipse 90i upright microscope (up to 600x magnification, DIC) and a Nikon TE2000-E inverted microscope (up to 400x magnification, phase contrast).

Amplification and sequencing

For PCR, 15 µl of clonal cultures were transferred to PCR-tubes, whereas from mixed cultures single cercomonad cells were picked with a tapered glass micro-pipette and transferred into PCR-tubes containing 10 µl ultrapure water. The tubes were frozen at -20 °C for storage. Subsequently a total volume of 35 µl of PCR mixture was added. The mixture

contained 5 µl of 0.1 µM forward and 5 µl of 0.1 µM reverse primers, 5 µl of 200 µM dNTPs, 5 µl Thermo Scientific Dream Taq Green Buffer, 0.3 µl of Dream Taq polymerase (Thermo Fisher Scientific, Dreieich, Germany) and 14.7 µl of ultrapure water. General eukaryotic PCR primers EukA and EukB (Medlin et al. 1988) were used for amplification. For single cell amplifications, semi-nested re-amplifications were performed with nested primers 25F (Bass and Cavalier-Smith 2004) and 18S-nested-rev (Wylezich et al. 2002), with the same conditions as above. Three µl of the first PCR product was used as template. All amplification products were purified by adding 0.15 µl Exonuclease, 0.9 µl FastAP and 1.95 µl water to 8 µl of the final PCR product. Then heated for 30 min at 37 °C, and subsequently for 20 min at 85 °C. The sequencing of rDNA was done using Big Dye-Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Dreieich, Germany) and an ABI PRISM automatic sequencer. Primers used for sequencing were EukA, 590F, 1280F (Quintela-Alonso et al. 2011) and 25F (Bass and Cavalier-Smith 2004) for the forward strand and 600R, 1300R, EukB (Quintela-Alonso et al. 2011) and 18S-nested-rev (Wylezich et al. 2002) for the reverse strand.

Phylogenetic analyses

Obtained sequences were manually checked for sequencing errors before being assembled into one sequence contig using BioEdit v7.2.5 (Hall 1999). For phylogenetic analyses sequences were blasted (blastn 2.3.0) against the NCBI GenBank database and closely related sequences used in previous analyses (Bass et al. 2009b; Brabender et al. 2012; Ploch et al. 2016) were downloaded. If several similar sequences from the same environmental study were found, only one representative sequence was chosen. Cercomonads are divided into two main clades, clade A and clade B (Bass and Cavalier-Smith 2004; Cavalier-Smith and Chao 2003; Karpov et al. 2006). We split the two clades and treated them independently in the phylogenetic analyses in order to be able to use more unambiguously aligned sites and to receive more clear and well-defined maximum likelihood trees. Alignments were carried out using SeaView v4.6.1 (Gouy et al. 2010) using 1,563 sites (clade A) and 1,566 sites (clade B), which were to 69.74% and 63.67% invariant, respectively. The best fitting model GTR+I+G was determined by jModeltest v2.1.5 (Darriba et al. 2012) for both clades. With this model, phylogenetic trees were calculated in PhyML v3.1 (Guindon and Gascuel 2003) using 100 replicates for the bootstrap analysis and MrBayes v3.2.6 (Ronquist and Huelsenbeck 2003) (settings: mcmc ngen = 1 M, sample freq = 100, print freq = 100, diagn freq = 500; (Altekar et al. 2004)) for the Bayesian analysis. To define OTUs and separate them from others, we compared the most variable regions of the cercomonad 18S rDNA – V2, V4, V5 and V7 (Wuyts et al. 2000), to reduce artefactual effects of sequencing errors and intra-clonal polymorphism along the whole gene. In some cercomonads, different species can have identical V4 regions but differences in other variable regions (Bass et al. 2009b). Therefore, unique 18S-types were defined as

sequences with three or more nucleotide differences among the V2+V4+V5+V7 regions and we only described novel species if there were additional differences in a group of morphological characters observed by light microscopy in clonal cultures. The gene sequences were submitted in the NCBI database under the accession numbers listed in Table S1.

Statistical analyses

Canonical correspondence analysis (CCA) was used to discern the relationship between cercomonad species composition and their origin (i.e. phyllosphere, rhizosphere and plant species identity) using the software package CANOCO version 5.0 (ter Braak and Šmilauer 2012). OTU data were converted to presence/absence values and CCA was undertaken without downweighting of rare species. To rank environmental variables in their importance for being associated with the structure of the cercomonad community, we used a forward selection where the statistical significance of each variable was judged by a Monte-Carlo unrestricted permutation test with 9,999 permutations (ter Braak and Verdonschot 1995).

Results

Sampling and protist occurrence

In total, 84 protist strains were isolated and sequenced. Six sequences were not affiliated to Cercomonadida and excluded from further analysis, as well as three cercomonad sequences from strains isolated twice from the same plant and habitat. In total, 75 Cercomonadida strains were isolated and sequenced, representing 28 OTUs from the genera *Cercomonas* (9 OTUs), *Neocercomonas* (12 OTUs) and *Paracercomonas* (7 OTUs) (Table S2).

Thirty-six strains (15 OTUs) were isolated from the phyllosphere and 39 strains (19 OTUs) from the rhizosphere. Only six OTUs (23.1%) could be detected in both habitats while nine and 13 OTUs were exclusively isolated from the phyllosphere and rhizosphere, respectively. In addition, only two OTUs (*Cercomonas* sp. OTU3 and *Paracercomonas* sp. OTU23) were isolated from the phyllosphere and rhizosphere of the same plant individual.

Considering the different plant functional groups, 23 strains (15 OTUs) were isolated from the phyllosphere and rhizosphere of grass (*Poa* sp.), while 24 strains (14 OTUs) and 28 strains (16 OTUs) were isolated from legumes (*Trifolium* sp.) and forbs (*Plantago* sp.), respectively. Comparing the different plant species, only four out of 28 OTUs were shared by all three investigated plant species. The novel species *Neocercomonas epiphylla* (see below) was shared only among the phyllosphere, *Cercomonas* sp. OTU2 only among the rhizosphere and two OTUs (*Cercomonas* sp. OTU3 and *Paracercomonas* sp. OTU23) were shared among both plant organs.

Species concept

Knowledge on the morphological and genetic diversity within and between cercomonad species is still limited. We followed the 18S rDNA-based barcoding of species applied by Bass et al. (2009b). To define OTUs and separate them from others, we compared the most variable regions of the cercomonad 18S rDNA – V2, V4, V5 and V7 (Wuyts et al. 2000) among strains (as described in Methods), to reduce artefactual effects of sequencing errors and intra-clonal polymorphism along the whole gene.

Phylogenetic analysis and relations

Near full length 18S rDNA sequences were obtained from isolated cercomonads (Table S1). These are marked with bold letters in the maximum likelihood trees (Fig. 1, 2). The extended phylogenetic analysis including the novel results of the present study generally confirms the topology of previous studies. Cercomonads have been divided into two main clades, clade A and clade B (Bass and Cavalier-Smith 2004; Brabender et al. 2012; Cavalier-Smith and Chao 2003; Karpov et al. 2006). This was also unambiguously confirmed by our phylogenetic

studies. Within clade A the distinction between clade A1a and A1b into the genera *Cercomonas* and *Neocercomonas* was controversial. Cavalier-Smith and Karpov (2012) reintroduced the genus *Neocercomonas* for subclades A1b (emended from Ekelund et al. (2004)) and further created the genus *Filomonas* for subclade A1c. The high bootstrap values in our tree (Fig. 1) and in the tree of Scoble and Cavalier-Smith (2014) support the monophyly of *Neocercomonas*. However, the distinctness within clade A1 cercomonads is further complicated by the fact that the genus *Filomonas* does not always branch separately from *Cercomonas* and *Neocercomonas*. To avoid additional confusion we refer to *Neocercomonas* plus *Filomonas* in our tree (Fig. 1) according to Scoble and Cavalier-Smith (2014).

Forty-seven strains were closely affiliated to described species, all other strains (37.3%) did not form clear clusters with any sequences or were affiliated to database sequences that were obtained from comparative environmental screenings. Fifty-five of our 75 sequences are located in clade A and were separated into different subclades: twenty-seven grouped in the *Cercomonas* clade A1a1 and twenty-eight in *Neocercomonas* A1b subclades. In detail, six in clade A1b1, twenty-one in clade A1b2 and one sequence was in clade A1b3 (all subclades referring to Bass et al. (2009b), see also Fig. 1). Novel species *Neocercomonas tuberculata* and *Neocercomonas nitschei* fall in clade A1b1 and were represented by single strains. The closest cultured relatives of *Neocercomonas tuberculata* are *N*. *pigra*, *N*. *sphagnicola* and *N*. *magna*. The closest relatives of *Neocercomonas nitschei* are *N*. *jendrali* and the probably misidentified strain *Cercomonas* sp. “alexieffi” ATCC50395 (see Bass et al. (2009b)). *Neocercomonas epiphylla* fell into clade A1b2. Besides the type strain of *N*. *epiphylla*, eight other strains with identical 18S rDNA gene sequences and morphology were isolated from the phyllosphere across all sampled plant species. The closest described relative of *N. epiphylla* is *N. braziliensis*. Nearly all other sequences could be affiliated to already published sequences. However six of our isolates possess unambiguous differences in their 18S rDNA gene sequences. *Cercomonas* sp. OTU7 was represented by two strains, both isolated from the phyllosphere of *Plantago* sp. Phylogenetically it is a sister group of *C*. *kiaerdammane* and *C*. *pellucida* supported by high bootstrap values. Other OTUs (OTU8, 15, 17 and 20) were represented by single strains and could not be affiliated to known species or published sequences. *Neocercomonas* sp. OTU12 was isolated from the phyllosphere of *Trifolium* sp. and *Plantago* sp. and represented by four strains with identical sequences to that of KT251182 published by Ploch et al. (2016).

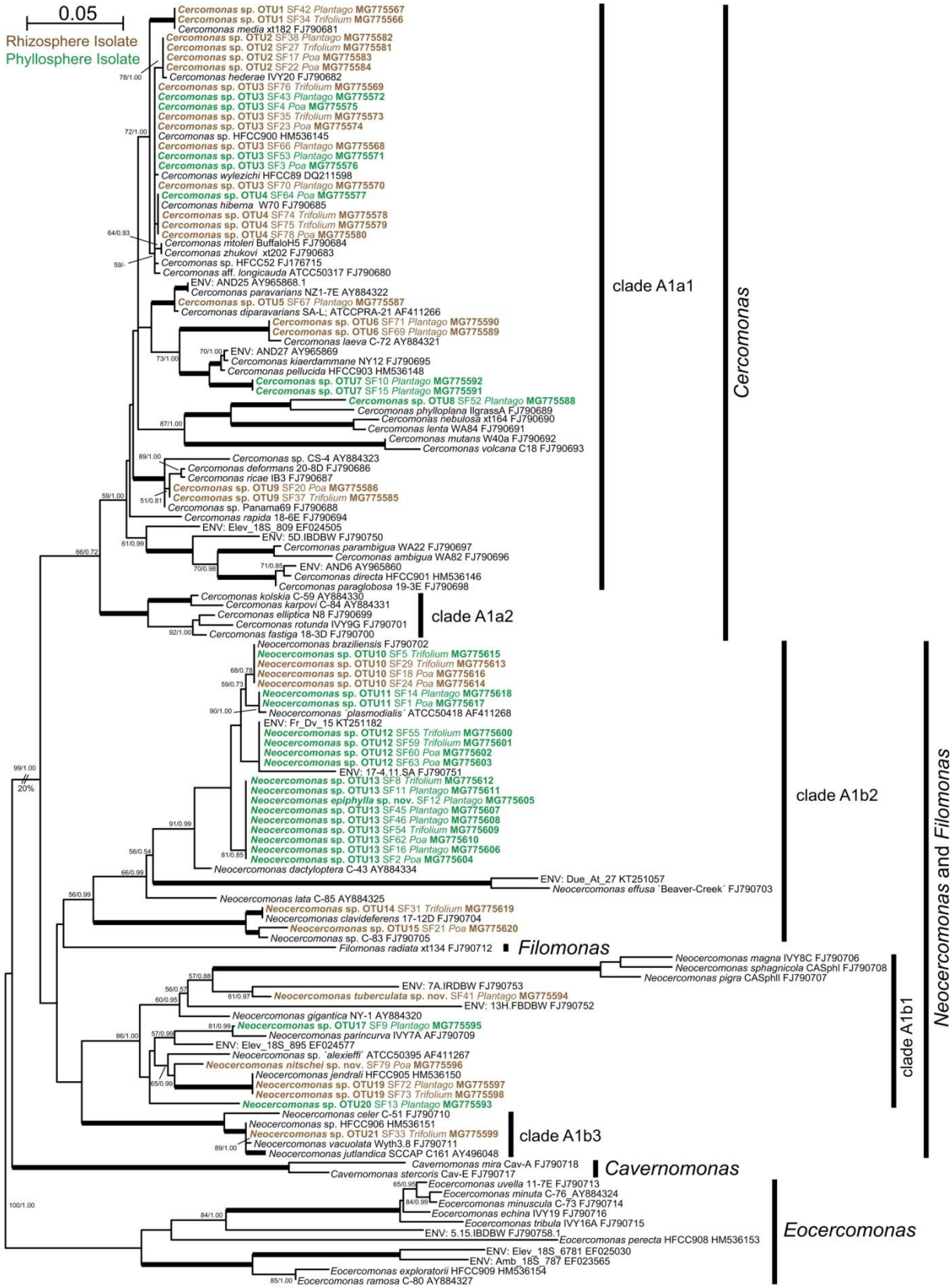

Figure 1 - SSU rDNA phylogeny of clade A cercomonads. Shown is the maximum likelihood (ML) 18S rDNA phylogenetic tree based on 120 sequences of clade A1 cercomonads from GenBank and this study, obtained by the PhyML GTR+I+G analysis using 1563 aligned nucleotide positions of which 69.74% were invariant. The support levels of the PhyML and the Bayesian analysis are shown on the respective branches (ML/BI) if support was over 50%/0.50. Branches with ML support of ≥ 95% are in bold. Newly obtained sequences from this study are in bold, marked in color according to their origin (green: phyllosphere-derived sequences; brown: rhizosphere-derived sequences). Interrupted branches (//) show 20% of their original length. Sequences from the genus *Eocercomonas* were selected as outgroup.

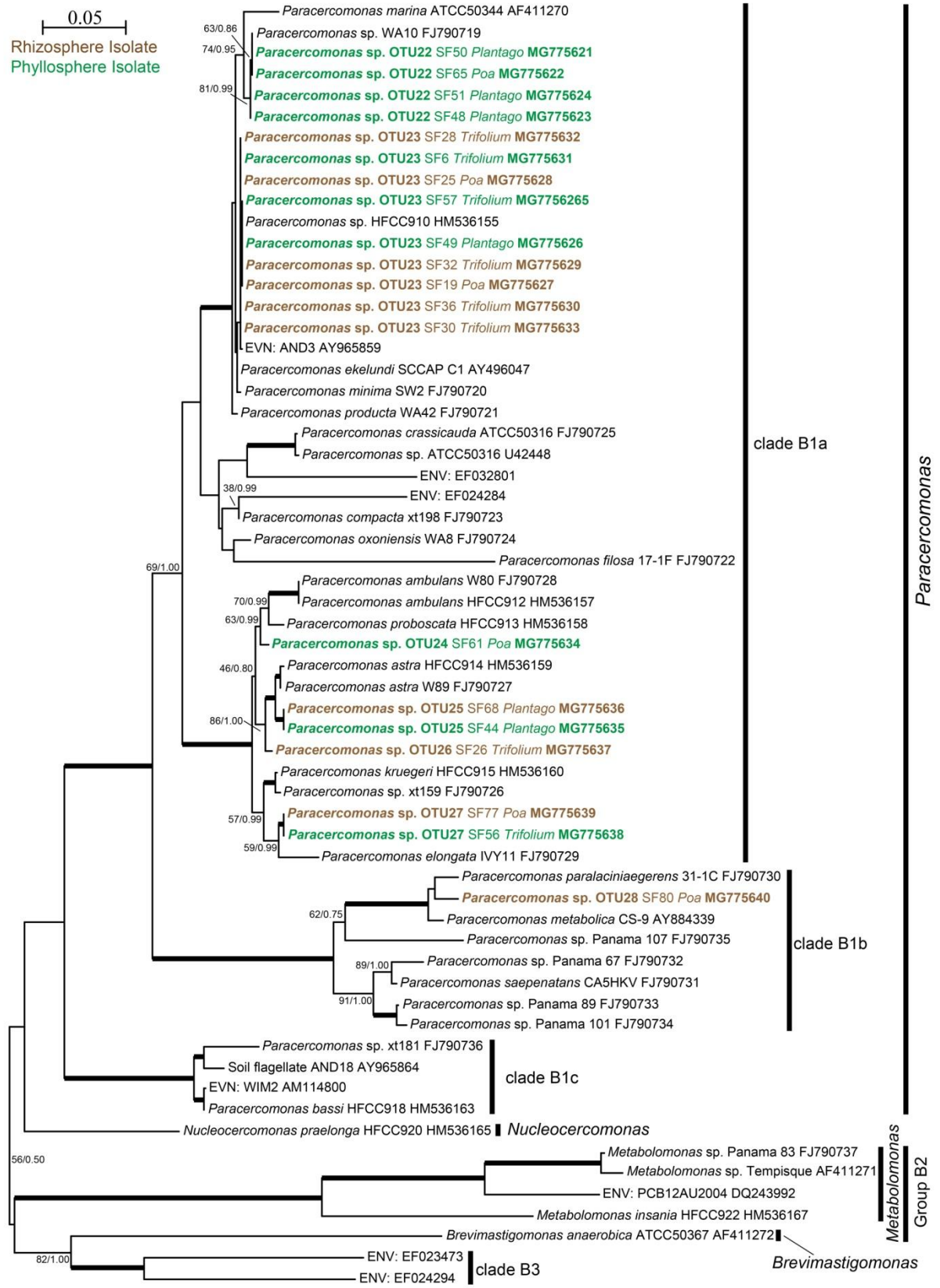

Figure 2 - SSU rDNA phylogeny of clade B cercomonads. Shown is the maximum likelihood (ML) 18S rDNA phylogenetic tree based on 61 sequences of clade B cercomonads from GenBank and this study, obtained by the PhyML GTR+I+G analysis using 1566 aligned nucleotide positions of which 63,67% were invariant. The support levels of the PhyML and the Bayesian analysis are shown on the respective branches (ML/BI) if support was over 50%/0.50. Branches with ML support of ≥ 95% are in bold. Newly obtained sequences from this study are in bold, marked in color according to their origin (green: phyllosphere-derived sequences; brown: rhizosphere-derived sequences). Sequences from clade B2 and B3 cercomonads were selected as outgroup.

Twenty of our sequences belong to clade B, nineteen to *Paracercomonas* clade B1a and one sequence to *Paracercomonas* clade B1b. Most strains could be affiliated to already published sequences. Nevertheless seven strains representing five *Paracercomonas* OTUs (OTU24-28) had unambiguously different sequences. *Paracercomonas* sp. OTU24 was represented as a single strain isolated from the phyllosphere of *Poa* sp. and branched next to *P. proboscata* and *P. ambulans*. OTU25 and 27 isolated from the phyllosphere and rhizosphere were represented by two strains and branched next to *P. astra* and *P. elongata*, respectively. Single strains OTU26 and 28 isolated from the rhizosphere grouped next to OTU25 and *P. metabolica*, respectively.

Statistical analysis – Cercomonad distribution in the phyllosphere and rhizosphere

The primary CCA axis (CCA1) explained 48.23% of the species-origin relationship of all 75 isolated strains, adding the second CCA axis (CCA2) increased the variance explained by 35.57%. Forward selection identified a statistical significance for phyllosphere-rhizosphere groupings ($F = 1.8$, $P = 0.002$) (Fig. 3), while plant functional groups ($F = 1.0$, $P = 0.341$) did not explain the cercomonad composition. When we tested the cercomonad species composition on genus level, the phyllosphere-rhizosphere groupings remained statistically significant for *Neocercomonas* ($F = 2.3$, $P < 0.001$) but could not be confirmed for the genera *Cercomonas* ($F = 1.6$, $P = 0.086$) and *Paracercomonas* ($F = 1.3$, $P = 0.236$).

In summary, the OTUs *Cercomonas* sp. OTU 7 and 8; *Neocercomonas* sp. OTU11, 12, 17 and 20; *Paracercomonas* sp. OTU22 and OTU24 as well as *N*. *epiphylla* were consistently isolated from the phyllosphere, while *Cercomonas* sp. OTU3, 4; *Neocercomonas* sp. OTU10 and *Paracercomonas* sp. OTU23, 25 and 27 were isolated from both the phyllosphere and rhizosphere. All other isolated OTUs were isolated exclusively from the rhizosphere (Fig. 3).

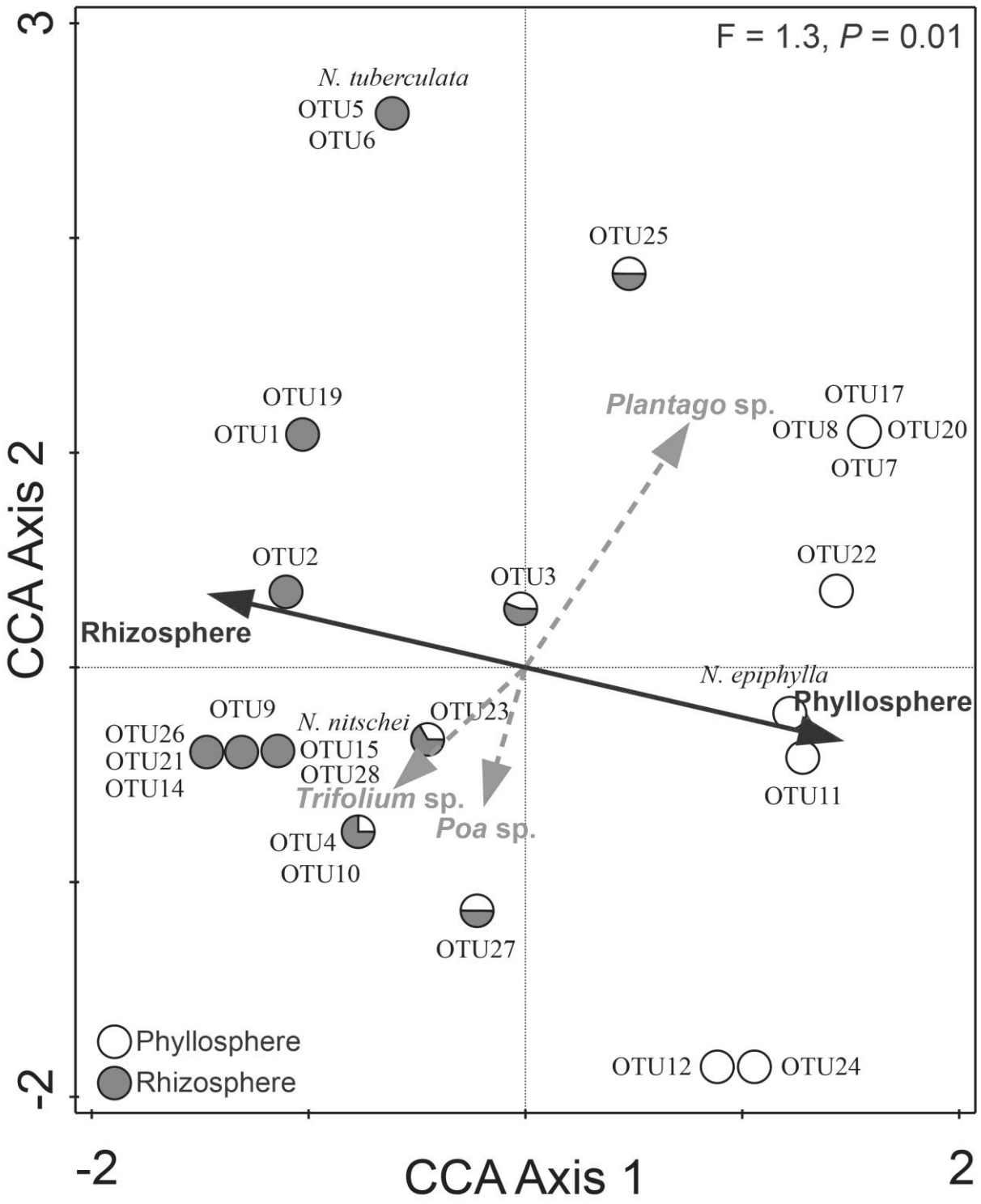

Figure 3 - Canonical correspondence analysis (CCA). CCA ordination biplot of environmental variables (i.e. phyllosphere, rhizosphere and plant species identity) and cercomonad data is shown. Black solid line arrows represent significant ($P < 0.05$), grey dashed line arrows not significant environmental variables. Global Monte-Carlo unrestricted permutation test value is shown in the upper right of the graph.

Diagnoses and description of Novel Species

***Neocercomonas tuberculata* sp. nov.** FLUES, BLOKKER, DUMACK ET BONKOWSKI

Diagnosis: *Neocercomonas* with a measured range in body length of 40.3-55.4 µm and body width of 30.2-38 µm. Anterior flagellum has a length of 48.8-54.2 µm, the posterior has a length of 47.6-49.5 µm. Cells most often surface attached, gliding, cells are bulky but elongated oval. Cells metabolic. Pseudopodia present, most often at posterior end, finger-like and bulbous. Nucleus attached to basal apparatus, therefore usually elongated, drop shaped, one rarely two spherical nucleoli. Several small contractile vacuoles are dispersed throughout the cell body. Cell plasm rich in large granules, evenly distributed in the cytoplasm. Small spherical “tubercles”, possibly extrusomes, in close proximity to the cell membrane. Cysts spherical with a diameter of approx. 25µm; the cystoplasm attaches closely to it.

Type generating strain: SF41 (Cologne, Germany; rhizosphere soil *Plantago* sp.; 2014).

Sequence of type generating strain (SSU rDNA): MG775594

Illustrations of type generating strain: Figure 4; Supplementary Video S1; this material constitutes the name-bearing type of the species.

Culture of type generating strain: A culture has been deposited in the Culture Collection of Algae and Protozoa; accession number CCAP 1250/3.

Etymology: tuberculata [LATIN] derived from tuberculatus (meaning tuberculum), referring to the tubercle-rich morphology of the cell membrane.

Zoobank registration number: urn:lsid:zoobank.org:act:2EAB9411-BD1C-48F9-9896-20C917EA8B5D

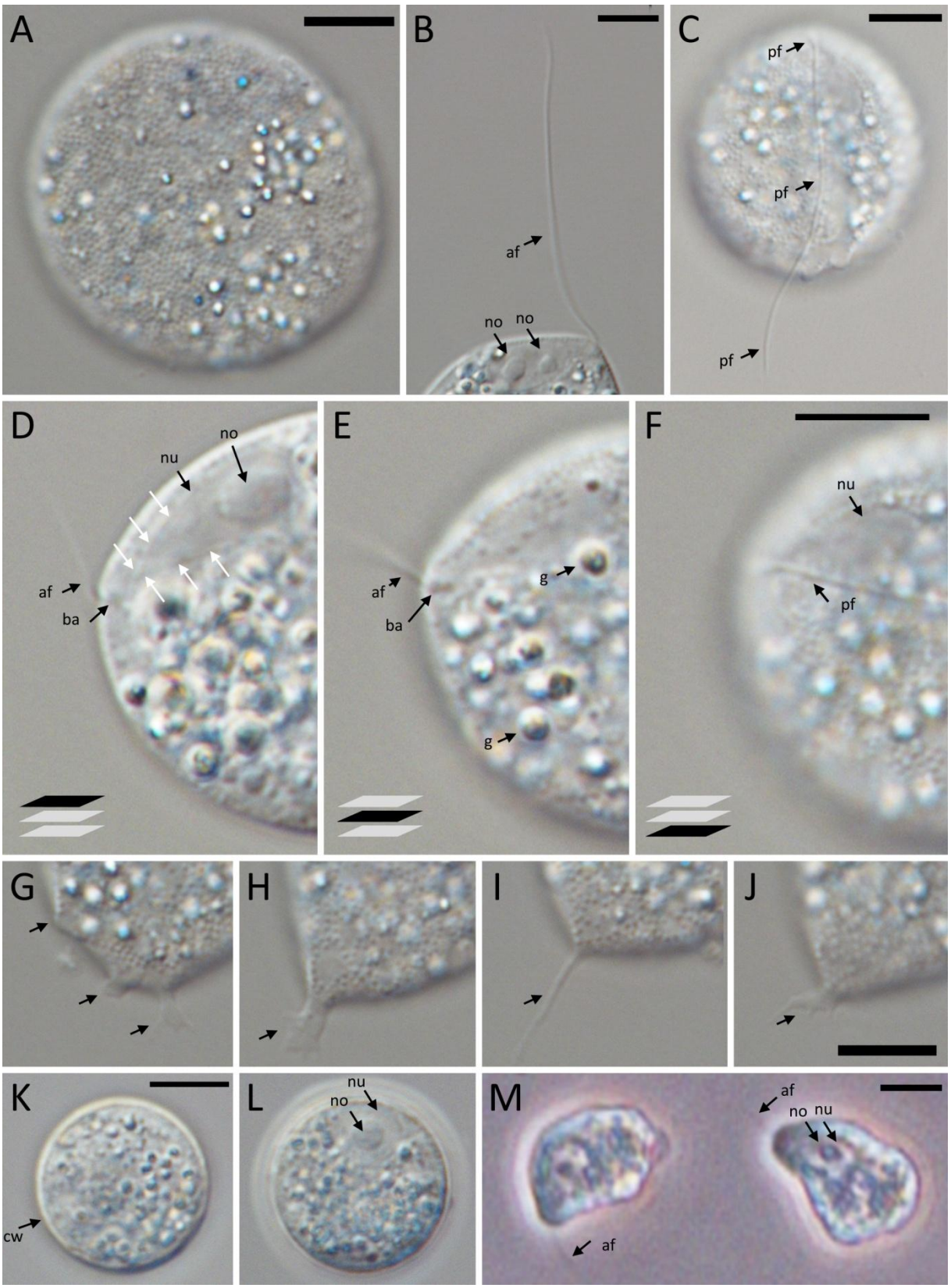

Figure 4 - General morphology of *Neocercomonas tuberculata* (SF41). **A.** overview of the cell focusing on the 'tubercle-rich' cell surface. **B, C.** length of the anterior (B) and posterior (C) flagellum. **D-F.** serial shots from the upper cell layer (D), over the middle layer (E) to the lower layer(F). **D.** the nucleus is connected to the basal apparatus, note the elongated shape of the nucleus with a pinpoint ending in direction of the basal apparatus, highlighted with white arrows. **E.** the basal apparatus in focus, shown is the origin of the anterior flagellum. **F.** the posterior flagellum in focus, its origin in the basal apparatus is shown. **G-J.** different posterior 'tails' of the same individual highlighted by black

arrows. **K-L.** Cyst in two different focus layers, surface (K) and interior with the nucleus (L). **M.** inverted images. Scale bars = 10 µm, the scaling for D-F, G-J and K-L are the same. A-L. differential interference contrast (DIC). M. phase contrast. Abbreviations: af = anterior flagellum; ba = basal apparatus; cw= cyst wall; g = granule; no = nucleolus; nu = nucleus; pf = posterior flagellum.

***Neocercomonas nitschei* sp. nov.** FLUES, BLOKKER, DUMACK ET BONKOWSKI

Diagnosis: *Neocercomonas* with a body length of 19.6-30.2 µm and a body width of 14.5-19.8 µm. Anterior flagellum has a length of 35-39.2 µm, the posterior flagellum is 32.3-38.8 µm. In directed movement, cells most often surface attached, gliding, ovoid anterior-posterior elongated. Cells metabolic. Pseudopodia present, usually one posterior, bulbous. Nucleus in actively moving cells could never be observed, but in stationary cells always spherical, one centric and spherical nucleolus. Often one posterior vacuole, contractile vacuoles have not been observed. Cell plasm with small granules, concentrated in the cell anterior. Cysts spherical but with rough cyst wall with a diameter of approx. 13 µm; with separated cell membrane.

Type generating strain: SF79 (Cologne, Germany; rhizosphere soil *Poa* sp.; 2014).

Sequence of type generating strain (SSU rDNA): MG775596

Illustrations of type generating strain: Figure 5; Supplementary Video S2; this material constitutes the name-bearing type of the species.

Culture of type generating strain: A culture has been deposited in the Culture Collection of Algae and Protozoa; accession number CCAP 1250/2.

Etymology: dedicated to Frank Nitsche for his valuable contribution and support during the cercomonad studies in recent years.

Zoobank registration number: urn:lsid:zoobank.org:act:1EEB1193-86EC-4362-884F-B5C77E125454

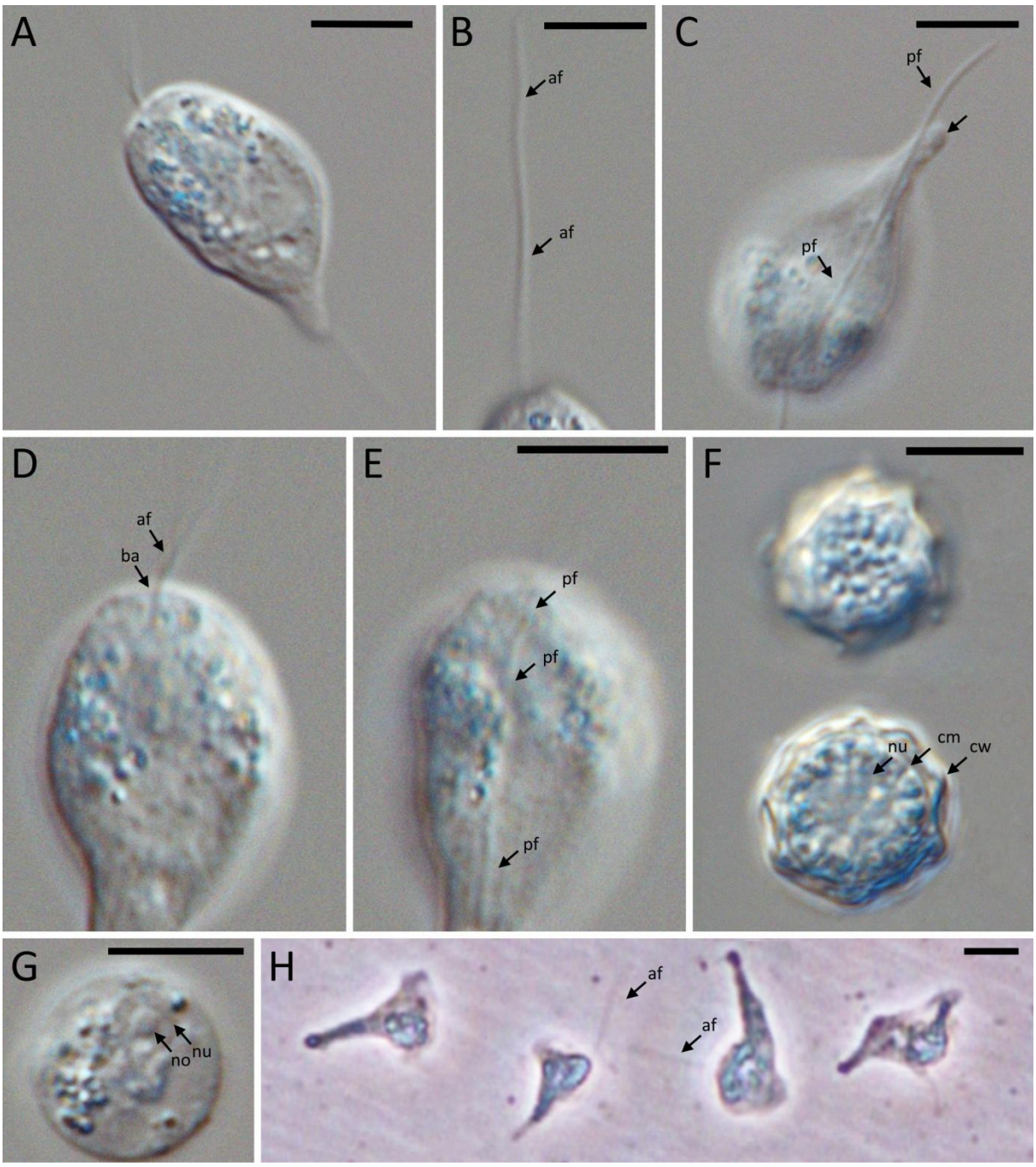

Figure 5 - General morphology of *Neocercomonas nitschei* (SF79). **A.** overview of the cell in the typical shape of directed movement. **B, C.** length of the anterior (B) and posterior (C) flagellum, the posterior tail is highlighted by an arrow. **D, E.** the same cell in two focus layers showing the basal apparatus and the connected flagella. **F.** same cyst in two different focus layers, notice the rough structure of the cyst wall and the visibly separated cell membrane. **G.** shows a roundish cell possibly due to cyst formation with a visible nucleus and one round central nucleolus. **H.** inverted images. Scale bars = 10 µm, the scaling for D, E are the same. A-G. differential interference contrast (DIC). H. phase contrast. Abbreviations: af = anterior flagellum; ba = basal apparatus; cm= cell membrane; cw= cyst wall; g = granule; no = nucleolus; nu = nucleus; pf = posterior flagellum.

***Neocercomonas epiphylla* sp. nov.** FLUES, BLOKKER, DUMACK ET BONKOWSKI

Diagnosis: *Neocercomonas* with a measured range in body length of 17.3-25.8 µm and body width of 12.4-20.4 µm. Anterior flagellum has a length of 23.1-26.5 µm, the posterior flagellum is 25.6-32.7 µm. Cells most often surface attached, gliding, flattened and most often triangular in shape, lateral ends often more flattened and thus quite transparent with only few or no granules. Cells highly metabolic. Pseudopodia present, lateral and posterior, finger-like and bulbous. Nucleus probably attached to basal apparatus, but always spherical, one centric and spherical nucleolus. Usually one contractile vacuole centric in the cell body. Cell plasm rich in small granules, concentrated in the anterior end and the cell center. Cysts spherical with a diameter of approx. 11 µm; the cystoplasm attaches closely to it.

Type generating strain: SF12 (Cologne, Germany; phyllosphere *Plantago* sp.; 2014). Other strains reported here (SF2, SF8, SF11, SF16, SF45, SF46, SF54 and SF62) indistinguishable by morphology and phylogeny.

Sequence of type generating strain (SSU rDNA): MG775605

Illustrations of type generating strain: Figure 6; Supplementary Video S3; this material constitutes the name-bearing type of the species.

Culture of type generating strain: A culture has been deposited in the Culture Collection of Algae and Protozoa; accession number CCAP 1250/1.

Etymology: epiphylla [GREEK] derived from *epi-* (meaning upon, near to, in addition) and *phyllon* or *phylla* (meaning leaf), referring to the association between *Neocercomonas epiphylla* and plant leaves.

Zoobank registration number: urn:lsid:zoobank.org:act:A97B03BA-420F-4F96-A457-62912D404974

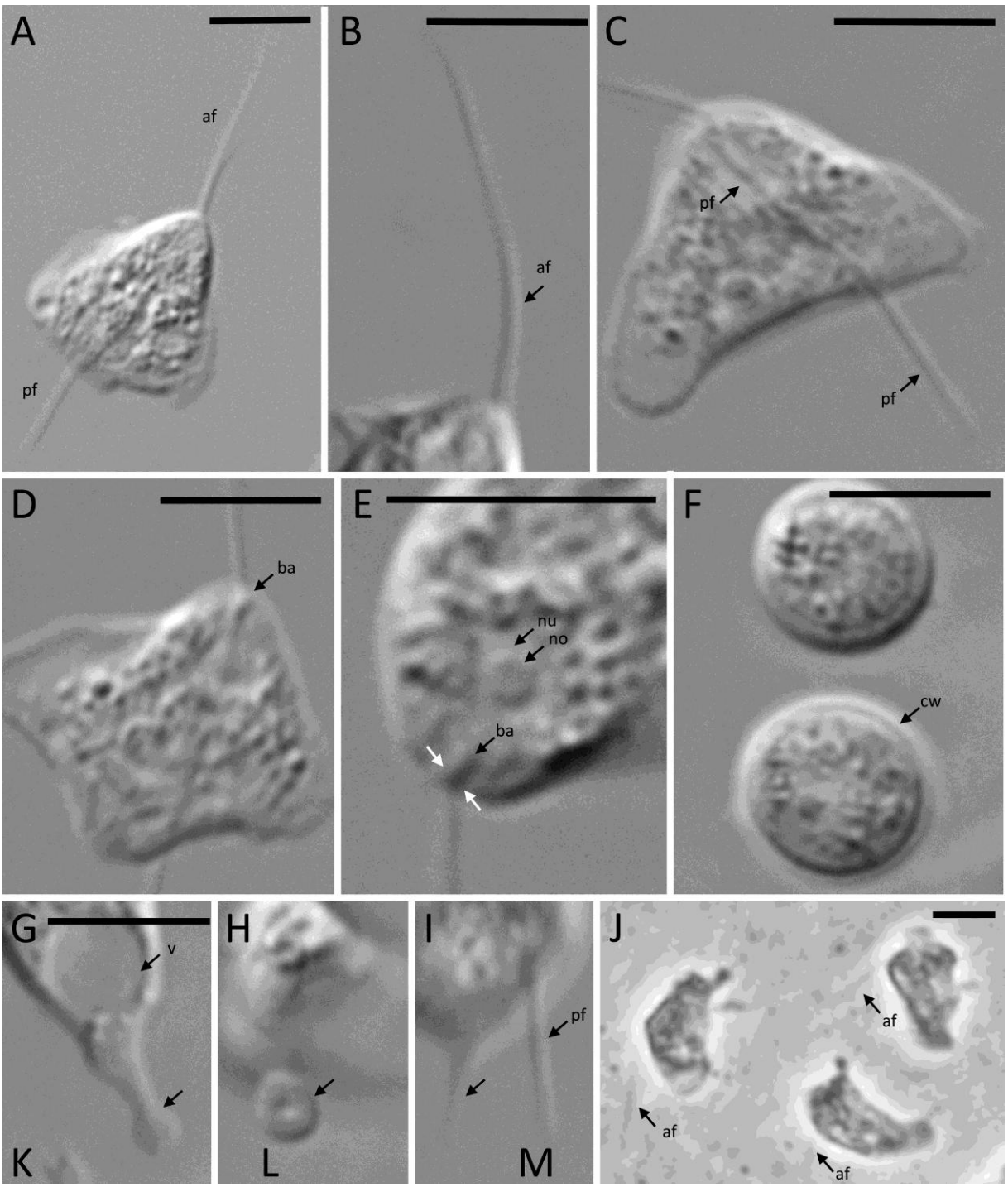

Figure 6 General morphology of *Neocercomonas epiphylla* (SF12). **A.** overview of the cell in the typical shape of directed movement. **B, C.** length of the anterior (B) and posterior (C) flagellum. **D, E.** the basalapparatus and connected flagellae. **D.** both the posterior flagellum and anterior flagellum originate from the same point. **E.** the basal apparatus is in focus, the origin of the anterior flagellum is seen (white arrows), additionally the spherical nucleus and nucleolus can be seen. **F.** same cyst in two different focus layers. **G-I.** different posterior 'tails' of the same individual are highlighted with an arrow. **J.** inverted images. Scale bars = 10 µm, the scaling for G-I is the same. A-I. differential interference contrast (DIC). J. phase contrast. Abbreviations: af = anterior flagellum; ba = basal apparatus; cw= cyst wall; g = granule; no = nucleolus; nu = nucleus; pf = posterior flagellum; v = vacuole.

Discussion

Cercomonads are an abundant and diverse group of heterotrophic flagellates in soil (Brabender et al. 2012) but also in the phyllosphere of plants where they potentially play a significant role as bacterial grazers (Flues et al. 2017). Similar to previous analyses using SSU rDNA sequence comparisons some of the basal branches within the Cercomonadida were not well supported. Nevertheless, by describing three novel species, adding gene sequences of ten new previously unknown cercomonad genotypes and nine genotypes matching those of previously described species, we contribute to a better resolution of the diversity of cercomonads. Several OTUs fall into formerly weakly resolved clusters. However, the scope of this study was to investigate whether cercomonad communities differ according to plant organ (rhizosphere vs. phyllosphere) or functional group (grass, legume, forb).

When we isolated the investigated cercomonads from our rhizosphere samples, several protist taxa from other protist groups were found, which is obvious for rhizosphere soil samples. To our surprise, we also found a vast diversity of protists in our phyllosphere samples. Several glissomonads from the genera *Neoheteromita*, *Sandona* and *Allapsa*, but also various taxa of Amoebozoa (Flamellidae, Hartmannellidae, Vannellidae) and colpodean ciliates were found. This suggests that the phyllosphere could act as an important habitat for protists, especially when we consider the large global surface area represented by leaves. The occurrence of several Glissomonadida (Howe et al. 2009) in the phyllosphere across all sampled plants supports findings of Ploch et al. (2016) and corroborates the assumption that leaf-associated Cercozoa must be considered to be an integral part of the phyllosphere microbiome (Ploch et al. 2016).

We identified 15 OTUs from the phyllosphere and 19 OTUs from the rhizosphere represented by 36 and 39 strains, respectively. Twelve out of 19 OTUs (63.2%) from the rhizosphere could be affiliated to described species or published sequences. In contrast, less than half of the detected cercozoan OTUs from the phyllosphere were assignable to described species or published sequences, illustrating the need for systematic taxonomic studies on the diversity of phyllosphere protists. OTUs of four clades (i.e. A1a1, A1b1, A1b2 and B1a) in Cercomonadida were associated to leaves within this study, whereas OTUs from clade A1b2 represented the major fraction of detected 18S types. Interestingly the leaf-associated Cercomonadida detected by Ploch et al. (2016) by culture-independent approaches also fall into clade A1b2, which indicates that this clade is associated to the phyllosphere. In addition to Ploch et al. (2016) we also isolated clade B cercomonads from the phyllosphere. Bass et al. (2009b) identified some Cercomonadida species that seemed to be specifically associated with leaf surfaces. *Neocercomonas magna*, *N. pigra* and *N.*

sphagnicola were all isolated from plant leaves and represent very large cells. Based on our study, cell size represents not an important trait of leaf-associated cercomonads. Nevertheless desiccation resistance and fast reproduction are considered as the most important traits to survive the harsh abiotic conditions in the phyllosphere (Mueller and Mueller 1970; Ploch et al. 2016), which most Cercomonadida possess.

Our data clearly show patterns in the cercomonad species composition of the phyllosphere and rhizosphere (Fig. 3). Only 23.1% of isolated OTUs could be detected in the phyllosphere as well as in the rhizosphere, indicating a deterministic assembly of cercomonads. We could not demonstrate that plant functional group identity affects the cercomonad species composition as known for bacteria (Laforest-Lapointe et al. 2016a; 2016b), suggesting that no plant species is preferentially colonized by protists. However, it is likely that protist communities on leaves are influenced by the biodiversity of their prey (Dumack et al. 2017a; Flues et al. 2017). Furthermore, leaf anatomical structures and plant secondary metabolites are also likely to affect protist colonization and composition in the phyllosphere. The novel described species *Neocercomonas epiphylla* was represented with nine strains and exclusively isolated from the phyllosphere across all sampled plant species, which indicates that *N. epiphylla* is suggestive of a protist preferentially associated with the phyllosphere. Also *Neocercomonas* sp. OTU12 was exclusively isolated from the phyllosphere of *Trifolium* sp. and *Plantago* sp. and represented with four strains with identical sequences to that of KT251182 published by Ploch et al. (2016). Ploch et al. (2016) identified this taxon in the phyllosphere of *Arabidopsis thaliana*, *Cardamine partensis*, *C. hirsuta* and *Draba verna* from two different locations in Germany. Further, a number of OTUs (OTU7, 8, 17, 20, 24), which were detected for the first time here might be also potential phyllosphere colonizers. However, further studies are needed for verification, since new species and lineages are found continuously and the total diversity of Cercozoa is still unrevealed.

Ecological aspects of protists in the phyllosphere

Our findings confirm the prevalent opinion that the cercomonad diversity is far from being completely revealed (Bass et al. 2009b; Brabender et al. 2012; Dumack et al. 2016a). Considering microbial communities on leaves that include bacteria but also filamentous fungi, yeasts, algae and protists (Lindow and Brandl 2003) it is surprising that protists predators up to now have gained little consideration in phyllosphere studies. The proven ability of protists to shape bacterial communities and functions (Bonkowski and Clarholm 2012; Flues et al. 2017; Rosenberg et al. 2009) and the ability to prey on a wide range of algae and yeasts from the phyllosphere (Dumack et al. 2017a) could lead to important repercussions on plant performance. Whether this results in positive or negative effects on plant performance needs to be elucidated in further studies and by direct investigations of the communities (i.e.

bacteria, algae, filamentous fungi, yeasts and protists). Our data indicate that the phyllosphere of plants contains complex food webs with different trophic levels. In addition, it is possible that certain phyllosphere protist species exist which have the potential to be implemented in agriculture and might provide a biological alternative for pathogen controls.

Supporting Information

Table S1 - Strains of isolated cercomonad OTUs and corresponding data

OTU	Strain	Taxonomic affiliation	CCAP accession	SSU rDNA accession	Sequence length	Habitat	Plant species
1	SF34	*C. media* FJ790681	-	MG775566	1,421	Rhizosphere	*Trifolium* sp.
	SF42	*C. media* FJ790681	-	MG775567	1,411	Rhizosphere	*Plantago* sp.
2	SF17	*C. hederae* FJ790682	-	MG775583	1,412	Rhizosphere	*Poa* sp.
	SF22	*C. hederae* FJ790682	-	MG775584	1,817	Rhizosphere	*Poa* sp.
	SF27	*C. hederae* FJ790682	-	MG775581	1,412	Rhizosphere	*Trifolium* sp.
	SF38	*C. hederae* FJ790682	-	MG775582	1,342	Rhizosphere	*Plantago* sp.
3	SF3	*C.* sp. HFCC900 HM536145	-	MG775576	1,817	Phyllosphere	*Poa* sp.
	SF4	*C.* sp. HFCC900 HM536145	-	MG775575	1,439	Phyllosphere	*Poa* sp.
	SF23	*C.* sp. HFCC900 HM536145	-	MG775574	1,435	Rhizosphere	*Poa* sp.
	SF35	*C.* sp. HFCC900 HM536145	-	MG775573	1,394	Rhizosphere	*Trifolium* sp.
	SF43	*C.* sp. HFCC900 HM536145	-	MG775572	1,809	Phyllosphere	*Plantago* sp.
	SF53	*C.* sp. HFCC900 HM536145	-	MG775571	1,817	Phyllosphere	*Plantago* sp.
	SF66	*C.* sp. HFCC900 HM536145	-	MG775568	1,819	Rhizosphere	*Plantago* sp.
	SF70	*C.* sp. HFCC900 HM536145	-	MG775570	1,819	Rhizosphere	*Plantago* sp.
	SF76	*C.* sp. HFCC900 HM536145	-	MG775569	1806	Rhizosphere	*Trifolium* sp.
4	SF64	*C. hiberna* FJ790685	-	MG775577	1,810	Phyllosphere	*Poa* sp.
	SF74	*C. hiberna* FJ790685	-	MG775578	1,810	Rhizosphere	*Trifolium* sp.
	SF75	*C. hiberna* FJ790685	-	MG775579	1,810	Rhizosphere	*Trifolium* sp.
	SF78	*C. hiberna* FJ790685	-	MG775580	1,810	Rhizosphere	*Poa* sp.
5	SF67	*C. diparavarians* AF411266	-	MG775587	1,815	Rhizosphere	*Plantago* sp.
6	SF69	*C. laeva* AY884321	-	MG775589	1,826	Rhizosphere	*Plantago* sp.
	SF71	*C. laeva* AY884321	-	MG775590	1,825	Rhizosphere	*Plantago* sp.
7	SF10	-	-	MG775592	750	Phyllosphere	*Plantago* sp.
	SF15	-	-	MG775591	1,344	Phyllosphere	*Plantago* sp.
8	SF52	-	-	MG775588	1,789	Phyllosphere	*Plantago* sp.
9	SF20	*C.* sp. Panama69 FJ790688	-	MG775586	1,818	Rhizosphere	*Poa* sp.
	SF37	*C.* sp. Panama69 FJ790688	-	MG775585	1,401	Rhizosphere	*Trifolium* sp.
10	SF5	*N. braziliensis* FJ790702	-	MG775615	1,815	Phyllosphere	*Trifolium* sp.
	SF18	*N. braziliensis* FJ790702	-	MG775616	1,425	Rhizosphere	*Poa* sp.
	SF24	*N. braziliensis* FJ790702	-	MG775614	770	Rhizosphere	*Poa* sp.
	SF29	*N. braziliensis* FJ790702	-	MG775613	1,397	Rhizosphere	*Trifolium* sp.
11	SF1	*N. ´plasmodialis´* AF411268	-	MG775617	1,816	Phyllosphere	*Poa* sp.
	SF14	*N. ´plasmodialis´* AF411268	-	MG775618	812	Phyllosphere	*Plantago* sp.
12	SF55	ENV: Fr_Dv_15 KT251182	-	MG775600	1,818	Phyllosphere	*Trifolium* sp.
	SF59	ENV: Fr_Dv_15 KT251182	-	MG775601	1,818	Phyllosphere	*Trifolium* sp.
	SF60	ENV: Fr_Dv_15 KT251182	-	MG775602	1,818	Phyllosphere	*Poa* sp.
	SF63	ENV: Fr_Dv_15 KT251182	-	MG775603	1,818	Phyllosphere	*Poa* sp.
13	SF12	*N. epiphylla*	1250/1	MG775605	1,734	Phyllosphere	*Plantago* sp.
	SF2	*N. epiphylla* MG775605	-	MG775604	1,418	Phyllosphere	*Poa* sp.
	SF8	*N. epiphylla* MG775605	-	MG775612	780	Phyllosphere	*Trifolium* sp.
	SF11	*N. epiphylla* MG775605	-	MG775611	719	Phyllosphere	*Plantago* sp.
	SF16	*N. epiphylla* MG775605	-	MG775606	1,393	Phyllosphere	*Plantago* sp.
	SF45	*N. epiphylla* MG775605	-	MG775607	1,818	Phyllosphere	*Plantago* sp.
	SF46	*N. epiphylla* MG775605	-	MG775608	1,818	Phyllosphere	*Plantago* sp.
	SF54	*N. epiphylla* MG775605	-	MG775609	1,818	Phyllosphere	*Trifolium* sp.
	SF62	*N. epiphylla* MG775605	-	MG775610	1,818	Phyllosphere	*Poa* sp.
14	SF31	*N. clavideferens* FJ790704	-	MG775619	1,378	Rhizosphere	*Trifolium* sp.
15	SF21	-	-	MG775620	1,813	Rhizosphere	*Poa* sp.
16	SF41	*N. tuberculata*	1250/3	MG775594	1,816	Rhizosphere	*Plantago* sp.
17	SF9	-	-	MG775595	1,813	Phyllosphere	*Plantago* sp.
18	SF79	*N. nitschei*	1250/2	MG775596	1,817	Rhizosphere	*Poa* sp.
19	SF72	*N. jendrali* HM536150	-	MG775597	1,817	Rhizosphere	*Plantago* sp.
	SF73	*N. jendrali* HM536150	-	MG775598	1,817	Rhizosphere	*Trifolium* sp.
20	SF13	-	-	MG775593	742	Phyllosphere	*Plantago* sp.

21	SF33	*N.* sp. HFCC906 HM536151	-	MG775599	1,357	Rhizosphere	*Trifolium* sp.
22	SF48	*P.* sp. WA10 FJ790719	-	MG775623	1,801	Phyllosphere	*Plantago* sp.
	SF50	*P.* sp. WA10 FJ790719	-	MG775621	1,790	Phyllosphere	*Plantago* sp.
	SF51	*P.* sp. WA10 FJ790719	-	MG775624	1,801	Phyllosphere	*Plantago* sp.
	SF65	*P.* sp. WA10 FJ790719	-	MG775622	1,798	Phyllosphere	*Poa* sp.
23	SF6	*P.* sp. HFCC910 HM536155	-	MG775631	1,802	Phyllosphere	*Trifolium* sp.
	SF19	*P.* sp. HFCC910 HM536155	-	MG775627	1,417	Rhizosphere	*Poa* sp.
	SF25	*P.* sp. HFCC910 HM536155	-	MG775628	1,395	Rhizosphere	*Poa* sp.
	SF28	*P.* sp. HFCC910 HM536155	-	MG775632	1,803	Rhizosphere	*Trifolium* sp.
	SF30	*P.* sp. HFCC910 HM536155	-	MG775633	1,005	Rhizosphere	*Trifolium* sp.
	SF32	*P.* sp. HFCC910 HM536155	-	MG775629	1,315	Rhizosphere	*Trifolium* sp.
	SF36	*P.* sp. HFCC910 HM536155	-	MG775630	1,423	Rhizosphere	*Trifolium* sp.
	SF49	*P.* sp. HFCC910 HM536155	-	MG775626	1,771	Phyllosphere	*Plantago* sp.
	SF57	*P.* sp. HFCC910 HM536155	-	MG7756265	1,801	Phyllosphere	*Trifolium* sp.
24	SF61	-	-	MG775634	1,809	Phyllosphere	*Poa* sp.
25	SF44	-	-	MG775635	1,802	Phyllosphere	*Plantago* sp.
	SF68	-	-	MG775636	1,807	Rhizosphere	*Plantago* sp.
26	SF26	-	-	MG775637	1,742	Rhizosphere	*Trifolium* sp.
27	SF56	-	-	MG775638	1,801	Phyllosphere	*Trifolium* sp.
	SF77	-	-	MG775639	1,808	Rhizosphere	*Poa* sp.
28	SF80	-	-	MG775640	1,801	Rhizosphere	*Poa* sp.

Table S2 - Presence / absence of cercomonad OTUs per habitat for all three plant species and summarized across all plant species. Numbers in brackets represent isolated strains. Total number of OTUs per habitat by plant species combination and summarized across all plant species are given, and number of unique OTUs per plant species and per habitat summarized across all plant species are given

OTU (most likely taxonomic affiliation)	***Poa* sp.**		***Trifolium* sp.**		***Plantago* sp.**		**summarized across all plant species**	
	Phyllo-sphere	Rhizo-sphere	Phyllo-sphere	Rhizo-sphere	Phyllo-sphere	Rhizo-sphere	Phyllo-sphere	Rhizo-sphere
OTU1 (*C. media* FJ790681)	0	0	0	1 (1)	0	1 (1)	0	1 (2)
OTU2 (*C. hederae* FJ790682)	0	1 (2)	0	1 (1)	0	1 (1)	0	1 (4)
OTU3 (*C.* sp. HFCC900 HM536145)	1 (2)	1 (1)	0	1 (2)	1 (2)	1 (2)	1 (4)	1 (5)
OTU4 (*C. hiberna* FJ790685)	1 (1)	1 (1)	0	1 (2)	0	0	1 (1)	1 (3)
OTU5 (*C. diparavarians* AF411266)	0	0	0	0	0	1 (1)	0	1 (1)
OTU6 (*C. laeva* AY884321)	0	0	0	0	0	1 (2)	0	1 (2)
OTU7	0	0	0	0	1 (2)	0	1 (2)	0
OTU8	0	1 (1)	0	1 (1)	0	0	0	1 (2)
OTU9 (*C.* sp. Panama69 FJ790688)	0	0	0	0	1 (1)	0	1 (1)	0
OTU10 (*N. braziliensis* FJ790702)	0	1 (2)	1 (1)	1 (1)	0	0	1 (1)	1 (3)
OTU11 (*N. ´plasmodialis´* AF411268)	1 (1)	0	0	0	1 (1)	0	1 (2)	0
OTU12 (ENV: Fr_Dv_15 KT251182)	1 (2)	0	1 (2)	0	1 (5)	0	1 (9)	0
OTU13 (*N. epiphylla* MG775605)	1 (2)	0	1 (2)	0	0	0	1 (4)	0
OTU14 (*N. clavideferens* FJ790704)	0	0	0	1 (1)	0	0	0	1 (1)
OTU15	0	1 (1)	0	0	0	0	0	1 (1)
OTU16 (*N. tuberculata* MG775594)	0	0	0	0	0	1 (1)	0	1 (1)
OTU17	0	0	0	0	1 (1)	0	1 (1)	0
OTU18 (*N. nitschei* MG775596)	0	1 (1)	0	0	0	0	0	1 (1)
OTU19 (*N. jendrali* HM536150)	0	0	0	1 (1)	0	1 (1)	0	1 (2)
OTU20	0	0	0	0	1 (1)	0	1 (1)	0
OTU21 (*N.* sp. HFCC906 HM536151)	0	0	0	1 (1)	0	0	0	1 (1)
OTU22 (*P.* sp. WA10 FJ790719)	1 (1)	0	0	0	1 (3)	0	1 (4)	0
OTU23 (*P.* sp. HFCC910 HM536155)	0	1 (2)	1 (2)	1 (4)	1 (1)	0	1 (3)	1 (6)
OTU24	1 (1)	0	0	0	0	0	1 (1)	0
OTU25	0	0	0	0	1 (1)	1 (1)	1 (1)	1 (1)
OTU26	0	0	0	1 (1)	0	0	0	1 (1)
OTU27	0	1 (1)	1 (1)	0	0	0	1 (1)	1 (1)
OTU28	0	1 (1)	0	0	0	0	0	1 (1)
Total number	7 (10)	10 (13)	5 (8)	11 (16)	10 (18)	8 (10)	15 (36)	19 (39)
Unique number	15		14		16		9	13

Chapter 2

Grazing of Leaf-Associated Cercomonads (Protists: Rhizaria: Cercozoa) Structures Bacterial Community Composition and Function

Chapter 2 - Grazing of leaf-associated Cercomonads (Protists: Rhizaria: Cercozoa) structures bacterial community composition and function

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Abstract

Preferential food selection in protists is well documented, but we still lack basic understanding on how protist predation modifies the taxonomic and functional composition of bacterial communities. We conducted feeding trials using leaf-associated cercomonad Cercozoa by incubating them on a standardized, diverse bacterial community washed from plant leaves. We used a shotgun metagenomics approach to investigate the taxonomic and functional changes of the bacterial community after five days protist predation upon bacteria. Predation-induced shifts in bacterial community composition could be linked to phenotypic protist traits. Protist reproduction rate, morphological plasticity and cell speed were most important in determining bacterial community composition. Analyses of co-occurrence patterns showed less complex correlations between bacterial taxa in the protist-grazed treatments with a higher proportion of positive correlations than in non-grazed controls, suggesting that predation reduced the influence of strong competitors. Protist predation influenced 14 metabolic core functions including membrane transport from which type VI secretion systems were in particular upregulated. In view of the functional importance of bacterial communities in the phyllosphere and rhizosphere of plants, a more detailed understanding of predator-prey interactions, changes in microbial composition and function, and subsequent repercussions on plant performance are clearly required.

Introduction

Heterotrophic protists are important predators of bacteria on plant surfaces (Bonkowski 2004; Rosenberg et al. 2009), and are known to substantially change both the structure and function of bacterial communities (Kreuzer et al. 2006; Matz and Kjelleberg 2005). Phyllosphere protists are characterized by diurnal life cycles usually with active periods of a few hours at nighttime when dew accumulates on plant leaves (Mueller and Mueller 1970). Although the occurrence of phyllosphere protists has long been recognized (Bamforth 1973), they have been studied until now in terms of their potential as human pathogens or vectors of bacterial pathogens on vegetables (Ciurea-Van Saanen 1981; Gourabathini et al. 2008; Napolitano and Collettieggolt 1984; Napolitano 1982; Rude et al. 1983; Vaerewijck and Houf 2014; Vaerewijck et al. 2011), while their function as bacterivores remained unknown. This is a thoughtful failing knowing that bacteria from the aboveground surface of plants are known to regulate leaf surface characteristics by the production of surfactants (Schreiber et al. 2005), phytohormones (Lindow and Brandl 2003) and toxins (Blanvillain et al. 2007).

Recently it was shown that cercozoan predators exhibit a high diversity in the plant phyllosphere (Ploch et al. 2016). Cercomonad Cercozoa are a diverse and ubiquitous group of terrestrial heterotrophic flagellates (Bass et al. 2009b; Brabender et al. 2012; Geisen et al. 2015a). With their ability to rapidly excyst, feed and multiply within hours (Ekelund 1996) cercomonads are perfectly adapted to the fluctuating environmental conditions in the phyllosphere, but little is known on their impact on the composition and function of leaf-associated bacterial communities.

Bacteria respond rapidly to protist predation by specific alterations in secondary metabolites, cell shape and microevolution (Friman et al. 2014; Jousset 2012; Jousset and Bonkowski 2010; Mazzola et al. 2009; Song et al. 2015; Young 2006), and there is compelling evidence that grazer-induced shifts in bacterial community composition and function are responsible for indirect effects of bacterivores on plant performance (Bonkowski and Clarholm 2012; Bonkowski and Brandt 2002; Krome et al. 2010; Phillips et al. 2003), but virtually nothing is known about interactions of phyllosphere protists with their bacterial prey.

Modern high throughput sequencing techniques now enable us to obtain detailed insights into these interactions by explaining variation in bacterial abundance and community composition at high taxonomic resolution. This is the first study to use shotgun metagenomic sequencing integrated with taxonomic and functional data to examine the structure and function of bacterial communities grazed by leaf-associated protists. We hypothesized that despite grazer-specific food preferences bacterial communities would show general responses to protist predation leading to changes in bacterial community structure and function as well as to specific shifts in the interaction pattern between bacterial lineages at the community scale. We explored the predation effects of two *Cercomonas* and one

Paracercomonas strains isolated from leaf surfaces on a standardized, but diverse bacterial community under controlled conditions using an experimental microcosm approach and shotgun metagenomic sequencing of the whole community.

Material and Methods

Protist isolation, identification and characterization

Three protist strains and a standardized bacterial community were isolated from plant leaf surfaces. Leaves of clover (*Trifolium repens*) and a grass (*Poa sp*.) were sampled from a grassland site on the campus of the University of Cologne, Germany (50°55´30.1"N 6°56´07.4"E) and transferred to the laboratory. Leaves were cut into 25 mm² pieces and each incubated in 1.5 ml Neff´s Modified Amoeba Saline (NMAS) (Page 1976) at room temperature for one week in 24-well plates. To establish monoclonal cultures from phyllosphere Cercozoa, cercomonad cells were picked manually with a tapered glass pipette using an inverted phase-contrast microscope (Nikon Eclipse TS100, Nikon, Tokyo, Japan), and transferred to 60 mm Petri dishes with NMAS containing one sterilized quinoa (*Chenopodium quinoa*) grain as carbon source for bacteria. Cercozoan strains were subcloned several times until free from other eukaryotes, and fed on the accompanying non-identified bacteria.

The isolated and cultivated cercozoan species were identified by their ribosomal 18S rRNA gene which we amplified and sequenced according to Brabender *et al*. (2012). Obtained sequences were aligned, as well as manually arranged in BioEdit 7.0.9.0 (Hall 1999) and subsequently blasted against NCBI GenBank database using Blastn search (Altschul et al. 1990). The protist isolates were sarcomonad bacterivorous flagellates from the phylum Cercozoa, including two *Cercomonas* species (*C. hederae* and *C. plasmodialis*) and one *Paracercomonas* species (*P. producta*). Taxonomic descriptions of these species can be found in Bass *et al*. (2009b) and Brabender *et al*. (2012), and the gene sequences have been submitted to the GenBank database under accession numbers KU926701 - KU926703.

Following Glucksman *et al*. (2010), we characterized a suite of traits for each cercomonad strain. Briefly, cell volume, plasticity, speed and reproduction rate were allocated an ordinal score based on observations of ten individual cells (Table S3). Cell volume was estimated assuming a generalized cone structure for each cell: $(1/3)\pi r^2 h$, where h = cell length in µm. Plasticity was a measure of the cell extent and shape distortions. Speed scores were assigned on the relative rate of movement of cells. Reproduction rates were calculated as described by Koch *et al*. (2005), where cell density was taken as the average number of cells per microscopic field (200x magnification) across four replicate microcosms counted at 24 h intervals after the point of inoculation for 5 days until the end of the incubation period. Each trait was standardized by the maximum value to obtain a scale from zero to one. We were interested in the effect of each trait on the bacterial communities and for each trait, the pairwise dissimilarity between protists was calculated as the Euclidean distance between the standardized traits measurements.

Establishment of monoxenic protist cultures

To eliminate the accompanying bacterial community in each of the protist cultures, we established monoxenic protist cultures prior to the start of the microcosm experiment. Protist cultures were washed three times in sterile NMAS. In particular, this includes a media change and centrifugation at 50 g for 10 min. Small volumes (5 µl) of the washed protist cultures were inoculated into culture flasks (T25, Sarstedt, Nümbrecht, Germany) containing 19 ml NMAS and 1 ml washed cells of *Escherichia coli* overnight culture (10^7 # / ml). Protists were allowed to grow for 7 days to appropriate densities of approximately > 2500 cells ml^{-1}, then repeatedly washed and re-inoculated into NMAS with washed cells of *E. coli*. In this manner, which we repeated five times in total, we diluted out the accompanying bacterial community of each protist strain, resulting in monoxenic protist cultures with *E. coli*.

Bacterial community and microcosm set-up

A diverse, natural bacterial community was obtained from 5 g of fresh leaves incubated at room temperature for 24 h at 60 rpm in 200 ml autoclaved liquid wheat grass medium (WG, 0.15 % w/v vacuum-dry wheat grass powder (Weizengras, Sanatur, Singen, Germany) in PJ medium (Prescott and James 1955)). The resultant bacterial community was subsequently filtered through 5 µm and 1.2 µm cellulose acetate filters (Sartorius, Göttingen, Germany) to remove particles, protozoa, and fungi (Bonkowski and Brandt 2002). The bacteria culture was inspected (200x magnification) prior the start of the microcosm experiment to ensure that the bacteria culture was uncontaminated by small protists. The experimental microcosms were 24-well plates (CLS3526, SIGMA-ALDRICH, MO, USA), each well containing 1 ml sterile WG and 50 µl of the bacterial community. The bacterial communities were incubated for three days at room temperature to develop prior protist inoculation. When we initiated the microcosm experiment approximately 10^2 cells of each protist strain were inoculated into four replicate microcosms and incubated for 5 days. At harvest, after 5 days of incubation we assessed the bacterial community composition using metagenomic profiles obtained by Illumina MiSeq sequencing (see below). Monitoring of reproduction rates ensured that protist growth was sufficient to affect the bacterial communities.

DNA extraction and sequencing

For cell harvest, bacteria were scraped off the bottom and sides of microcosm wells using disposable cell scrapers, subsequently transferred to 2 ml sterile screw cap micro tubes (Sarstedt, Nümbrecht, Germany) and pelleted by centrifugation at 10 000 g for 10 min. 500 µl of the supernatant was removed from each sample and tubes were stored at -80°C. Subsequently, samples were freeze dried for 24 h at -56°C. DNA was extracted using the UltraClean Soil DNA Extraction kit (MO-BIO Laboratories Inc., Carlsbad, CA, USA) and DNA content was assessed with the Qubit 2.0 instrument applying the Qubit dsDNA HS Assay

(Life Technologies, Invitrogen, Darmstadt, Germany). 50 ng of the genomic DNA (gDNA) was used to construct Nextera libraries (Illumina) and sequenced, using 250-bp paired-end sequencing, on a MiSeq platform (Illumina) by the Sequencing Service of the University of Exeter, Exeter, UK.

Annotation of metagenomic sequences and data analysis

Sequences were trimmed by quality (minimum of 20) and length (minimum of 50 bp) using prinseq-lite (Schmieder and Edwards 2011). The quality-controlled unassembled DNA sequences were annotated with the Metagenomics Rapid Annotation (MG-RAST) pipeline version 3.6. (Meyer et al. 2008). BLASTX was used with a minimum alignment length of 50 bp and an *E*-value cut-off of $E < 1 \times 10^{-5}$ (Dinsdale et al. 2008). Taxonomic and functional profiles were generated using the normalized abundance of sequence matches to the RDP and SEED Subsystems database, respectively. A table of the frequency of hits to each individual taxa (taxonomy) or metabolic subsystem (function) for each metagenome was generated and normalized by dividing by the total number of hits to remove bias indifference in read length and sequencing efforts. To identify hits, the RDP database (Cole et al. 2009) was used for the taxonomic assignment to species level at ≥97% sequence identity, while the SEED Subsystems database (Overbeek et al. 2005) was used to generate metabolic profiles. Metagenomes of microbial communities were deposited at the MG-RAST platform (Table S1). To control for potential errors due to unclassified bacterial species across treatments, we removed species-level phylotypes which were unclassified at the genus level and we analyzed the taxonomic dataset at increasing levels (i.e., phylum, class, order, family, genus, species).

Subsequent data visualization and analyses were performed in R version 3.1.1 (R Core Team 2014) unless otherwise specified. Non-metric multidimensional scaling (NMDS) plots were used to visualize the structure among microcosms, using the taxonomic and functional abundance matrix generated as described above. The plots were generated from Bray-Curtis dissimilarities index matrices of the 16 samples. The grazing effect of protists on the bacterial communities was analyzed with three different non-parametric analyses for multivariate data ($\alpha = 0.05$). Analysis of Similarities (ANOSIM) (Clarke 1993), Multiple Response Permutation Procedure (MRPP) (Mielke and Berry 2001) and Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson 2001) using Bray-Curtis dissimilarity were employed to test for differences in bacterial community structure and function across treatments. All tests and ordination plots were performed using the vegan package (Oksanen et al. 2015), and each test was permuted 999 times.

If significant differences in the bacterial community composition were detected with any global ANOSIM, MRPP, or PERMANOVA test, pairwise post hoc tests using PERMANOVA and the General Linear Model (GLM) procedure (McCullagh and Nelder 1989) were performed to determine which bacterial phylotypes changed in the protist treatments in comparison to the non-protist controls. We further expected that the composition of the bacterial community was determined by the suite of traits of the protist strains and significant association between trait dissimilarity matrices and the taxonomic profile dissimilarity matrix was tested using Mantel tests (Legendre and Legendre 2012) from the vegan package.

To determine statistical differences of functional profiles between the protist samples and the non-protist control, the Statistical Analysis of Metagenomic Profiles (STAMP) software package was used (Parks and Beiko 2010). *P*-values were calculated in STAMP using the two sided Fisher´s Exact test (Fisher 1958), while confidence intervals were calculated using the Newcombe-Wilson method (Newcombe 1998). False discovery rate was corrected for using the Benjamini-Hochberg FDR method (Benjamini and Hochberg 1995).

Bacterial co-occurrence patterns and network visualization

Co-occurrence analyses provide useful information for characterizing interactions of microbes at the community scale and bacterial co-occurrence analyses and network visualizations were performed to compare and better understand the taxonomic relations within the grazed and ungrazed microbial communities and to analyze how bacterial co-occurrence patterns were influenced by protist predation. For this purpose, we utilized microbial co-occurrence pattern analyses as described by Williams *et al*. (2014) using pairwise correlations analysis of the taxonomic abundance matrices. We evaluated co-occurrence between all pairs of bacterial species within each treatment using Spearman´s correlation coefficient for which a positive coefficient (Spearman´s rho > 0) indicates positive co-occurrence (i.e., positive interaction between microbes). Negative correlations (indicative of competitive interactions) were also included and we considered negative and positive co-occurrence relationships based on strength of correlation (i.e., $P < 0.05$ from the Spearman´s correlation) at values less than -0.95 or greater than 0.95, taking into account all replicates. To test for differences in bacterial community co-occurrence patterns and to analyze how bacterial co-occurrence patterns were influenced by protist predations, we generated a dissimilarity matrix consisting of Spearman correlation coefficient distances (1-(correlation coefficient)) representing co-occurrence between all pairs of bacterial species within and between the non-protist control and the protist treatment, respectively, using the bioDist package (Ding et al. 2015). Following Williams *et al*. (2014) the calculation of these distances

produces a matrix where microbial taxa rather than treatments were compared to one another, and we used PERMANOVA (Anderson 2001) to test for differences in co-occurrence patterns at the bacterial community level across treatments.

Microbial co-occurrence networks of treatments were visualized using Cytoscape v. 3.2.1 software (Shannon et al. 2003), where the mean taxonomic abundance of bacterial metagenomes is represented at genus level by nodes, and edges showing the union of negative and positive co-occurrence (less than -0.95 or greater than 0.95 with $P < 0.05$) of the bacterial communities at species level. We indicated positive co-occurrence with green and negative co-occurrence with red edges, whereas the edge widths indicate the proportion of species correlations between two genera. Nodes were clustered on class level based on their current taxonomy, and loops indicating co-occurrence relationships of bacterial species within the same genus were removed for reduced network complexity. Bacterial genera and classes which were favored or suppressed by the presence of protists were indicated by green and red nodes, respectively. Finally, bacterial genera which went extinct were represented by nodes with dotted outlines.

Results

Taxonomic and metabolic profiling of metagenomes

A total of 15 367 606 sequences, with an average length of 452 bases were obtained from the 16 microcosms samples (Table S1). Using a cut-off of $E < 1 \times 10^{-5}$ and 50-bp minimal alignment length on the MG-RAST server (Meyer et al. 2008), an average of 91% of sequences were predicted as protein-coding genes. Proteobacteria (85 ± 3%) and Bacteroidetes (13 ± 3%) represented the highest percentages of matches to the RDP database for all samples. Other phyla (Actinobacteria, Gemmatimonadetes, Verrucomicrobia) were represented by < 1% sequences matches. Proteobacteria were composed of Gammaproteobacteria (77.2 ± 4.8%), Betaproteobacteria (6.7 ± 1.7%) and Alphaproteobacteria (1.5 ± 0.6%), whereas Bacteroidetes mainly constituted of Sphingobacteriia (9.0 ± 2.7%) and Flavobacteriia (3.8 ± 0.7%) (Fig. S1).

To predict shifts in bacterial functions, the functional profiles of the protist treatments and the ungrazed non-protist control were analyzed according to the SEED subsystems database. The most prevalent metabolic core function for all samples was amino acids and derivatives, while bacterial genes coding for core metabolic functions including carbohydrate metabolism, protein metabolism and clustering-based subsystems were also abundant in the metagenomes (Table S2).

Comparison of taxonomic and metabolic profiles - Effects of protist predation

In order to quantify the differences in bacterial community structure and function between cercomonad treatments and the ungrazed non-protist control, the taxonomic and functional abundance profiles of bacterial communities were used to compute a Bray-Curtis dissimilarity matrix, and analyzed by non-metric multidimensional scaling (NMDS) (Fig. 1). NMDS revealed clear differences in the microbial community structure and function between the protist and non-protist treatments. All three protist isolates significantly affected the bacterial community composition at the class level compared to the control (Fig. 1A) (PERMANOVA *C. hederae* $F_{1,12} = 16.08$, $P = 0.001$; *C. plasmodialis* $F_{1,12} = 4.07$, $P = 0.044$; *P. producta* $F_{1,12} = 5.63$, $P = 0.02$). These findings were confirmed for bacterial ranks from class to genus level, while at the bacterial species level grazer-specific effects dominated and only *C. hederae* and *P. producta* were still found to affect bacterial composition ($F_{1,12} = 3.38$, $P = 0.001$ and $F_{1,12} = 1.95$, $P = 0.031$, respectively), but not *C. plasmodialis* ($F_{1,12} = 1.22$, $P = 0.241$). Pairwise comparison of taxonomic profiles from each protist treatment with the ungrazed non-protist control using General Linear Models (GLM) (McCullagh and Nelder 1989) showed that Alphaproteobacteria ($P = 0.013$), Betaproteobacteria ($P < 0.001$) and Sphingobacteriia ($P < 0.001$) decreased by half in presence of *C. hederae*, whereas

Gammaproteobacteria ($P < 0.001$) increased by 15% and Opitutae ($P = 0.023$) doubled. Similarly, Gammaproteobacteria increased and Sphingobacteriia decreased also in presence of *C. plasmodialis* and *P. producta*. All other bacterial classes were only marginally or not affected by protist predation.

Specific predation-induced shifts in bacterial community composition were also clearly seen at the genus level (PERMANOVA *C. hederae* $F_{1,12} = 9.95$, $P = 0.001$; *C. plasmodialis* $F_{1,12} = 3.51$, $P = 0.032$; *P. producta* $F_{1,12} = 3.56$, $P = 0.024$). GLM revealed that in the *C. hederae* treatment 9 genera were suppressed and 2 favored. Within Alphaproteobacteria, *Rhizobium* ($P = 0.021$) and *Acetobacter* ($P = 0.048$) were reduced by over 84% while *Brevundimonas* ($P = 0.021$) completely disappeared. Among Betaproteobacteria the genera *Burkholderia* ($P = 0.037$), *Comamonas* ($P = 0.034$), *Duganella* ($P = 0.002$), *Janthinobacterium* ($P < 0.001$) and *Zoogloea* ($P = 0.027$) decreased by at least 62%, also *Pedobacter* ($P < 0.001$), belonging to the Sphingobacteriia class was reduced by half. Despite these strong reductions, Gammaproteobacteria were positively affected by *C. hederae* where the genera *Pseudomonas* ($P < 0.001$) increased by 16% and *Stenotrophomonas* ($P = 0.041$) tripled (Fig. 2). Among the class Opitutae, *Diplosphaera* appeared to increase by factor of 3.5 and *Opitutus* by 87%, but due to variation among treatments with only marginal significance ($P = 0.071$ and $P = 0.091$, respectively). In the *C. plasmodialis* treatment 4 genera were suppressed and 3 favored. Among Alphaproteobacteria, *Rhizobium* ($P = 0.035$) completely disappeared in presence of *C. plasmodialis*. Within Betaproteobacteria, the relative abundance of *Burkholderia* ($P = 0.016$) and *Comamonas* ($P = 0.021$) decreased to a quarter, whereas *Herbaspirillum* ($P = 0.008$) increased by 61%. *Pedobacter* ($P < 0.001$) from the Sphingobacteriia class declined by 38%. Within Gammaproteobacteria *Pseudomonas* ($P = 0.027$) responded with an increase of 8% in presence of *C. plasmodialis,* and *Diplosphaera* ($P = 0.044$) among Opitutae, increased by a factor of 4 (Fig. 2). Also the relative abundance of *Zymomonas* (Alphaproteobacteria) doubled and *Duganella* as well as *Zoogloea* (Betaproteobacteria) was reduced by half, albeit with marginal significance ($P = 0.055$, $P = 0.051$ and $P = 0.056$, respectively). *P. producta* changed the read abundance of 3 genera. Alphaproteobacteria were not affected, but *Burkholderia* ($P = 0.021$) among Betaproteobacteria decreased by 70%, whereas *Kinetoplastibacterium* ($P = 0.044$) increased by factor of 2.8 (Fig. 2). Moreover, *Janthinobacterium* was reduced by a quarter and *Rhodoferax* tripled, but this trend could statistically not be verified ($P = 0.096$ and $P = 0.068$, respectively). Concordant with the other two protist treatments, *Pedobacter* ($P = 0.002$) was also reduced (-30%) in presence of *P. producta*. Among Gammaproteobacteria again *Pseudomonas* tended to increase by 7% ($P = 0.056$) and *Stenotrophomonas* by a factor of 2.6 albeit with marginal significance ($P = 0.083$).

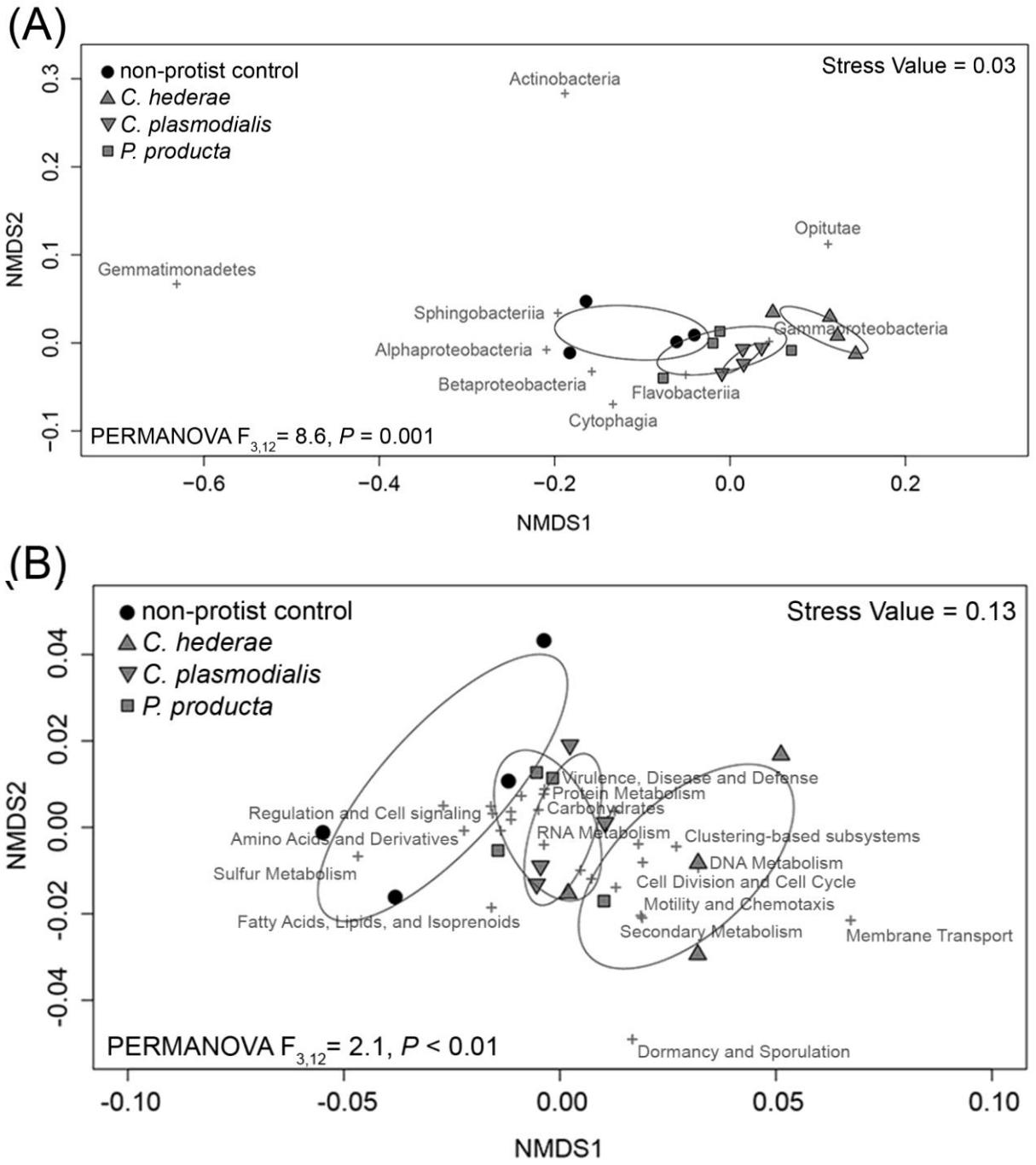


Figure 1 - Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities among 16 samples from microcosm experiment. (**A**) Taxonomic analyses using relative abundance based on RDP bacterial matches at class level. (**B**) Functional analyses using relative abundance based on SEED bacterial matches at subsystem level 1. Ellipses indicate 95% confidence limits of standard errors. PERMANOVA values are shown in the down left and stress values are shown in the upper right of the graphs.

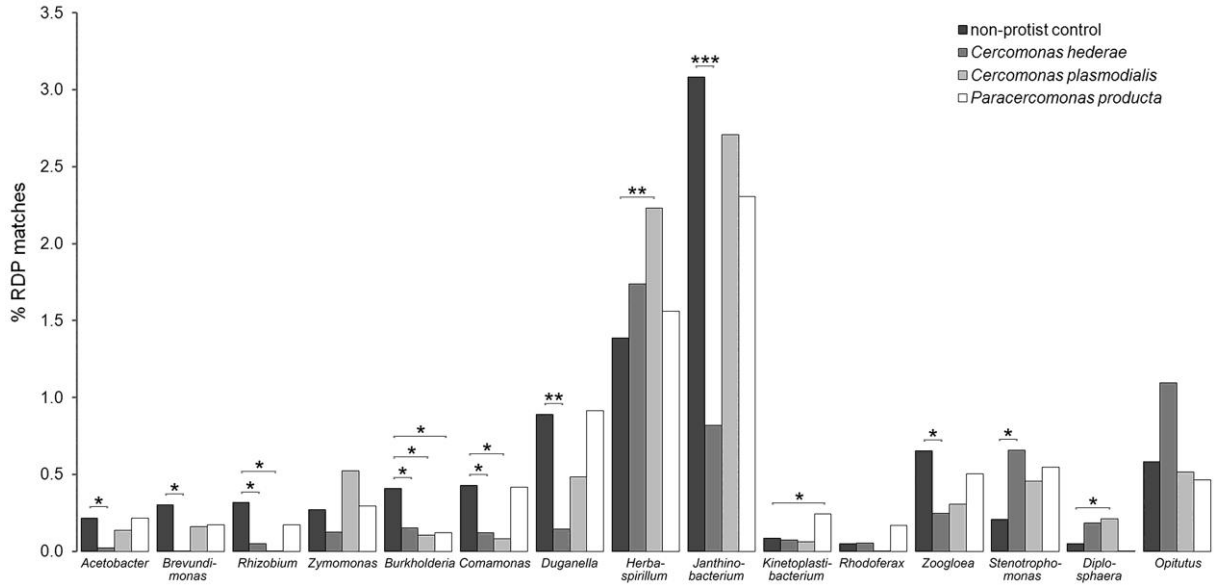


Figure 2 - Comparison of taxonomic profiles at genus level taxonomy from microcosm experiment. Frequency distribution (relative % of bacterial RDP matches) of taxonomy in the non-protist control and the protist samples.

Subsequently we indentified the phenotypic traits of cercomonads associated with grazing preferences. Among the four protist traits tested using Mantel tests, reproduction rate ($r = 0.23$, $P = 0.048$), plasticity ($r = 0.23$, $P = 0.047$), and protist cell speed ($r = 0.23$, $P = 0.042$), but not protist cell volume ($r = 0.02$, $P = 0.374$) affected the grazer-impact on bacterial taxonomic profiles. These associations remained statistically significant from bacterial genus to class level, but could be confirmed on species level only with marginal significance (i.e., $r = 0.18$, $P < 0.09$).

Protist predation significantly affected bacterial functional profiles (Fig. 1B). Most functional cores were represented by < 5% of sequence matches to the SEED subsystem database (Fig. 3A-C). Pairwise comparison of functional profiles from each protist treatment with the ungrazed non-protist control using the Statistical Analysis of Metagenomic Profiles (STAMP) software package (Parks and Beiko 2010) revealed a differential representation of 27 functional cores among the protist treatments. A total of 14 functional cores were equally over- or underrepresented among the three cercomonad treatments. Among them, seven functional cores including membrane transport and DNA metabolism were overrepresented relative to the control, whereas seven functional cores including amino acids and derivatives and carbohydrate metabolism were underrepresented (Fig. 3D-F). Finer levels (subsystem level) of resolution within the functional core membrane transport revealed that the type VI protein secretion system (T6SS) was significantly upregulated in the cercomonad treatments, while the ungrazed control had an overrepresentation of sequences matching uni-, sym- and antiporters, protein translocation across cytoplasmic membrane and type VIII protein secretion system (T8SS) (Fig. 4A-C). Additionally, within the functional core

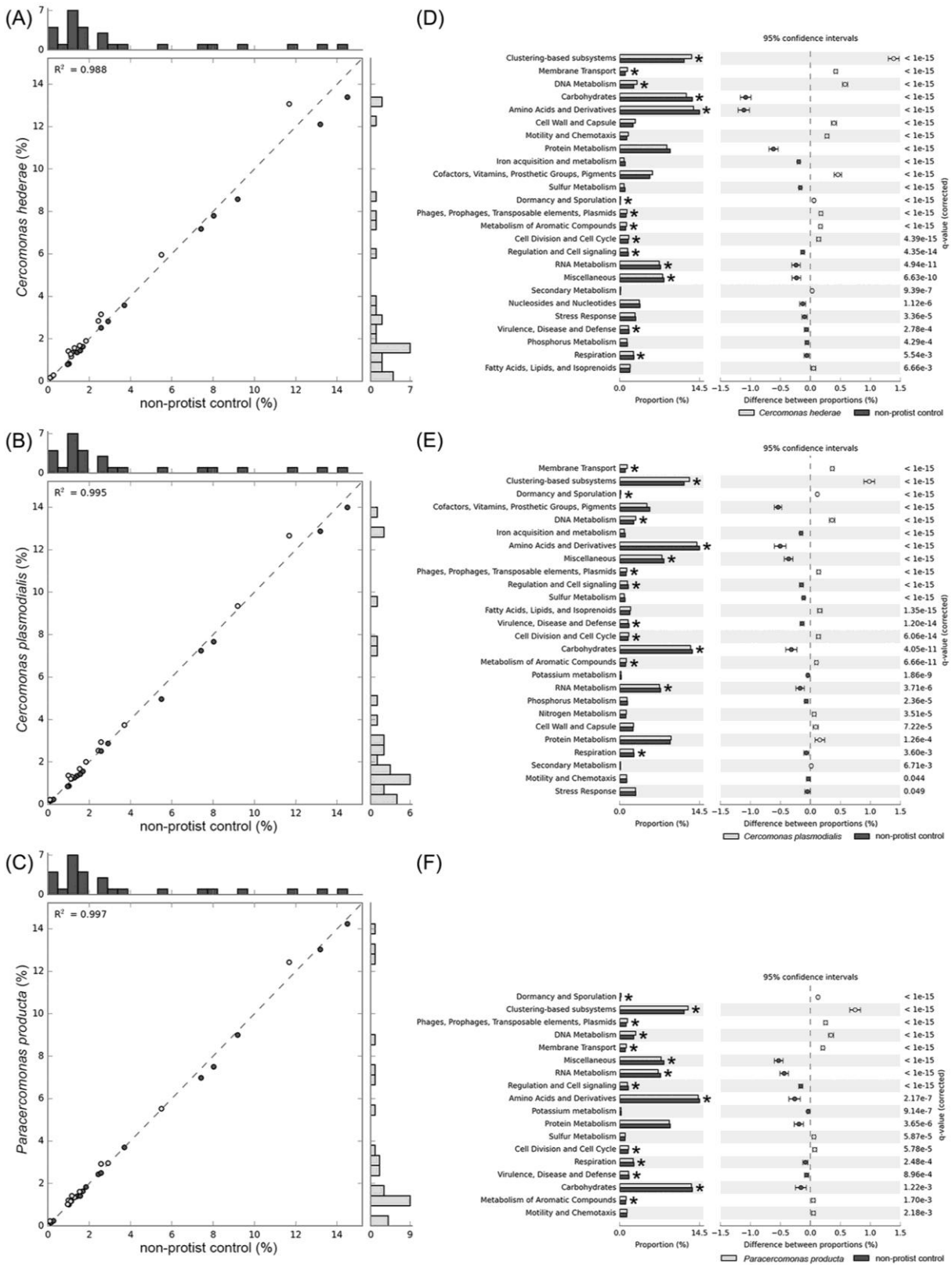


Figure 3 - Comparison of functional profiles between protist samples (grey) and non-protist control (black). (**A-C**) Scatter plots showing differences for functional categories (SEED subsystem level 1). (**D-F**) Functional categories (SEED subsystem level 1) statistically different between protist samples and non-protist control. Categories overrepresented in the protist treatments (grey) correspond to positive differences between proportions and categories overrepresented in the non-protist control treatment (black) correspond to negative differences between proportions. Asterisks (*) indicate categories equally enriched or depleted among protist samples (*P*-value < 0.05). Corrected *P*-values (q-values) were calculated using Benjamini-Hochberg false discovery rate approach (*P* < 0.05).

DNA metabolism, subsystem level DNA repair was upregulated in the protist treatments, while DNA replication and metabolism were overrepresented in the ungrazed control (Fig. 4D-F). Also within the functional core amino acids and derivatives, subsystem level branched-chain amino acids were overrepresented in the protist treatments (Fig. S2A). Other subsystem levels of functional cores were only marginally affected by protist predation (Fig. S2).

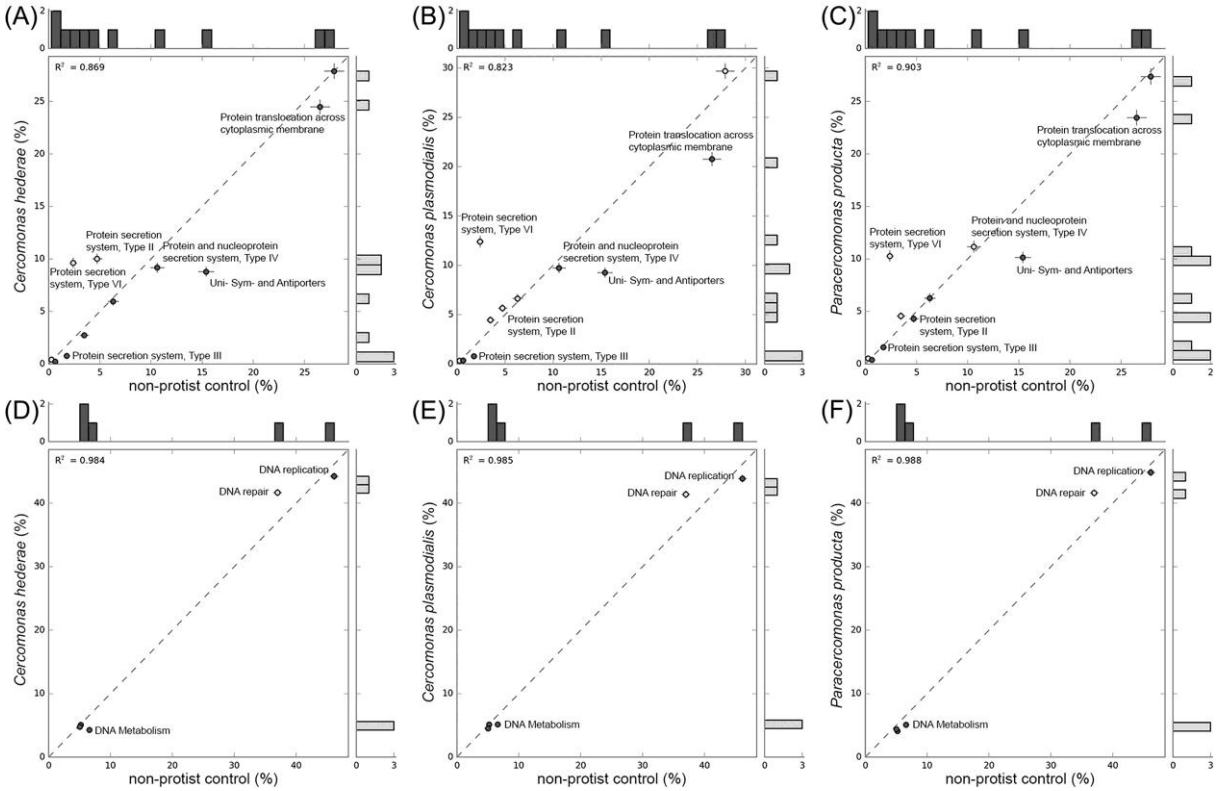


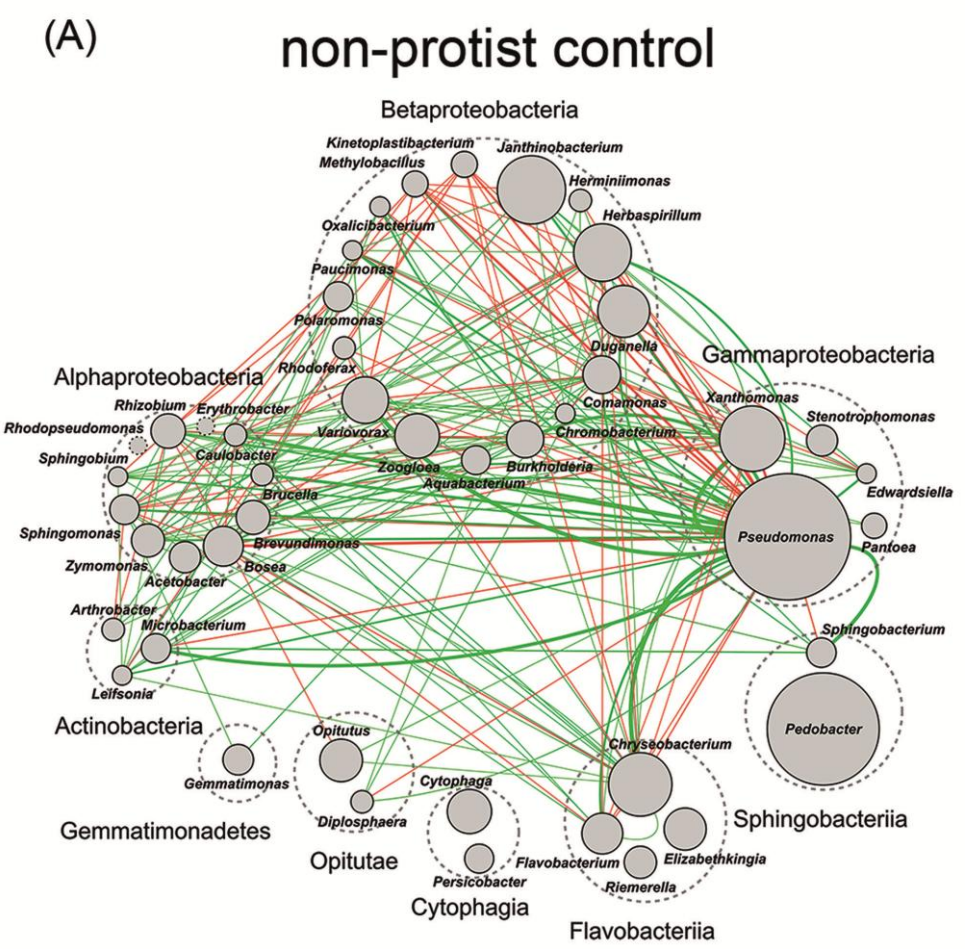
Figure 4 - Comparison of functional profiles between protist samples (grey) and non-protist control (black). (**A-C**) Scatter plot showing differences for membrane transport (SEED subsystem level 2). (**D-F**) Scatter plot showing differences for DNA metabolism (SEED subsystem level 2).

Differences in microbial co-occurrence patterns among treatments and co-occurrence networks

Co-occurrence relationships are ecologically important patterns that reflect interactions between organisms within biological communities and can provide valuable information for characterizing ecological interactions of microbes at the community scale (Williams et al. 2014). Thus bacterial co-occurrence analyses and network visualizations were performed to better understand the taxonomic relations within the grazed and ungrazed microbial communities. We evaluated co-occurrence between bacterial species within each treatment using Spearman´s correlation coefficients for which a positive coefficient (Spearman´s rho > 0.95 with $P < 0.05$) indicates positive co-occurrence and vice versa, taking into account all replicates. To analyze how bacterial co-occurrence patterns were influenced by protist

predation, we generated a dissimilarity matrix consisting of Spearman correlation coefficient distances (1-(correlation coefficient)) representing co-occurrence between all pairs of bacterial species within and between the non-protist control and the protist treatment, subsequently we used PERMANOVA to test for differences in co-occurrence patterns at the bacterial community level across treatments.

The non-protist control treatment showed 622 significant bacterial species correlations from which 79% were positive. In presence of protist grazers the total number of significant bacterial species correlations declined by 15% and 30% from 622 to 529 (grazed by *P. producta*) and 438 (grazed by *C. plasmodialis*), respectively; while the proportion of positive bacterial species correlations increased from 79% to 85% (Table 1). When we quantified differences in bacterial community co-occurrence across treatments using PERMANOVA and Spearman´s distance, both *Cercomonas* treatments showed a clear difference in co-occurrence patterns in comparison to the non-protist control (*C. hederae* $F_{1,444} = 10.90$, $P < 0.001$; *C. plasmodialis* $F_{1,444} = 8.10$, $P < 0.001$), while the predation-impact of *Paracercomonas* was not so strong ($F_{1,444} = 2.31$, $P = 0.086$). In the non-protist control, the top five bacterial genera that presented most correlations on species level were *Pseudomonas*, *Chryseobacterium*, *Bosea*, *Burkholderia* and *Brucella*, while *Arthrobacter* and *Herbaspirillum* gained a high number of correlations in the protist treatments. Patterns due to the pattern of predation-induced shifts in microbial interactions became clearly visible in networks of bacterial co-occurrence, where the taxonomic abundance was represented at the genus level (Fig. 5).


(A)
non-protist control
Betaproteobacteria
Kinetoplastibacterium
Janthinobacterium
Methylobacillus
Herminiimonas
Herbaspirillum
Oxalicibacterium
Paucimonas
Polaromonas
Duganella
Rhodoferax
Alphaproteobacteria
Gammaproteobacteria
Rhizobium
Erythrobacter
Comamonas
Xanthomonas
Stenotrophomonas
Rhodopseudomonas
Variovorax
Chromobacterium
Sphingobium
Caulobacter
Zoogloea
Burkholderia
Aquabacterium
Edwardsiella
Brucella
Sphingomonas
Pseudomonas
Pantoea
Brevundimonas
Zymomonas
Bosea
Acetobacter
Arthrobacter
Microbacterium
Sphingobacterium
Leifsonia
Pedobacter
Actinobacteria
Opitutus
Chryseobacterium
Gemmatimonas
Cytophaga
Sphingobacteriia
Diplosphaera
Gemmatimonadetes
Opitutae
Flavobacterium
Elizabethkingia
Persicobacter
Riemerella
Cytophagia
Flavobacteriia
(B)
Cercomonas hederae
Betaproteobacteria
Kinetoplastibacterium
Janthinobacterium
Methylobacillus
Herminiimonas
Herbaspirillum
Oxalicibacterium
Paucimonas
Polaromonas
Duganella
Rhodoferax
Alphaproteobacteria
Gammaproteobacteria
Comamonas
Rhizobium
Erythrobacter
Xanthomonas
Stenotrophomonas
Variovorax
Chromobacterium
Rhodopseudomonas
Zoogloea
Burkholderia
Sphingobium
Caulobacter
Aquabacterium
Edwardsiella
Brucella
Sphingomonas
Pseudomonas
Pantoea
Brevundimonas
Zymomonas
Bosea
Acetobacter
Arthrobacter
Microbacterium
Sphingobacterium
Leifsonia
Pedobacter
Actinobacteria
Chryseobacterium
Opitutus
Gemmatimonas
Cytophaga
Sphingobacteriia
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Flavobacterium
Elizabethkingia
Persicobacter
Riemerella
Cytophagia
Flavobacteriia

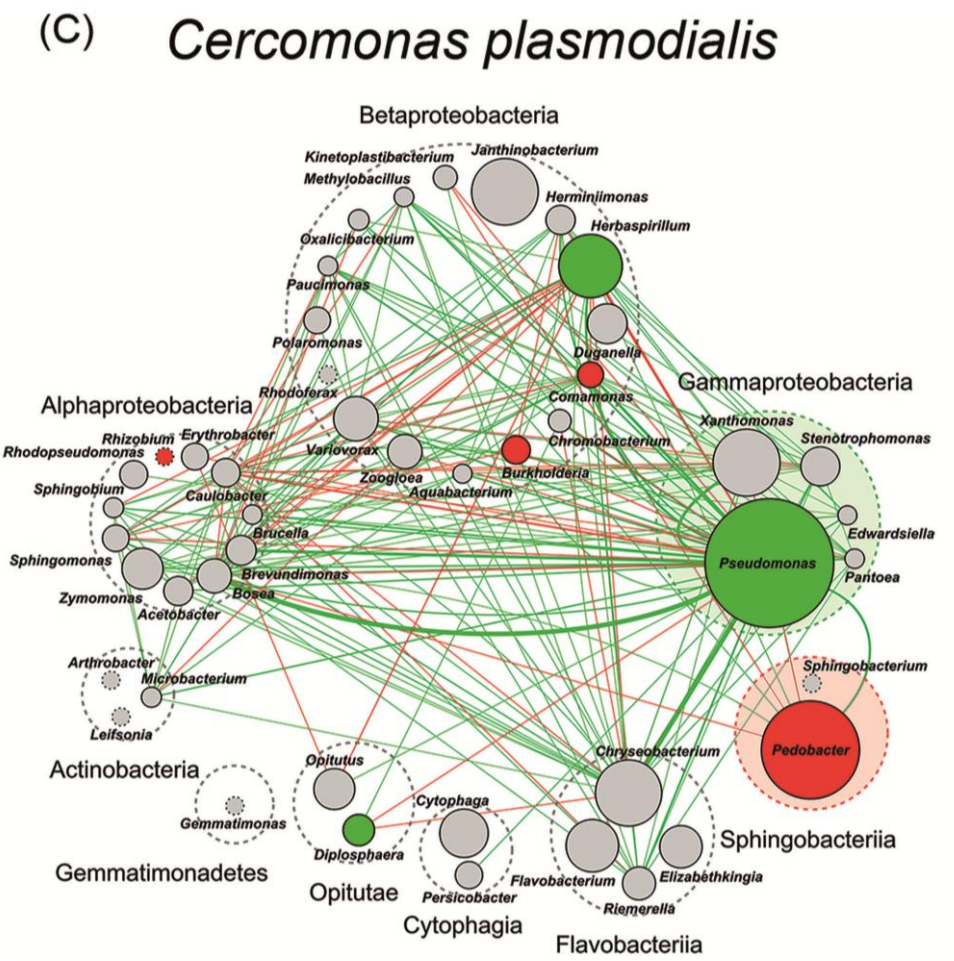

(C) Cercomonas plasmodialis
Betaproteobacteria
Kinetoplastibacterium
Janthinobacterium
Methylobacillus
Herminiimonas
Herbaspirillum
Oxalicibacterium
Paucimonas
Polaromonas
Duganella
Rhodoferax
Comamonas
Alphaproteobacteria
Gammaproteobacteria
Xanthomonas
Rhizobium
Erythrobacter
Stenotrophomonas
Rhodopseudomonas
Variovorax
Chromobacterium
Zoogloea
Burkholderia
Sphingobium
Aquabacterium
Caulobacter
Edwardsiella
Brucella
Sphingomonas
Pseudomonas
Pantoea
Brevundimonas
Bosea
Zymomonas
Acetobacter
Arthrobacter
Sphingobacterium
Microbacterium
Pedobacter
Leifsonia
Chryseobacterium
Actinobacteria
Opitutus
Cytophaga
Gemmatimonas
Sphingobacteriia
Diplosphaera
Flavobacterium
Elizabethkingia
Gemmatimonadetes
Opitutae
Persicobacter
Riemerella
Cytophagia
Flavobacteriia
(D) Paracercomonas producta
Betaproteobacteria
Kinetoplastibacterium
Janthinobacterium
Methylobacillus
Herminiimonas
Oxalicibacterium
Herbaspirillum
Paucimonas
Polaromonas
Duganella
Alphaproteobacteria
Rhodoferax
Gammaproteobacteria
Rhizobium
Erythrobacter
Comamonas
Xanthomonas
Stenotrophomonas
Rhodopseudomonas
Variovorax
Chromobacterium
Zoogloea
Burkholderia
Sphingobium
Aquabacterium
Caulobacter
Edwardsiella
Brucella
Sphingomonas
Pseudomonas
Pantoea
Brevundimonas
Bosea
Zymomonas
Acetobacter
Arthrobacter
Sphingobacterium
Microbacterium
Pedobacter
Leifsonia
Chryseobacterium
Actinobacteria
Opitutus
Gemmatimonas
Cytophaga
Sphingobacteriia
Diplosphaera
Gemmatimonadetes
Flavobacterium
Elizabethkingia
Opitutae
Riemerella
Persicobacter
Cytophagia
Flavobacteriia

Figure 5 - Microbial co-occurrence networks based on correlation analysis from taxonomic profiles of microcosm treatments. The relative abundance of bacterial metagenomes are represented on genus level by nodes, whereas pseudomonads are represented on a log scale for illustration reasons. A connection shows the union of negative or positive co-occurrence of the bacterial communities on species level and stands for strong (Spearman´s $r < -0.95$ or $r > 0.95$) and significant ($P < 0.05$) correlation. We indicated positive co-occurrences with green and negative co-occurrences with red edges, whereas the edge widths indicate the proportion of species correlations between two genera. Nodes were clustered on class level based on their current taxonomy and loops that indicate co-occurrence relationships of bacterial species from the same genus were removed. Bacterial genera and classes which were favored or suppressed by the presence of protist are indicated with green and red nodes, respectively. Further, bacterial genera which were not present in the treatment are represented by nodes with dotted outlines.

Table 1 – Number of correlations as inferred by Spearman. The total numbers of pairwise correlations as well as significant correlations ($P < 0.05$) among taxonomic profiles (species level) based on RDP bacterial matches are shown.

	non-protist control	*C. hederae*	*C. plasmodialis*	*P. producta*
Pairwise correlations	10519	8023	7881	10355
Significant correlations	622	458	438	529
Significant positive correlations	490	386	372	451
Significant negative correlations	132	72	66	78

Discussion

Leaf-associated microbial communities are considered to be species rich, but are generally less diverse than rhizosphere or bulk soil communities (Vorholt 2012). The taxonomic profiles of our bacterial communities were dominated by Proteobacteria and Bacteroidetes and showed similarity to typical phyllosphere communities on genus level (Bai et al. 2015; Rastogi et al. 2013; Vorholt 2012), despite cultivation in microcosm wells.

Protist predation significantly altered bacterial community composition (PERMANOVA $F_{3,12}$ = 8.6, P = 0.001) and led to specific changes at class level from Alphaproteobacteria, Betaproteobacteria and Sphingobacteriia towards Gammaproteobacteria and Opitutae (Fig. 1A). *C. hederae* was the strongest bacterial grazer (Table S3), while *C. plasmodialis* and *P. producta* only significantly influenced Gammaproteobacteria and Sphingobacteriia. These data support earlier general findings that Alpha- and Betaproteobacteria are less resistant to grazing (Boenigk et al. 2004; Murase et al. 2006; Rosenberg et al. 2009), highlighting the importance of 'top-down' processes for structuring bacterial communities. When we tested at the genus level, our data indicate that losses in bacterial numbers of one taxon were compensated by other genera. Bacterial gains and losses were however not random but showed surprisingly regular patterns. The bacterial taxa *Burkholderia, Zoogloea* (Betaproteobacteria) and *Pedobacter* (Sphingobacteriia) were consistently suppressed while *Pseudomonas* increased in all protist treatments, indicating clearly deterministic patterns of bacterial community assembly when subjected to predation. In addition both *Cercomonas* species favored *Diplosphaera* (Opitutae), but strongly suppressed *Rhizobium* (Alphaproteobacteria), and also *Comamonas* and *Duganella* thus leading to a general and strong reduction of Betaproteobacteria. Especially the strong decrease in *Duganella* and *Janthinobacterium* in presence of *C. hederae* was surprising, since both genera contain taxa producing violacein (Choi et al. 2015), which is highly toxic to several protists (Matz and Kjelleberg 2005). The other protist taxa did not proliferate as well as *C. hederae* on the bacterial communities. At the end of the experiment, total biomass of *C. hederae* compared to *C. plasmodialis* and *P. producta* increased by factors of 1.9 and 3.1, respectively (Table S3). Consequently, their predation-induced responses of the other bacterial genera were more variable in magnitude, but the general direction of the response, being positive or negative, was the same for all protist strains tested.

These shifts in bacterial community composition could be linked to functional traits of the predators. Most importantly, multiplication rates of cercomonads were a major predictor of shifts in bacterial community composition. Multiplication rate is a function of efficiency in prey capture and grazing resistance to predators. Concordant with findings of Glucksman *et al*. (2010), we could clearly link prey capture efficiency to the mode of protist movement

(plasticity and cell speed), but we could not detect an effect of predator size (i.e., cell volume) as for other protists (Boenigk et al. 2004; Glücksman et al. 2010; Pfandl et al. 2004). Since only one *Paracercomonas* species was included in the experiment, we could not differentiate whether its low predation impact was species or genus specific. *P. producta* might have a more specialized feeding niche or is more vulnerable to bacterial defences (Jousset 2012).

Bacterial co-occurrence networks clearly changed in presence of predators. The lower number of correlations in the protist treatments and the parallel increase in the relative amount of positive correlations between bacterial taxa indicate that predation reduced the influence of strong competitors, leading to weaker interaction strength in grazed communities (Fig. 5). *Pseudomonas*, *Chryseobacterium* and *Bosea* were consistently found among the top five bacterial genera that had most correlations on species level. *Arthrobacter* and *Herbaspirillum* showed a high number of correlations exclusively in the protist treatments. Interestingly *Arthrobacter* and *Herbaspirillum* did not belong to the most abundant genera, indicating an increasing competitive advantage of formerly subordinate species in response to predation (Bell et al. 2010; Jousset et al. 2008).

Our data clearly show consistent patterns in transcriptional changes of the grazed bacterial communities, but due to the general lack of information how these affect microbial function, we will discuss only impacts on membrane transport, DNA and amino acid metabolism. Membrane transport and DNA metabolism were overrepresented in the protist treatments and most influenced by protist predation. Within membrane transport an overrepresentation of uni-, sym- and antiporters, protein translocation across cytoplasmic membrane and T8SS was seen in the ungrazed non-protist control. Various strains of bacteria are able to produce a unique class of functional amyloids termed curli, which are secreted via the T8SS and critical for biofilm formation, host cell adhesion, and colonization of inert surfaces. The downregulation of T8SS in the protist treatments might indicate a reduced surface colonization in response to predation. Residing in the water column can be expected to reduce predation risk by surface feeding protists such as *Cercomonas* and *Paracercomonas*; however, the relevance of this strategy for leaf habitats may be limited. Moreover, we found a high abundance of sequences affiliated with T6SS in the protist treatments. T6SS have been found in a quarter of all proteobacterial genomes, including animal, plant, human pathogens, as well as soil, environmental or marine bacteria (Bingle et al. 2008; Cascales 2008). Early studies of T6SS focused on its role in the pathogenesis of higher organisms, more recent studies suggested a role in the defense against protists and competing bacteria (Coulthurst 2013; Hood et al. 2010; Schwarz et al. 2010). The overrepresentation of T6SS in the protist treatments suggests that the grazed bacterial communities upregulated their T6SS to defend themselves against protist predation by the injection of antimicrobial toxins. Finer levels (subsystem level) of resolution within the functional core DNA metabolism revealed an overrepresentation of DNA repair in the protist treatments, whereas DNA replication and

metabolism were overrepresented in the ungrazed control. An upregulation of DNA repair could indicate a response of some bacteria to the upregulation of T6SS of competitor bacterial cells. The core metabolic function in each of our microcosm treatments was related to amino acids and derivatives; and on finer levels (subsystem level) an overrepresentation of branched-chain amino acids was seen in the protist treatments compared with the non-protist control (Fig. S2A). The enzymatic repertoire for the catabolism of branched-chain amino acids has been reported for a wide variety of bacteria (Massey et al. 1976), but particularly in Pseudomonads (Stanier et al. 1966). The strong enhancement of pseudomonads in the protist treatments thus might have shifted the physiological capacity of the whole bacterial community.

Bass *et al*. (2009b) and Howe *et al*. (2009) identified some Cercozoa clades that seemed to be specifically associated with the phylloplane habitat. Is protist grazing on phyllosphere bacteria functionally important? The genus *Pseudomonas* contains important phyllosphere pathogens, such as *P. syringae* (Hirano and Upper 1990), as well as strains with potential plant growth-promoting (Lugtenberg et al. 2013) and biocontrol activities (Haas and Defago 2005). In a review Lindow (2006) reports an unpublished experiment where *C. cucullus* reduced numbers of inoculated *P. syringae* on bean leaves by two orders of magnitude, showing that i) potential pathogens could be substantially reduced, and ii) pseudomonads may not be favored by all protist taxa, or at least consumed when predators have little food choice. Also the genus *Burkholderia* contains both, important leaf pathogens (Compant et al. 2008) and strains beneficial for plant growth (Mahenthiralingam et al. 2008). As well as genera like *Herbaspirillum* and *Stenotrophomonas* which are known to enhance plant growth (Ryan et al. 2009; Schmid et al. 2006) were favored by protist grazing. Our data confirm significant shifts in abundance and co-occurrence of all these bacterial taxa and therefore it is likely that protist grazers may have indirect effects on plant performance that are not considered yet in studies on the assembly and function of the phyllosphere microbiome. Further, our results clearly demonstrate that leaf-associated protists can have a significant impact on the taxonomic composition, as well as on the physiological function of bacterial communities with potential effects on plant performance. Since microbial communities on leaf surfaces are spatially highly structured (Esser et al. 2015), they offer ideal conditions for group selection processes (Goodnight 2011).

In our experiment protists had specific feeding preferences as well as complementary grazing effects on the bacterial communities. These effects were confirmed at the bacterial class and genus level, structured the bacterial community composition and function as well as changed competitive relationships of subordinate bacterial genera. Accordingly, protist predation produced reproducible patterns in bacterial community assembly. Such reproducible shifts in bacterial community composition are a fundamental prerequisite for predation-induced indirect effects on plant performance. The bacterial genera consistently

favored (e.g. *Pseudomonas*) or reduced (e.g. *Burkholderia*) by protist grazers are known to contain both important leaf pathogens as well as bacteria beneficial to plant growth. Further analysis and direct investigations on the inhabiting phyllosphere communities (i.e., bacteria, protozoa, fungi) are needed to better understand the mechanisms by which predation-induced changes in bacterial community structure and function caused by leaf-associated protists leads to repercussions on plant performance. The strong upregulation of T6SS in protist treatments suggests that prokaryotes evolved and activate direct defenses against protists by the injection of toxins or effector molecules into their predators. Only few transcriptional changes of the grazed bacterial communities could be so clearly interpreted. However, our results clearly confirm that the composition and function of natural bacterial communities might not be understood if protist grazers are not considered (Trap et al. 2016).

Supporting Information

Table S1 - Number of sequencing reads, base pairs, reads assigned to SEED Subsystems, percentages of predict proteins and taxonomic abundances of the bacteria domain after quality control on MG-RAST pipeline.

Sample ID	MG-RAST ID	Sample	N. of sequence reads	Mean sequence length	Total bp*	N. of predict Subsystems functions*	% of predicted proteins*	Taxonomic abundances of bacteria*
CHa	4551357.3	*Cercomonas hederae* Sample A	999,612	439±64	378,187,120	957,860	91.7	1,547,005
CHb	4551359.3	*Cercomonas hederae* Sample B	526,769	454±66	219,827,807	507,216	88.6	829,589
CHc	4551361.3	*Cercomonas hederae* Sample C	1,115,838	445±64	432,818,930	1,052,834	89.9	1,665,576
CHd	4551363.3	*Cercomonas hederae* Sample D	1,051,911	459±65	442,766,119	1,034,560	91.1	1,630,505
CPa	4551373.3	*Cercomonas plasmodialis* Sample A	1,012,837	449±64	399,302,062	985,595	91.1	1,596,453
CPb	4551375.3	*Cercomonas plasmodialis* Sample B	710,553	466±68	293,893,606	675,761	90.0	1,115,836
CPc	4551377.3	*Cercomonas plasmodialis* Sample C	1,222,650	443±64	469,477,032	1,148,968	90.1	1,884,907
CPd	4551379.3	*Cercomonas plasmodialis* Sample D	862,672	464±66	368,354,076	856,294	91.0	1,398,323
PPa	4551381.3	*Paracercomonas producta* Sample A	1,045,232	448±64	413,380,170	1,013,052	90.7	1,677,846
PPb	4551383.3	*Paracercomonas producta* Sample B	1,016,260	455±66	428,265,125	1,016,661	91.9	1,658,452
PPc	4551385.3	*Paracercomonas producta* Sample C	1,060,419	445±63	417,388,657	1,030,147	90.8	1,714,438
PPd	4551387.3	*Paracercomonas producta* Sample D	958,514	457±66	405,639,781	954,819	91.5	1,577,850
CTRLa	4551365.3	non-protist control Sample A	900,254	446±64	356,866,618	869,110	89.7	1,470,151
CTRLb	4551367.3	non-protist control Sample B	931,751	458±65	396,067,988	924,635	90.8	1,521,934
CTRLc	4551369.3	non-protist control Sample C	1,009,379	449±64	400,290,018	990,344	91.4	1,669,697
CTRLd	4551371.3	non-protist control Sample D	942,955	459±66	400,411,517	945,873	91.8	1,514,022

* Post Quality Control

Taxonomic hits were generated using data from the M5NR ontology with a minimum alignment length of 15 bp and an *E*-value cut-off of $E < 1 \times 10^{-5}$.

Table S2 - Relative proportions of matches to a given subsystem hierarchy level 1.

Subsystem hierarchy level 1	**non-protist control**	***Cercomonas hederae***	***Cercomonas plasmodialis***	***Paracercomonas producta***
Amino Acids and Derivatives	0.1450	0.1339	0.1399	0.1424
Carbohydrates	0.1319	0.1211	0.1287	0.1303
Clustering-based subsystems	0.1168	0.1307	0.1266	0.1243
Protein Metabolism	0.0919	0.0858	0.0935	0.0900
Miscellaneous	0.0803	0.0780	0.0767	0.0750
RNA Metabolism	0.0742	0.0718	0.0724	0.0698
Cofactors, Vitamins, Prosthetic Groups, Pigments	0.0550	0.0596	0.0496	0.0552
Nucleosides and Nucleotides	0.0371	0.0358	0.0374	0.0370
DNA Metabolism	0.0258	0.0315	0.0294	0.0292
Cell Wall and Capsule	0.0245	0.0284	0.0254	0.0243
Stress Response	0.0292	0.0282	0.0287	0.0296
Respiration	0.0258	0.0252	0.0251	0.0249
Fatty Acids, Lipids, and Isoprenoids	0.0185	0.0190	0.0200	0.0183
Cell Division and Cell Cycle	0.0154	0.0168	0.0167	0.0161
Virulence, Disease and Defense	0.0170	0.0163	0.0156	0.0163
Motility and Chemotaxis	0.0129	0.0157	0.0126	0.0134
Regulation and Cell signaling	0.0157	0.0144	0.0142	0.0141
Membrane Transport	0.0099	0.0142	0.0136	0.0120
Phosphorus Metabolism	0.0141	0.0135	0.0134	0.0139
Phages, Prophages, Transposable elements, Plasmids	0.0116	0.0134	0.0130	0.0142
Metabolism of Aromatic Compounds	0.0111	0.0128	0.0121	0.0116
Nitrogen Metabolism	0.0114	0.0116	0.0120	0.0116
Iron acquisition and metabolism	0.0102	0.0083	0.0087	0.0100
Sulfur Metabolism	0.0096	0.0079	0.0085	0.0102
Potassium metabolism	0.0027	0.0029	0.0023	0.0024
Secondary Metabolism	0.0016	0.0019	0.0017	0.0016
Dormancy and Sporulation	0.0010	0.0016	0.0022	0.0023
Photosynthesis	0.0000	0.0000	0.0000	0.0000

Hits were generated by blasting sequences of 16 metagenomes (12 from protist treatment and 4 from non-protist control) to the MG-RAST subsystem database with a minimum alignment length of 50 bp and an *E*-value cut-off of $E < 1 \times 10^{-5}$. Relative representation in the metagenomes was calculated by dividing the number of hit to each category by the total number of hits to all categories.

Table S3 - Protist traits used in the study.

Species	Length (µm)	Volume (µm³)	Plasticity	Speed (µm/sec)	Reproduction rate (d^{-1})	Final abundance (cells/well)	Final total biomass* (µg dry wt)
C. hederae	15.56	76.12	0.71	0.77	3.06	570,066	25.25
C. plasmodialis	14.46	229.13	0.82	2.67	1.32	99,700	13.30
P. producta	6.81	15.89	0.74	0.82	2.49	892,572	8.25

*Total protist biomass was calculated using the conversion factor of 0.582 pg dry wt / µm³ according to Gates et al. (1982)

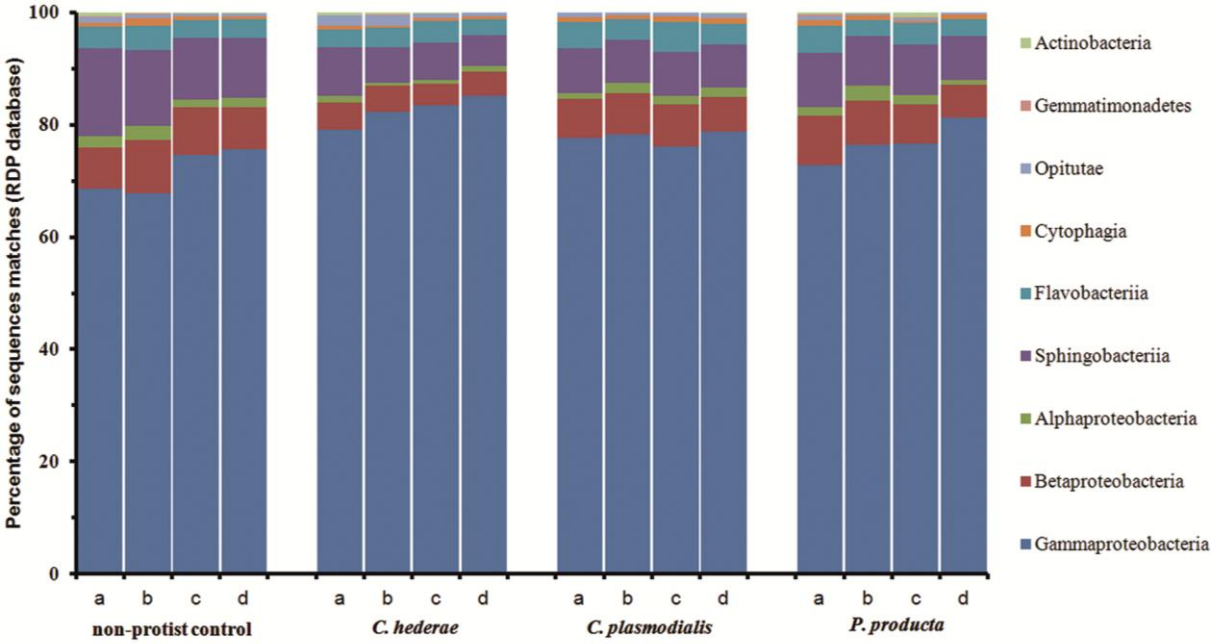
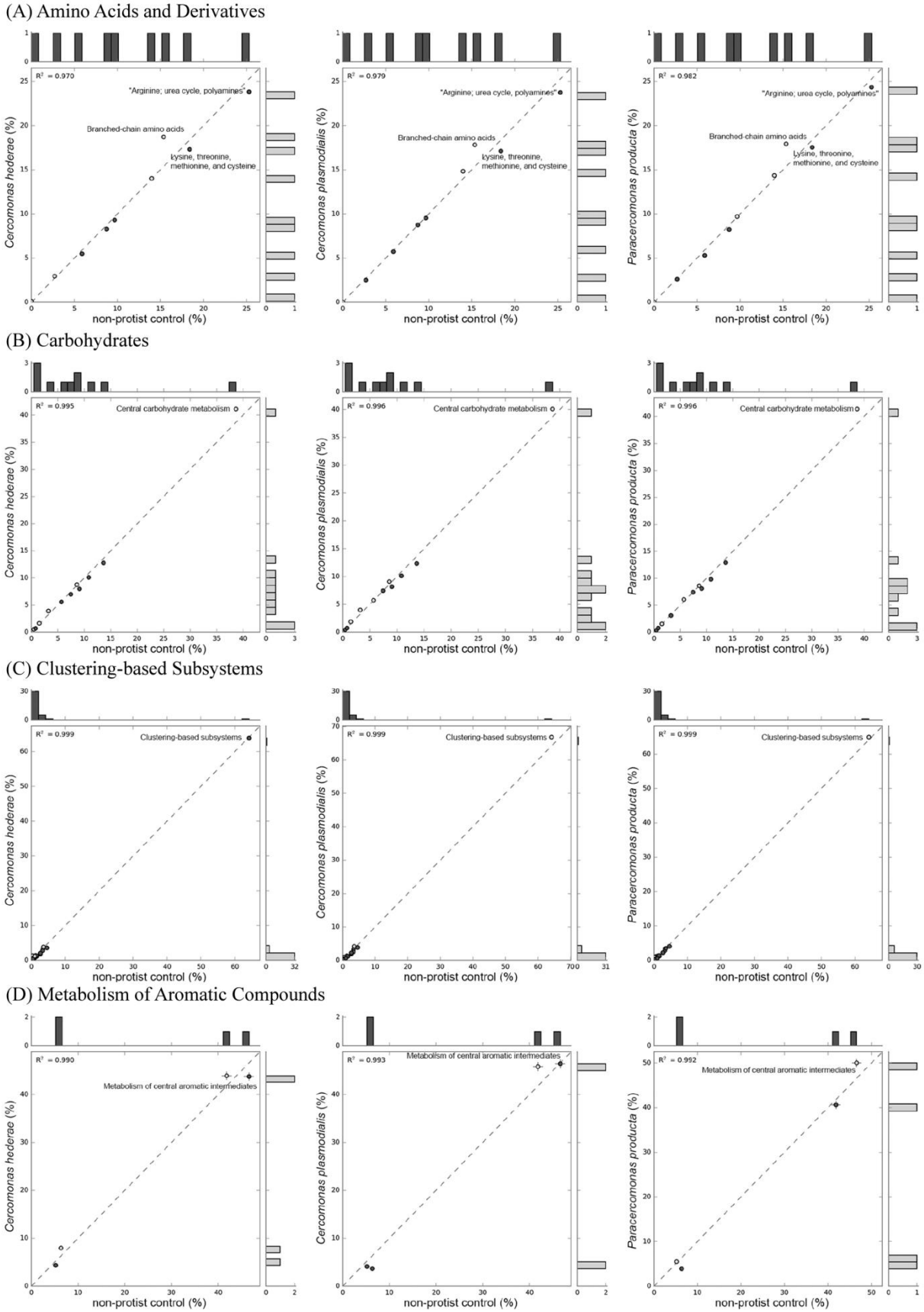
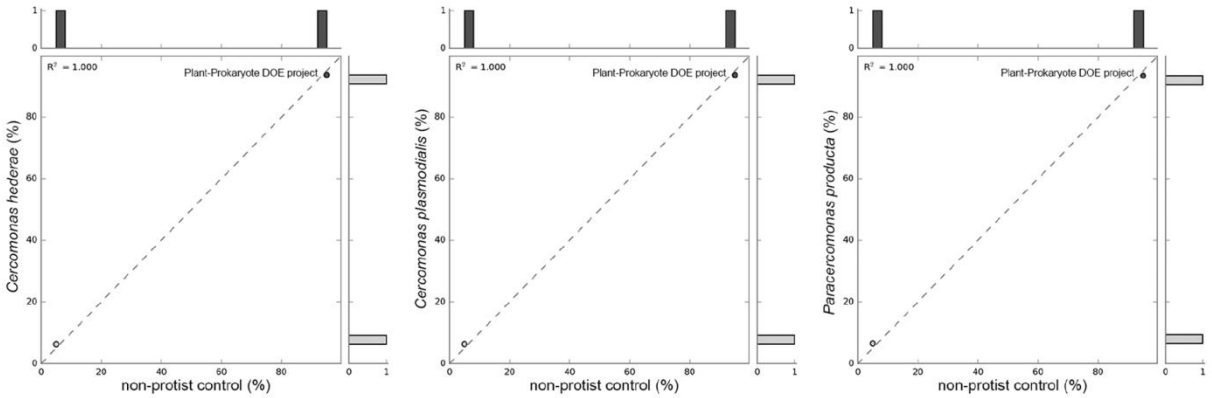


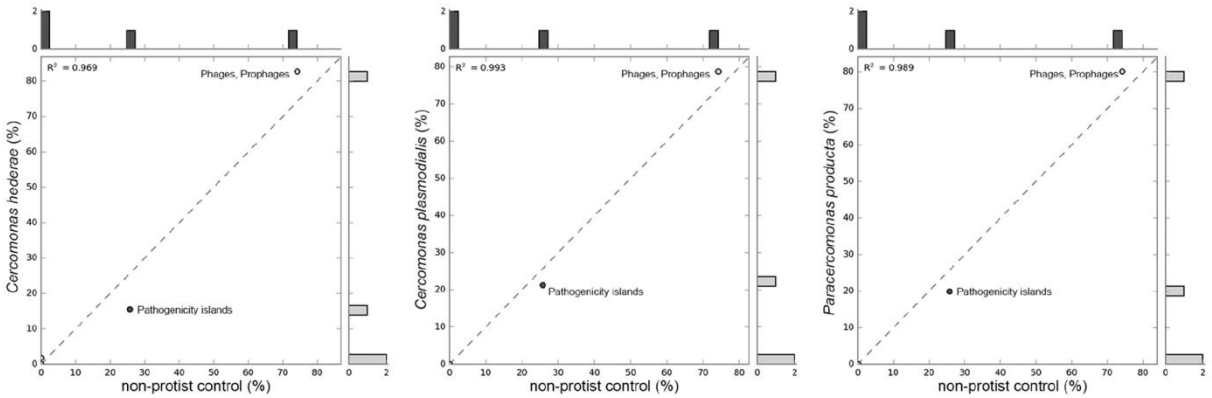
Figure S1 - Taxonomic affiliation of metagenomic reads from microcosm experiment evaluated by BLASTX analysis against the RDP database using MG-RAST v 3.6. software. Shown is the bacterial community composition of individual samples at class level. Samples are labeled on the x-axis by their identifier.


(A) Amino Acids and Derivatives
R² = 0.970
"Arginine; urea cycle, polyamines"
Branched-chain amino acids
Lysine, threonine, methionine, and cysteine
Cercomonas hederae (%)
non-protist control (%)
R² = 0.979
Cercomonas plasmodialis (%)
R² = 0.982
Paracercomonas producta (%)
(B) Carbohydrates
R² = 0.995
Central carbohydrate metabolism
R² = 0.996
(C) Clustering-based Subsystems
R² = 0.999
Clustering-based subsystems
(D) Metabolism of Aromatic Compounds
R² = 0.990
Metabolism of central aromatic intermediates
R² = 0.993
R² = 0.992

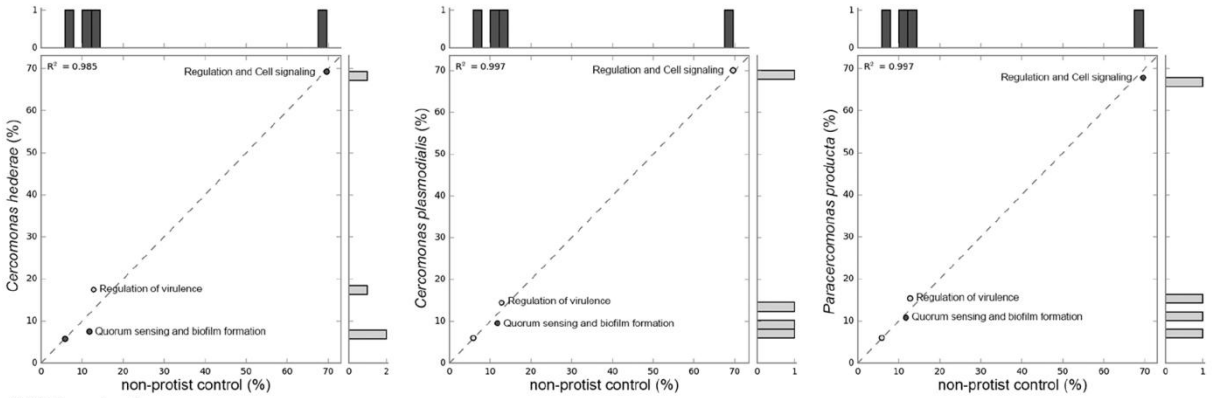
(E) Miscellaneous



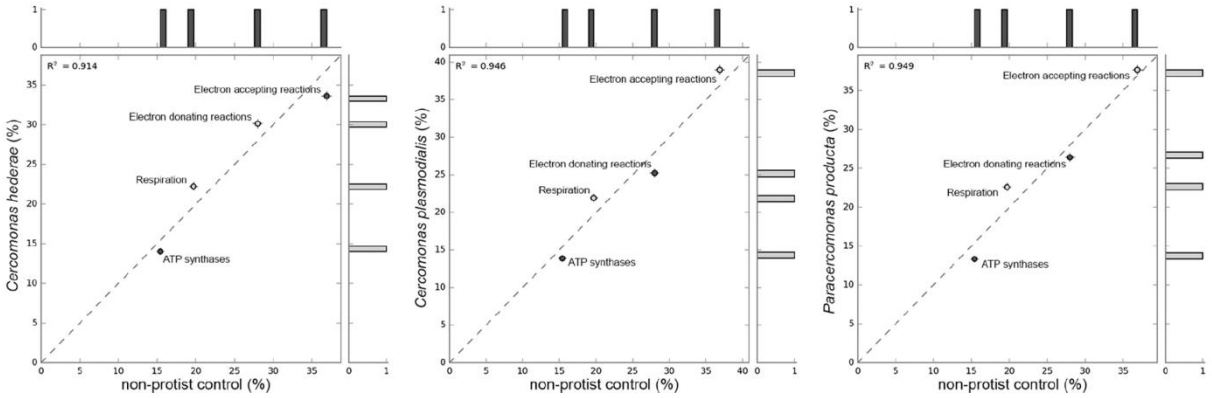
(F) Phages, Prophages, Transposable Elements, Plasmids



(G) Regulation and Cell Signaling



(H) Respiration



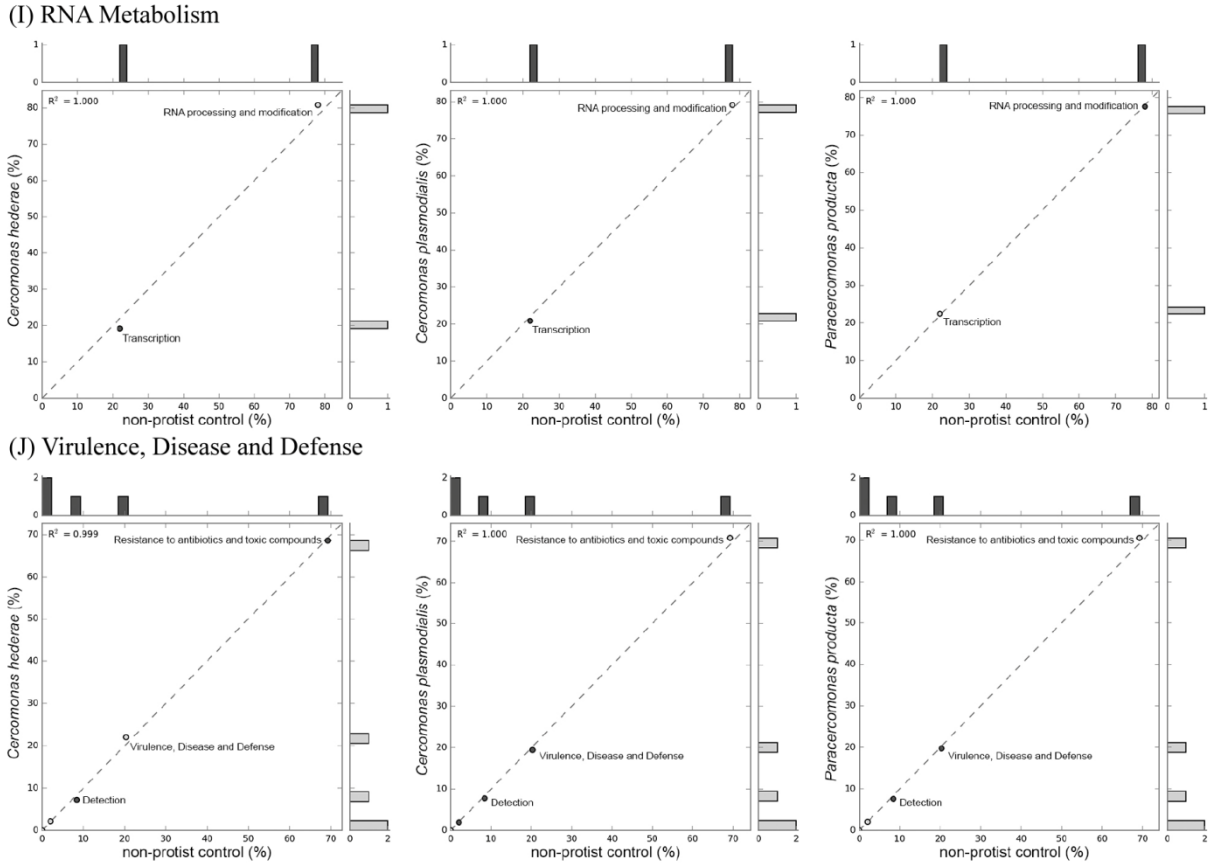


Figure S2 - Functional profiles of protist samples (grey) and non-protist control (black) of (A) amino acids and derivatives, (B) carbohydrates, (C) clustering-based subsystems, (D) metabolism of aromatic compounds, (E) miscellaneous, (F) phages, prophages, transposable elements, plasmids, (G) regulation and cell signaling, (H) respiration, (I) RNA metabolism and (J) virulence, disease and defense (SEED subsystem level 2). The data was calculated for metagenomes from microcosm experiment and compared to SEED database using a maximum E-value cut-off of E < 1 x 10-5 and minimum alignment length of 50 bp.

Chapter 3

Spatial and Temporal Dynamics between Leaf-Associated Cercomonad Cercozoa and Phyllosphere Bacteria on Bean Leaves (*Phaseolus vulgaris*)

Chapter 3 - Spatial and Temporal Dynamics between Leaf-Associated Cercomonad Cercozoa and Phyllosphere Bacteria on Bean Leaves (*Phaseolus vulgaris*)

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Introduction

The phyllosphere (Last 1955) comprises the leaf surface of plants which is a microbial habitat characterized by a multitude of plant-microbe interactions with an impact on plant performance (Vorholt 2012). Investigations on the factors controlling the composition, colonization and dispersion of bacteria in the plant phyllosphere traditionally focus solely on bacterial adaptations to the phyllosphere environment or on direct bacterial competition for space and nutrients. While many plant-microbe interactions such as plant-pathogen interactions (Beattie and Lindow 1995; Hirano and Upper 2000), adaptations of bacteria to their host plant (Blanvillain et al. 2007; Knief et al. 2011) or interactions with the leaf surface (Esser et al. 2015; Krimm et al. 2005; Schreiber et al. 2005) are well known, only little information on the interactions of bacteria and eukaryotes on leaf surfaces exist. However, complex interactions are expected to occur in the phyllosphere between various microorganisms, including bacteria, fungi and protozoa (Lindow 2006; Vorholt 2012). Furthermore, the phyllosphere exhibits a complex and heterogeneous environment where intra- and interspecific competition for resources are influenced by environmental stresses such as UV radiation, temperature changes and desiccation (Leveau 2006). Leaves posses a variety of anatomical features, such as stomata, trichomes, veins and grooved junctions of epidermal cells and these influence bacterial colonization of leaf surfaces (Leveau and Lindow 2001; Monier and Lindow 2005; 2004; Yadav et al. 2005). Accordingly, the spatial

pattern of bacteria is non-random and bacteria are preferentially distributed along these structural features (Hunter et al. 2010; Kong et al. 2010; Monier and Lindow 2004; Yadav et al. 2005; Yu et al. 2014).

Although the occurrence of protists in the phyllosphere has been noted (Gourabathini et al. 2008; Mueller and Mueller 1970; Ploch et al. 2016; Vaerewijck et al. 2014; Vaerewijck et al. 2011) their influence on phyllosphere bacterial communities was virtually unknown. One of the most difficult questions to answer is how protist grazing influences bacterial competition for space and nutrients on leaves, and how bacterivore protists influence bacterial function in the phyllosphere.

We hypothesize that bacterial grazers, such as leaf-associated cercomonads, are as important for structuring the spatial and temporal dynamics of phyllosphere bacteria as protozoa have been shown to structure rhizosphere bacteria (Kreuzer et al. 2006; Rosenberg et al. 2009). We investigated the spatial and temporal dynamics between leaf-associated cercomonad Cercozoa and bacterial strains of *Pantoea eucalypti*. First we studied if cercomonads graze and reproduce on *Pantoea eucalypti* and second we performed experiments on bean leaves (*Phaseolus vulgaris*) to investigate their spatial and temporal interactions by direct examinations using epifluorescence microscopy.

Material and Methods

Bacterial strains and culture conditions

Pantoea eucalypti (formerly known as *Pantoea agglomerans* and before that *Erwinia herbicola*) (Brady et al. 2009) is an indigenous member of the plant microbial community as it colonizes the phyllosphere of various plants (Lindow and Brandl 2003). We used *Pantoea eucalypti* strain 299R (also known as *Erwinia herbicola* 299R) (Brandl and Lindow 1996) and its GFP (pFRU48) and DsRed (pFRU97) constitutive labeled reporter strains for our study (Tecon and Leveau 2012). Microcosm experiments used *P. eucalypti* strain 299R (pRfbX3-gfp) in addition (Tecon and Leveau 2016). This strain forms multicellular capsule-embedded clusters known as symplasmata and the plasmid pRfbX3-gfp drives the expression of GFP only in capsule-embedded clusters of progeny cells (Tecon and Leveau 2016).

All bacterial strains were routinely grown at 30°C on Luria–Bertani (LB) agar plates or in LB liquid cultures supplemented with 50 µg kanamycin per ml. Bacterial cells in midexponential phase were harvested by centrifugation at 2500 *g* for 10 min and the supernatant was discarded. The cells were washed twice in 25% M9 minimal medium (Sambrook and Russell 2001) devoid of a carbon source and resuspended in the same medium. All bacteria were diluted in 25% M9-medium to the desired concentrations of 10^5 or 10^7 individuals per ml, while for microcosm experiments 0.01% D-fructose was added as a carbon source for bacterial growth. Bacterial suspensions were used immediately.

Cercozoa

To investigate the interaction dynamics between leaf-associated cercomonads and *P*. *eucalypti*, we chose two *Cercomonas* species (*C*. *hederae* and *C*. *plasmodialis*) and one *Paracercomonas* species (*P*. *producta*) which were isolated from the phyllosphere (see Chapter 2 for protist isolation, identification and characterization).

To substitute the accompanying bacteria with *P*. *eucalypti* in each of the protist cultures, we established monoxenic protist cultures prior to the start of the microcosm and bean leaf experiments. Protist cultures were washed three times in sterile Neff´s Modified Amoeba Saline (NMAS) (Page 1976) with intermediate centrifugation at 50 g for 10 min and change of the medium. Small volumes (5 µl) of the washed protist cultures were inoculated into culture flasks (T25, Sarstedt, Nümbrecht, Germany) containing 19 ml NMAS and 1 ml washed cells of a *P*. *eucalypti* strain 299R (pFRU48, pFRU97 or pRfbX3-gfp) overnight culture (10^7 #/ml). Protists were allowed to grow for 7 days to appropriate densities, then repeatedly washed and re-inoculated in NMAS with washed cells of *P*. *eucalypti* strain 299R. In this manner,

after five replicated washing and culturing steps, we substituted the accompanying bacteria of each protist strain, resulting in monoxenic protist cultures with *P. eucalypti.*

Immediately before microcosm and bean leaf experiments, cercomonads were washed three times with NMAS and resuspended in 25% M9 minimal medium devoid of carbon source as mentioned above.

Microcosm set-up

Microcosms were Frame-Seal Incubation Chambers (SLF-0601, Bio-Rad, CA, USA) mounted on microscopy slides covered with microscopy glass cover slips. Each chamber containing 60 µl *P. eucalypti* strain*,* diluted in 25% M9-medium with 0.01% D-fructose to the concentration of 10^5 individuals/ml. Subsequently 5 µl with approximately 10^2 cells of each protist strain were inoculated into four replicate microcosms and incubated for 5 days. Protist cell density was taken as the average number of active excysted cells per chamber across the four replicate microcosms counted at 24 h intervals after the point of inoculation for 5 days until the end of inoculation period using phase-contrast microscopy. Monitoring of activity and reproduction ensured that protist grew sufficient to affect *P. eucalypti* in bean leaf experiments. *C. plasmodialis* and *P. producta* were eliminated for the bean leaf experiments because of insufficient growth on *P. eucalypti* (see below).

Plant inoculation and growth conditions

All plant experiments were conducted with 3-week-old bean plants (*Phaseolus vulgaris* variety Blue Lake Bush 274) grown in a greenhouse and incubated under controlled conditions in the laboratory. Only the primary leaves were used in our experiment and round sections of approximately 80 mm² (~ 10 mm diameter) were marked on leaves using a waterproof permanent marker. The bacteria were applied by drop inoculation where we pipetted a 10 µl drop of the desired *P. eucalypti* suspension (10^5 or 10^7 bacteria per ml) in the centre of marked sections on the leaves. When we initiated the protist treatment, another drop of 5 µl with approximately 10^2 protist cells were co-inoculated into the drop on the leaf containing the bacterial suspension. Inoculated plants were transferred to a moist chamber followed by incubation of the plants at close to 100% relative humidity at 21°C (12-h photoperiod) for 4 or 6 days (first trial).

In subsequent trials we drop inoculated *P. eucalypti* (10^5 bacteria per ml) with co-inoculated protists (10^2 cells) on the bean leaves and incubated them for 24 h in the moist chamber.

Leaf sample preparation, epifluorescence microscopy and image analysis

After incubation, we cut leaf circles (7 mm diameter) out of marked drop-inoculated sections with a cork borer and mounted them on microscope slides with 10 µl of Aqua Poly/Mount solution (Polyscience Inc., Warrington PA, USA) and a glass cover slip.

From each leaf section, we took 5 micrographs at random positions using an Axio Imager.M2 epifluorescent microscope (Carl Zeiss AG, Oberkochem, Germany) using 20x objective (EC Plan-NEOFLUAR 20x/0.5, Zeiss) and a 40x objective (EC Plan-NEOFLUAR 40x/0.75, Zeiss). Fluorescent images were sequentially recorded with an AxioCam MRm monochrome camera (Zeiss), using a rhodamine filter cube (exciter: 546/12; emitter: 607/80; beamsplitter 560) and a GFP filter cube (exciter: 470/40; emitter: 525/50; beamsplitter 495) with various exposure times. We also took phase-contrast images of all samples to visualize the leaf surface structure. To account for the topography of the leaf surface, we took all images as 3D 'z-stacks', i.e. several shots of the same area at different focal planes.

Saved images were combined using an Axiovision routine. Subsequently bacterial abundances and locations (x-/y-coordinates) of all individuals were determined using macros created with the program Axiovision (version 4.8, Zeiss, Germany) in order to automate the image analysis following Tecon and Leveau (2012).

Leaf washings and protists observation

Since inoculated protists are invisible on the bean leaf surface by microscopy, inoculated leaf samples were washed after the incubation period to verify the activity of excysted protists. After incubation, we cut leaf circles (7 mm diameter) out of marked drop-inoculated sections with a cork borer and transferred them to Eppendorf tubes containing 1 ml NMAS. After vortexing for 1 minute, the tube was centrifuged at 2500 g for 2 minutes and supernatant was discarded. 65 µl from the remaining suspension was transferred to Frame-Seal Incubation Chambers (SLF-0601, Bio-Rad, CA, USA) mounted on microscopy slides covered with glass cover slips and inspected for active protists using a Axio Imager.M2 microscope (Carl Zeiss AG, Oberkochem, Germany) (up to 400x magnification, phase-contrast).

To quantify if encysted *C. hederae* washed from leaves will excyst at favorable conditions and to exclude possible side effects of bean leaf metabolites, leaf washings were transferred to 60 mm petri dishes with NMAS containing on sterilized quinoa (*Chenopodium quinoa*) grain as carbon source for bacteria and incubated at room temperature for one week. Subsequently we checked the cultures for active protist with an inverted microscope (Carl Zeiss AG, Oberkochem, Germany) (up to 400x magnification, phase-contrast).

Results and Discussion

Grazing and reproduction of cercomonads on *P. eucalypti* 299R

All cercozoan strains were successfully feeding on *P. eucalypti* as illustrated for *C. plasmodialis* and *C. hederae* in Figure 1 and 2.

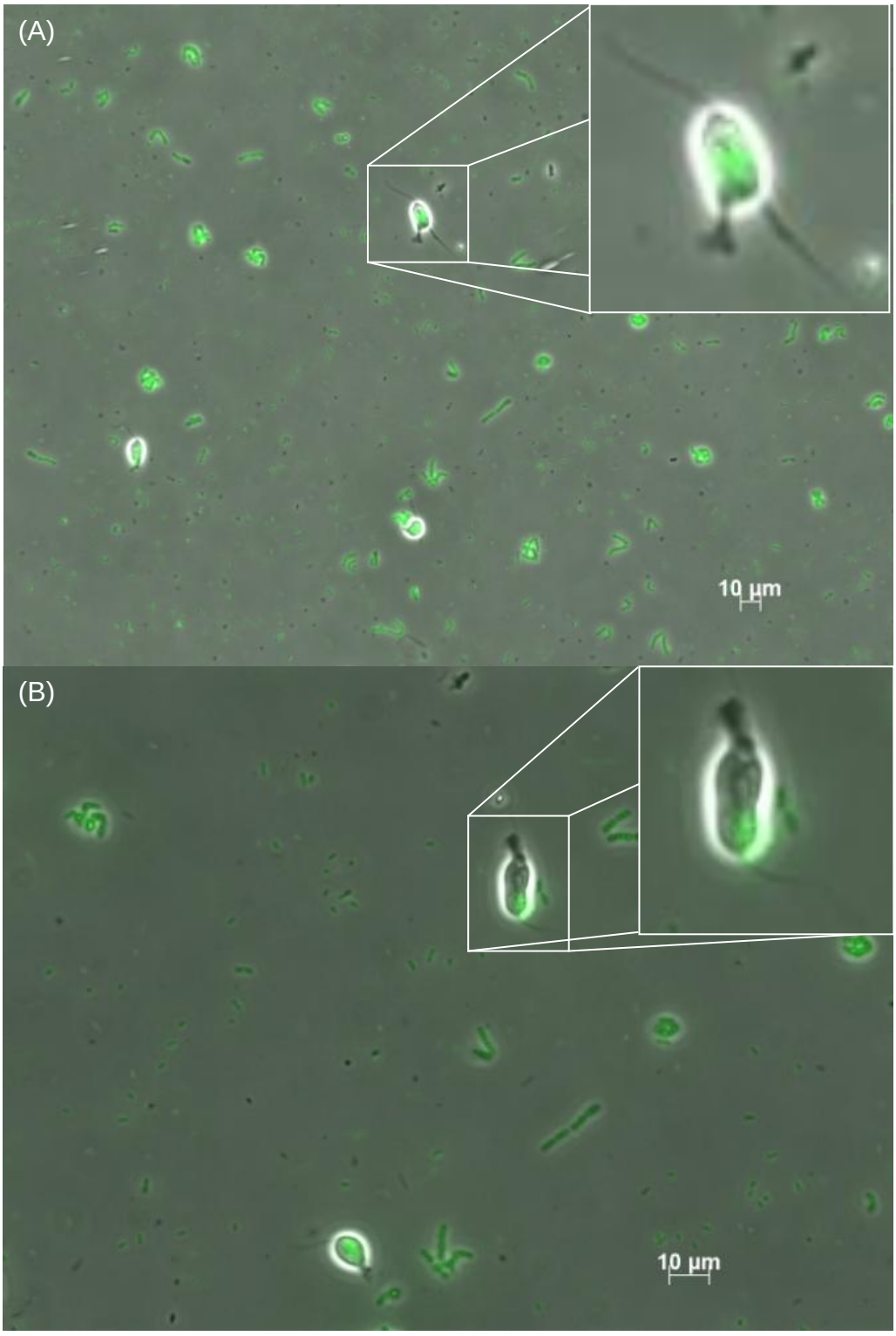


Figure 1 - (**A**) and (**B**). *Cercomonas plasmodialis* consumed *Pantoea eucalypti* 299R (pFRU48), seen by green fluorescence of protist cells (zoomed in area).

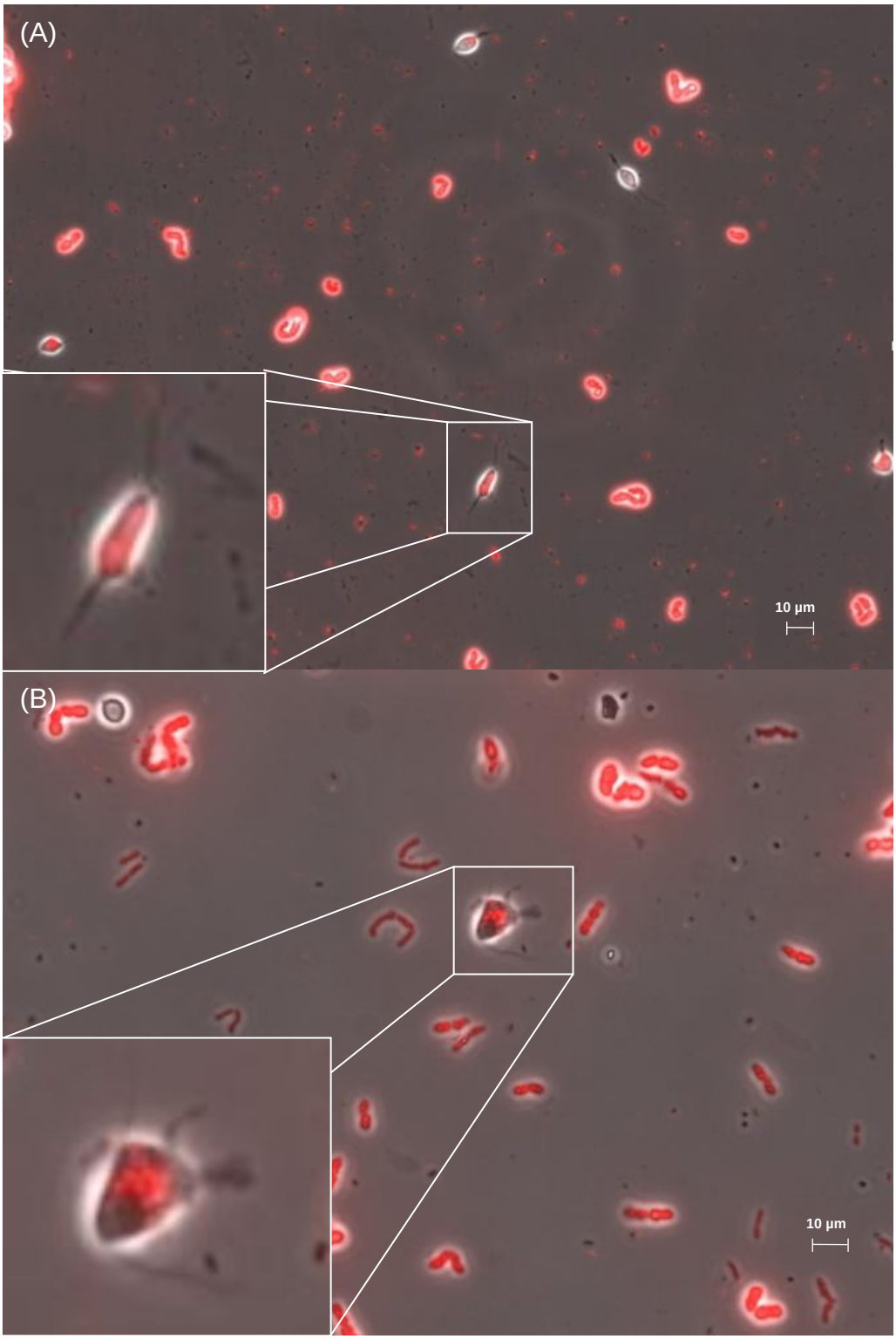


Figure 2 - (**A**) and (**B**). *Cercomonas hederae* consumed *Pantoea eucalypti* 299R (pFRU97), seen by red fluorescence of protist cells (zoomed in area).

In contrast, none of the three protist strains consumed, or were able to consume capsule-embedded bacterial clusters of *Pantoea eucalypti* 299R (pRfbX3-gfp). We never observed a fluorescent signal inside the cercozoan cells (Figure 3). Well developed bacterial

capsule-embedded clusters are probably just too large to be engulfed by cercozoans, indicating relevance of colony formation to reduce predation risk by surface feeding Cercozoa.

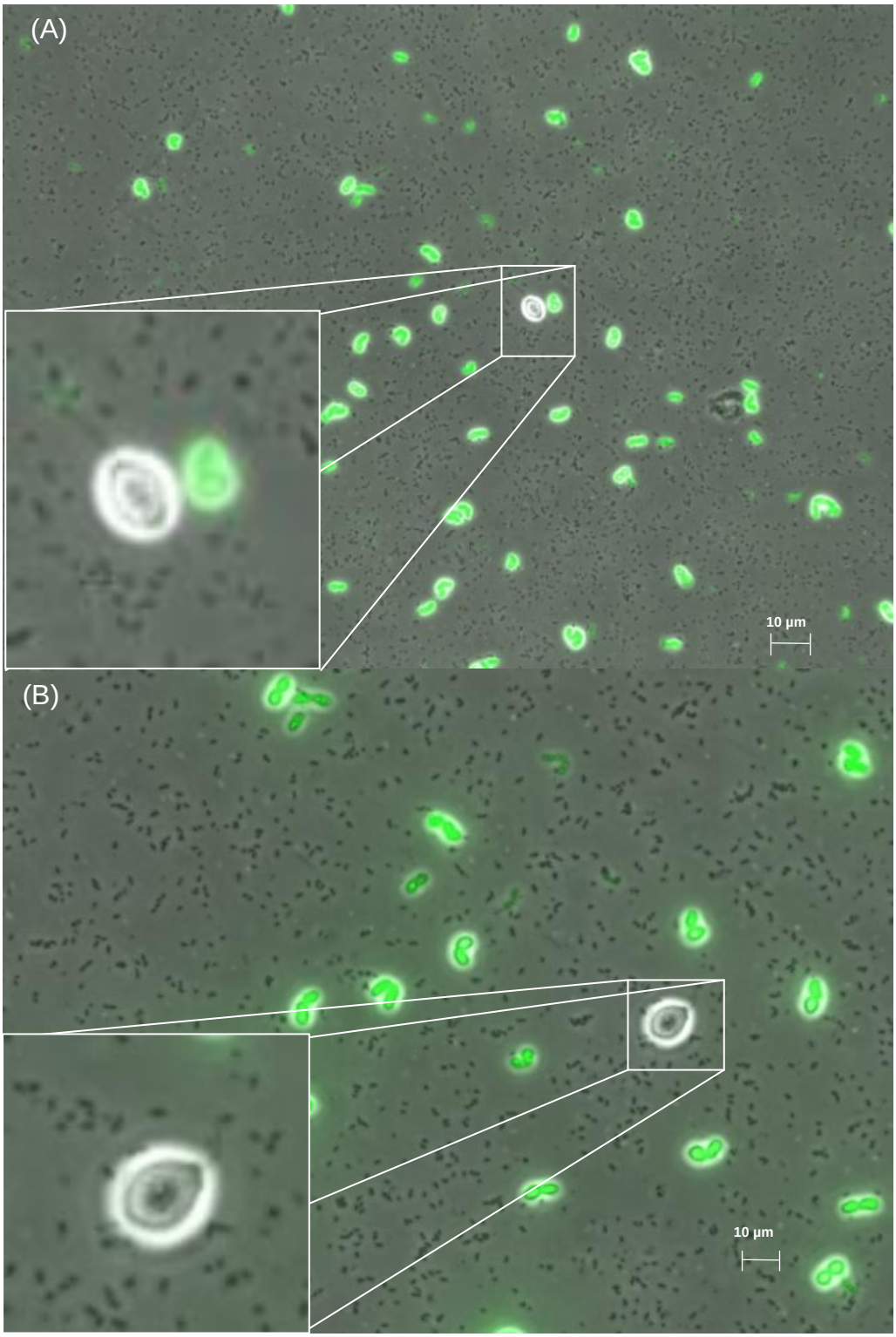


Figure 3 - (**A**) and (**B**). *C. hederae* and fluorescent bacterial capsule-embedded clusters and non-fluorescent single cells of *Pantoea eucalypti* 299R (pRfbX3-gfp). *C. hederae* did not ingest any fluorescent bacterial clusters of *Pantoea eucalypti* 299R (pRfbX3-gfp) as demonstrated by the lack of fluorescence (zoomed in area). They grazed on non-fluorescent single cells instead.

All three cercozoan strains successfully grazed on *P. eucalypti* single cells (Figure 1 and 2), but activity and reproduction of *C. hederae* was much higher than of *C. plasmodialis* and *P. producta* to affect *P. eucalypti* populations on bean leaves (Figure 4).

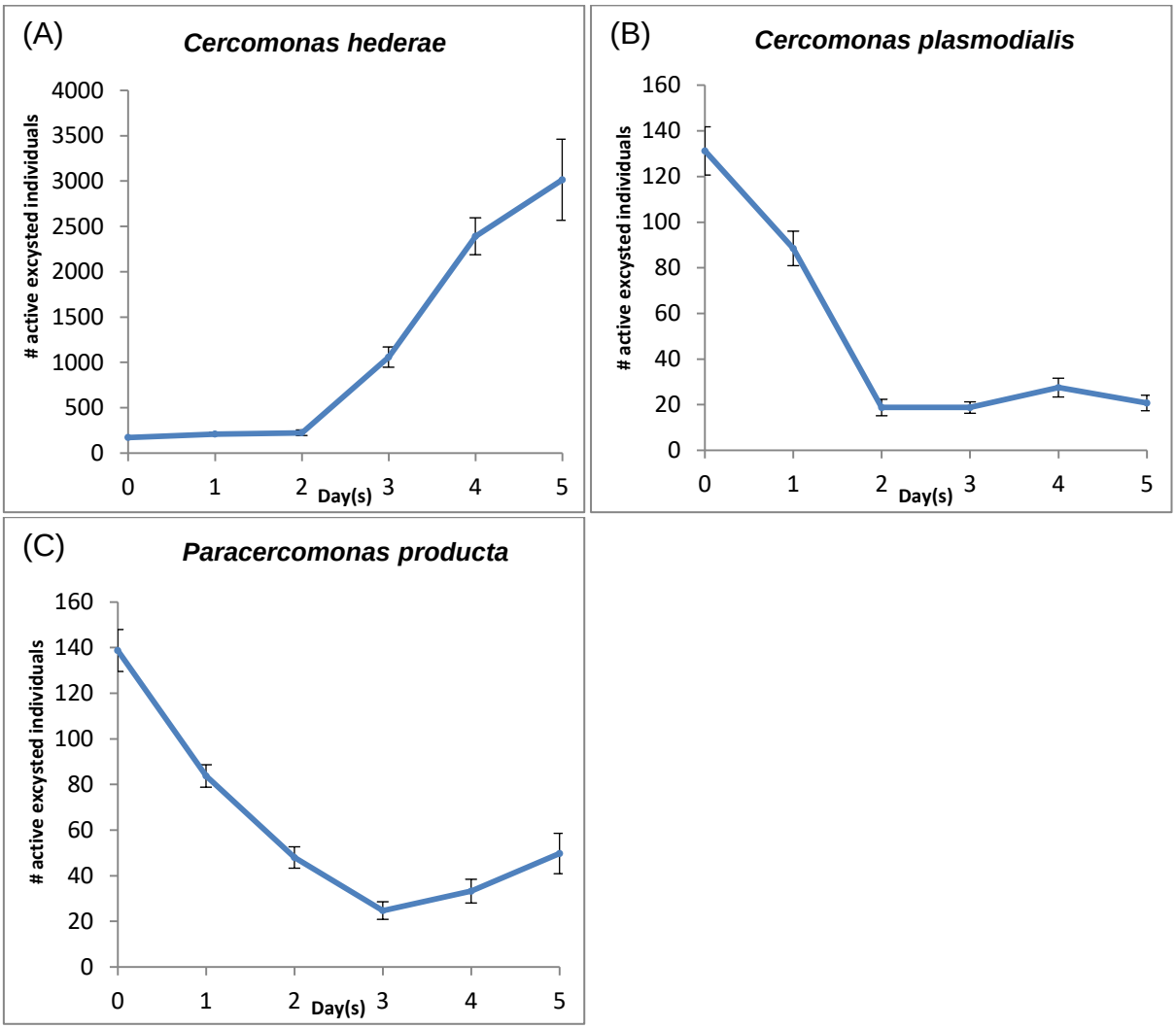


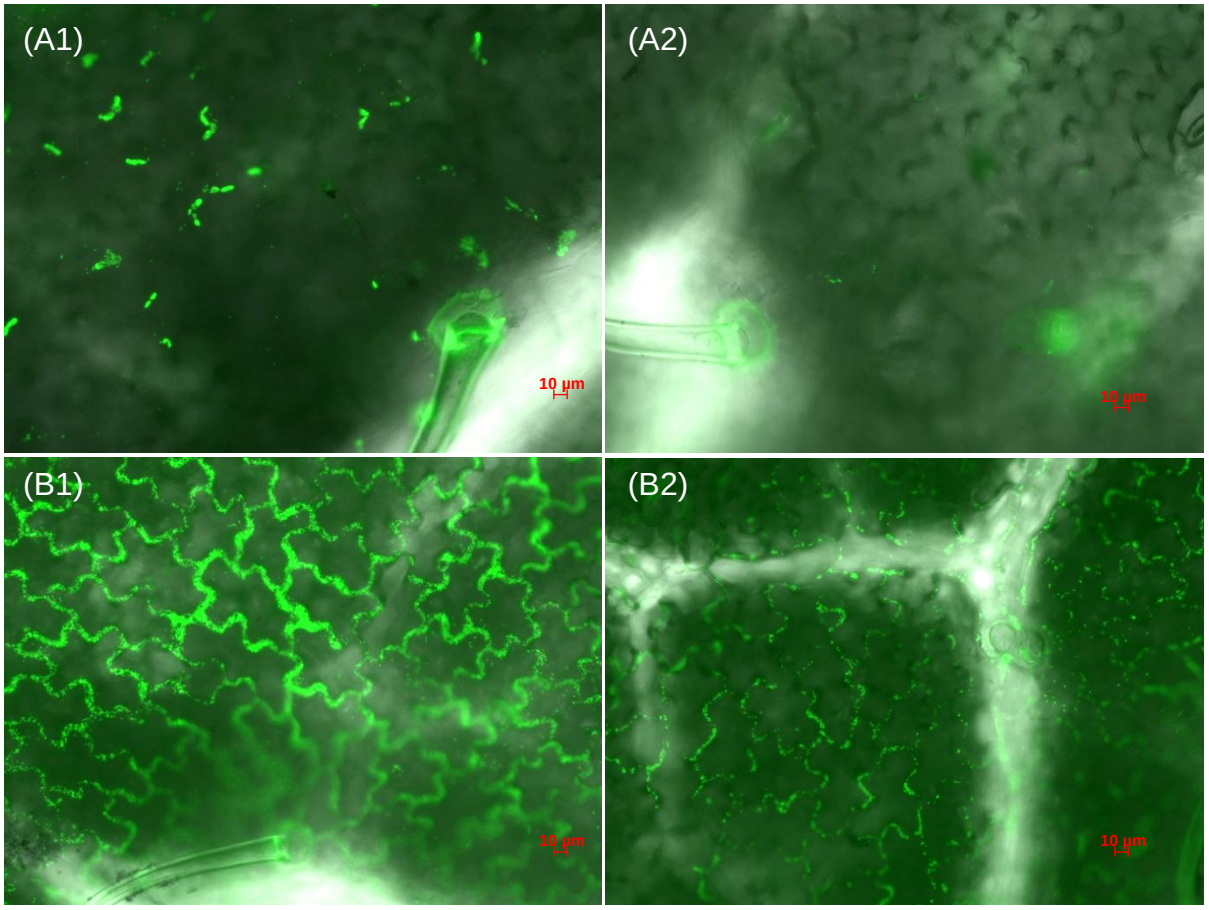
Figure 4 - Mean (±SD) cell densities of active excysted cercozoan strains within four replicate microcosms are shown. (**A**) *C. hederae* with *P. eucalypti* 299R (pFRU48). (**B**) *C. plasmodialis* with *P. eucalypti* 299R (pFRU48). (**C**) *P. producta* with *P. eucalypti* 299R (pFRU48).

Experiments on bean leaves

Inoculated bean plants were incubated in a moist chamber at high relative humidity (Figure 5).

Figure 5 - Moist chamber incubation with inoculated bean plants.

In non-protist controls, bacterial cells occurred preferentially along cell junctions of epidermal cells and along veins, while bases of trichomes were less colonized (Figure 6). These data support earlier findings that phyllosphere bacteria are associated with specific leaf anatomical structures (Kong et al. 2010; Leveau and Lindow 2001; Monier and Lindow 2005; 2004; Yadav et al. 2005).



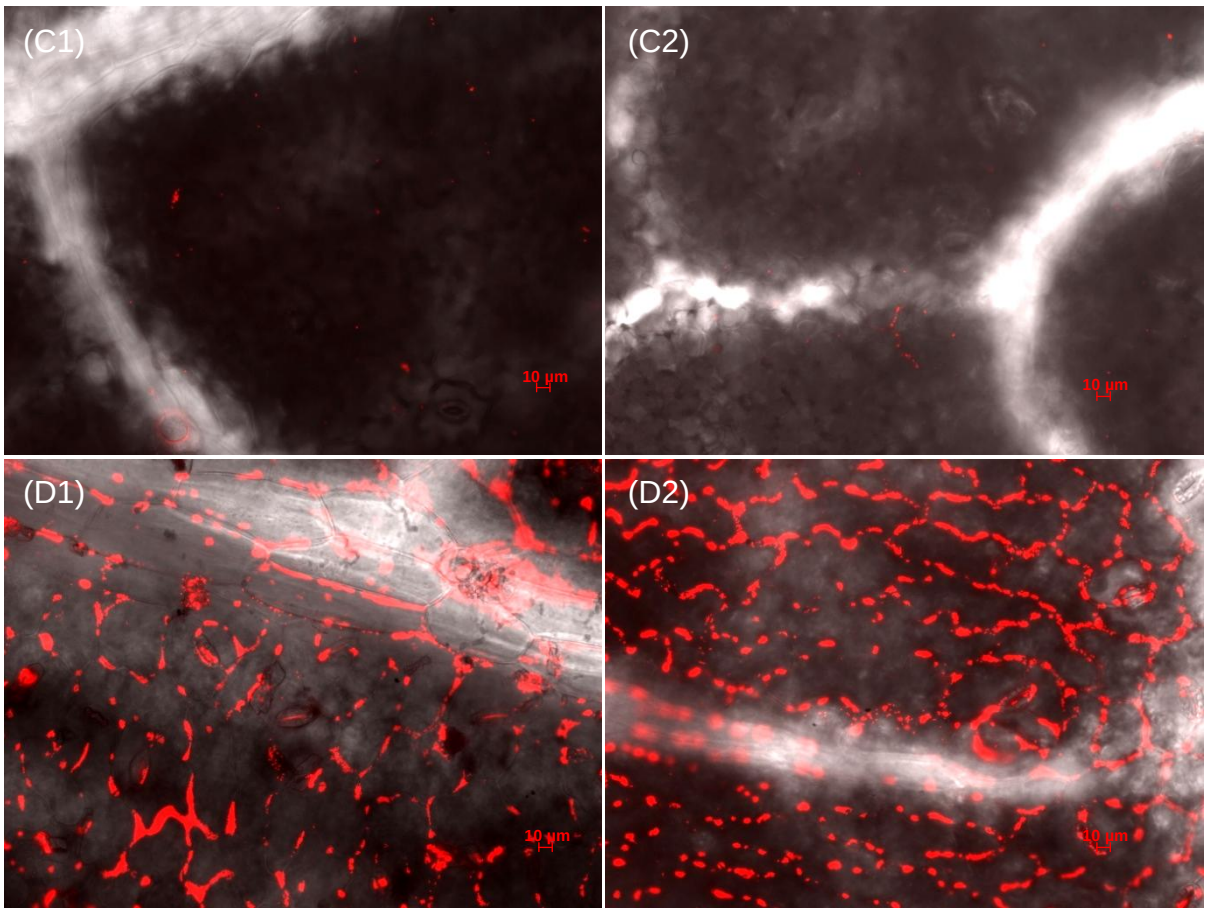
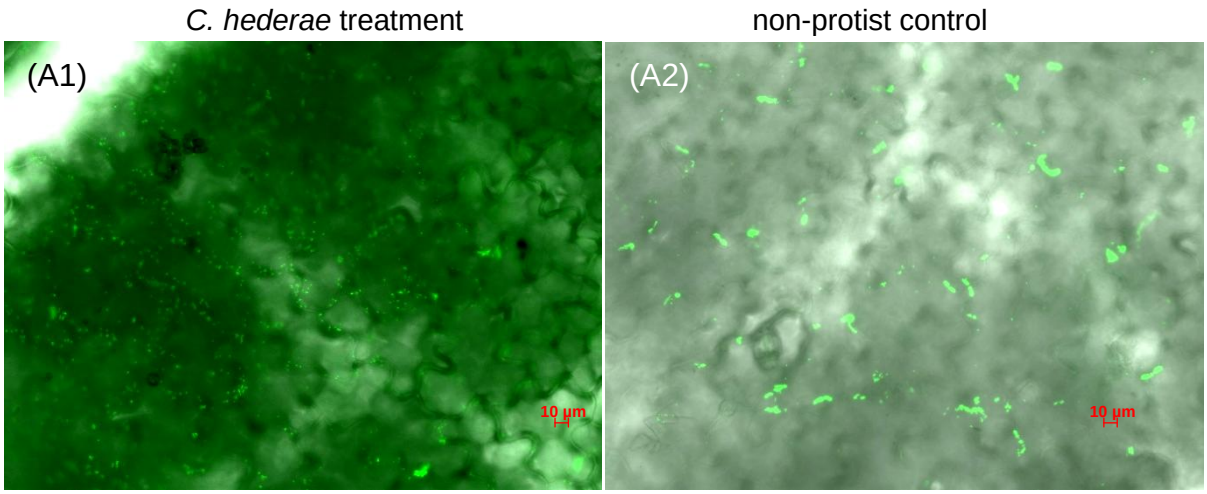


Figure 6 - Cells of *Pantoea eucalypti* strain 299R on bean leaves observed by epifluorescence microscopy: (**A1**+**A2**) low inoculation concentration (10^5 #/ml) of strain 299R (pFRU48) after 4 days incubation; (**B1**+**B2**) high inoculation concentration (10^7 #/ml) of strain 299R (pFRU48) after 4 days incubation; (**C1**+**C2**) low inoculation concentration (10^5 #/ml) of strain 299R (pFRU97) after 6 days incubation; (**D1**+**D2**) high inoculation concentration (10^7 #/ml) of strain 299R (pFRU97) after 6 days incubation.

Co-inoculated *P. eucalypti* did not show differences in the spatial distribution and cell density of bacterial aggregates in comparison to the non-protist control (Figure 7). Not even an effect of *C. hederae* on bacterial abundance in treatments with low bacterial concentration of 10^5 bacteria per ml could be observed (Figure 7 C1+C2).



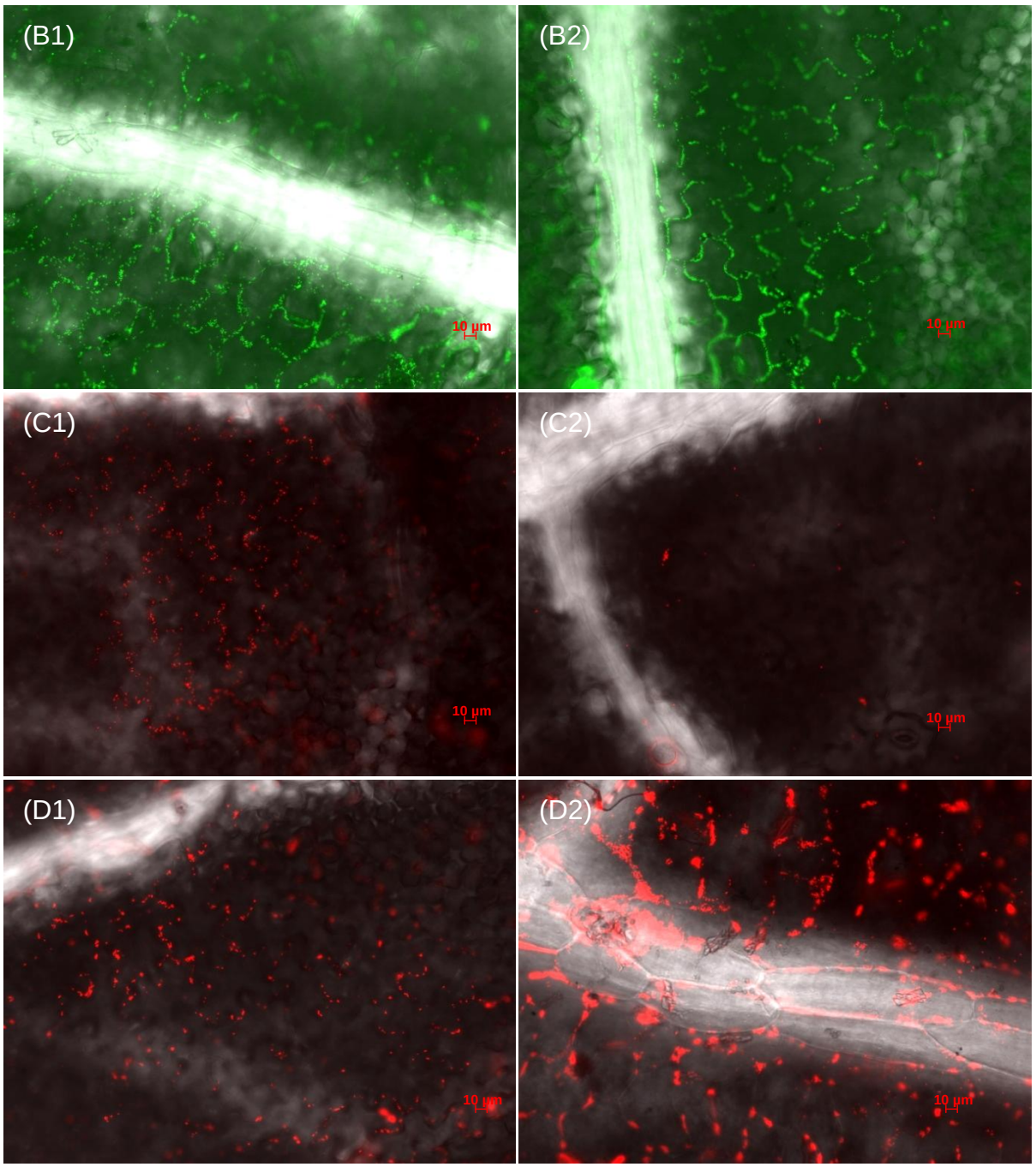
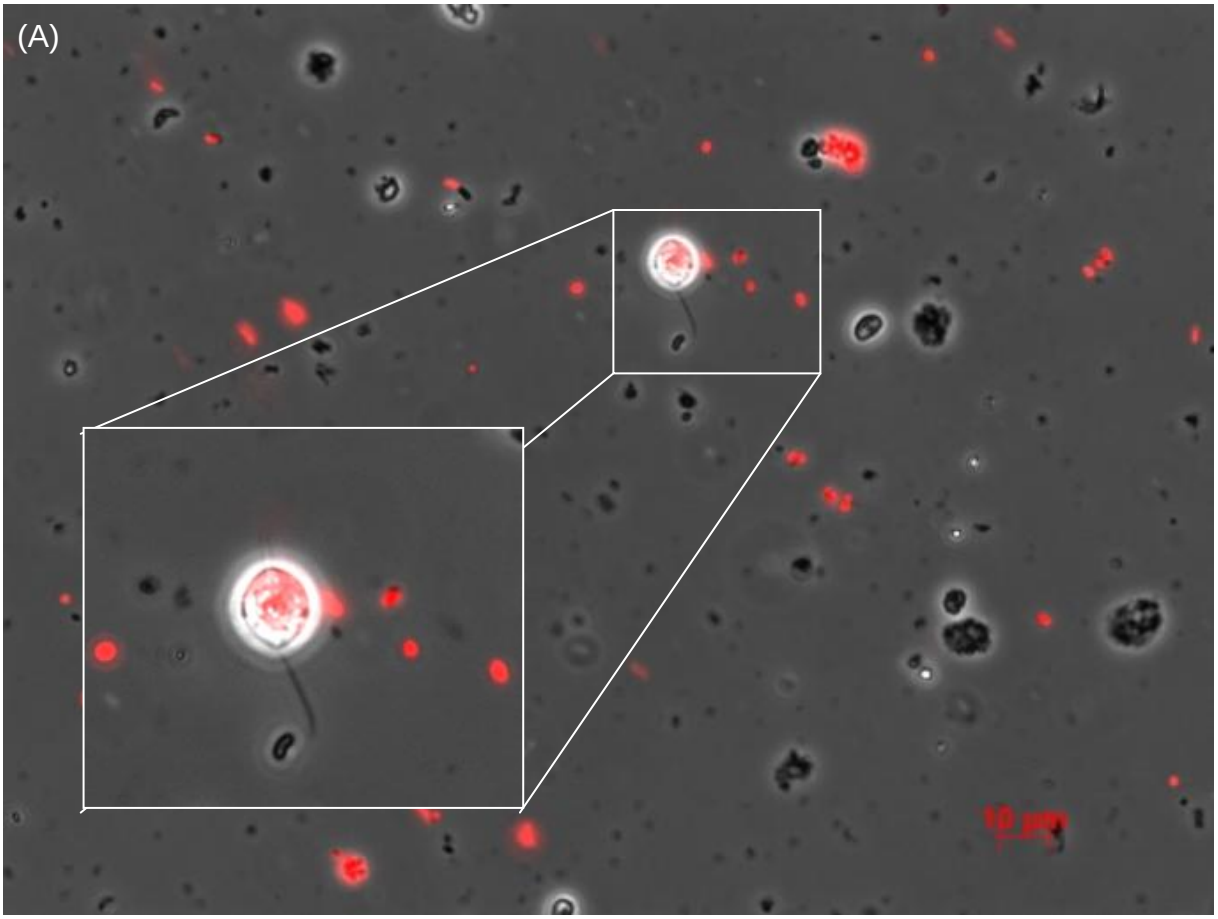


Figure 7 - Cells of *Pantoea eucalypti* strain 299R on bean leaves in presence and absence of *C. hederae* observed by epifluorescence microscopy: (**A1**) low inoculation concentration (10^5 #/ml) of strain 299R (pFRU48) after 4 days incubation in presence of *C. hederae* and (**A2**) in absence of *C. hederae*. (**B1**) high inoculation concentration (10^7 #/ml) of strain 299R (pFRU48) after 4 days incubation in presence of *C. hederae* and (**B2**) in absence of *C. hederae*. (**C1**) low inoculation concentration (10^5 #/ml) of strain 299R (pFRU97) after 6 days incubation in presence of *C. hederae* and (**C2**) in absence of *C. hederae*. (**D1**) high inoculation concentration (10^7 #/ml) of strain 299R (pFRU97) after 6 days incubation in presence of *C. hederae* and (**D2**) in absence of *C. hederae*.

Only encysted *C. hederae* were observed in the leaf washings. We observed that inoculated drops (10-15 µl) on the leaves remained for about 24 - 36 hours until they were fully evaporated when kept in moist chambers at high relative humidity, indicating that it was most likely that *C. hederae* encysted because of insufficient moisture on bean leaves. We checked

the leaf washing cultures after incubation of one week for active individuals and observed that in every sample *C. hederae* excysted and replicated well.

In a second trial, inoculated drops were hardly evaporated after 24 h of incubation and we washed the leaf samples to investigate them for active *C. hederae* individuals. We could prove that *C. hederae* was active and feeding on *P. eucalypti* cells when the drop remained on the leaf surface (Figure 8A). Subsequently we let inoculated drops slowly desiccate (additional 24 h incubation in the moist chamber) and observed that only protist cysts could be isolated from the leaf samples after desiccation. Additionally, the marked spots on leaves with desiccated bacteria and protist inoculums were rewetted with 10 µl 25% M9-medium and incubated for additional 24 h. Again active *C. hederae* were observed after washing of leaf samples (Figure 8B).



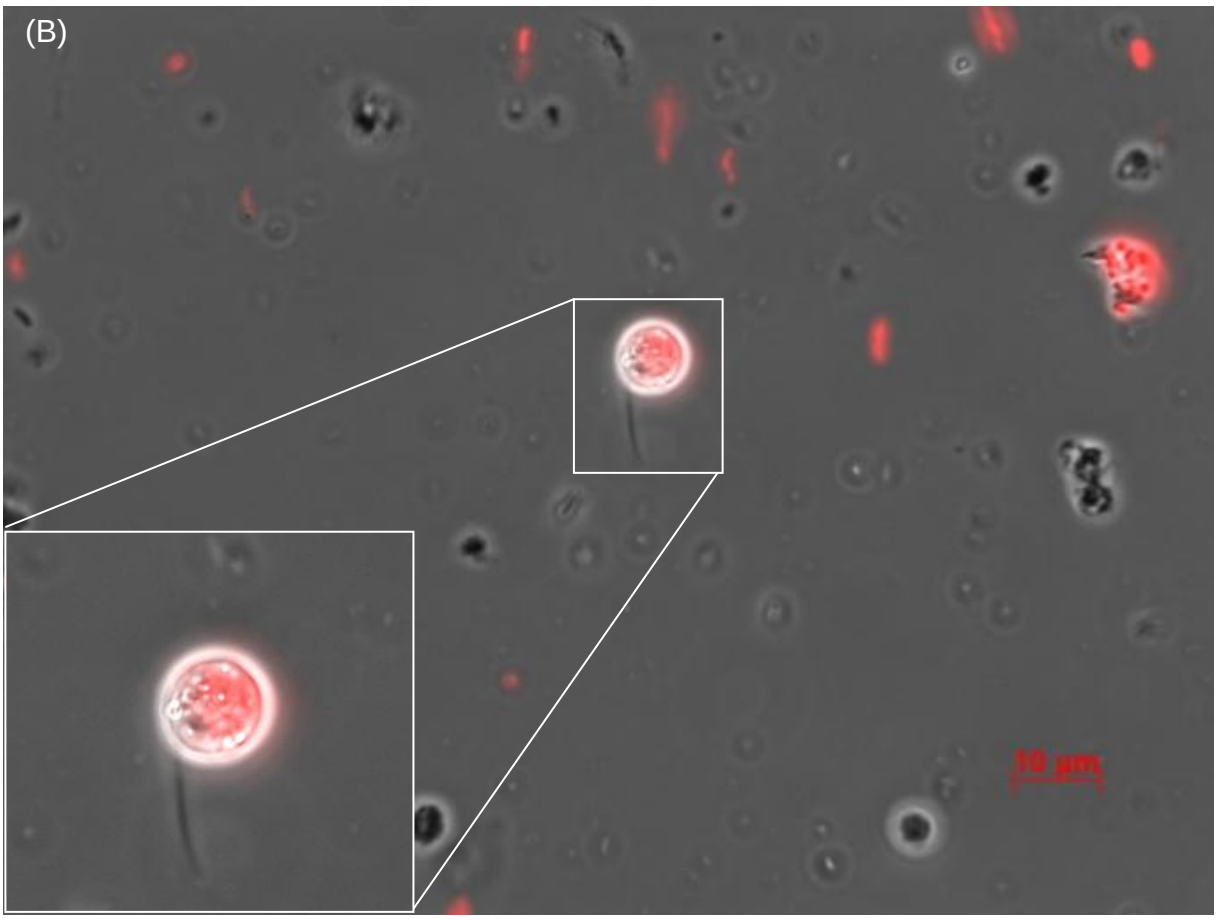


Figure 8 - Active and excysted *Cercomonas hederae* individuals with consumed *Pantoea eucalypti* 299R (pFRU 97), seen by red fluorescence of protist cells (zoomed in area): (**A**) active *C. hederae* individual isolated from the bean phyllosphere after 24 h co-incubation; (**B**) active *C. hederae* individual isolated from the bean phyllosphere after desiccation of inoculums and rehydrated.

In a third trial, we quantified the spatial distribution and cell density of *P. eucalypti* after 24 h incubation in order to observe a grazing effect of *C. hederae*. A portion of *P. eucalypti* cells remained in the liquid phase of inoculated drops, which overlaid settled bacterial cells during the preparation of leaf samples for epifluorescence microscopy (Figure 9). Therefore we could not observe a clear distribution pattern of bacterial cells and therefore cell densities could not be reliably quantified. Residing in the water column can be expected to reduce predation risk by surface feeding *Cercomonas*. Even after slow evaporation of the inoculated drops during incubation (~48 h), bacterial cells remained in the liquid phase and overlaid settled cells on the leaf surface during desiccation. No clear spatial distribution and grazing effects of *C. hederae* therefore were observable (Figure 10). The same approach also failed with *C. plasmodialis* and *P. producta*.

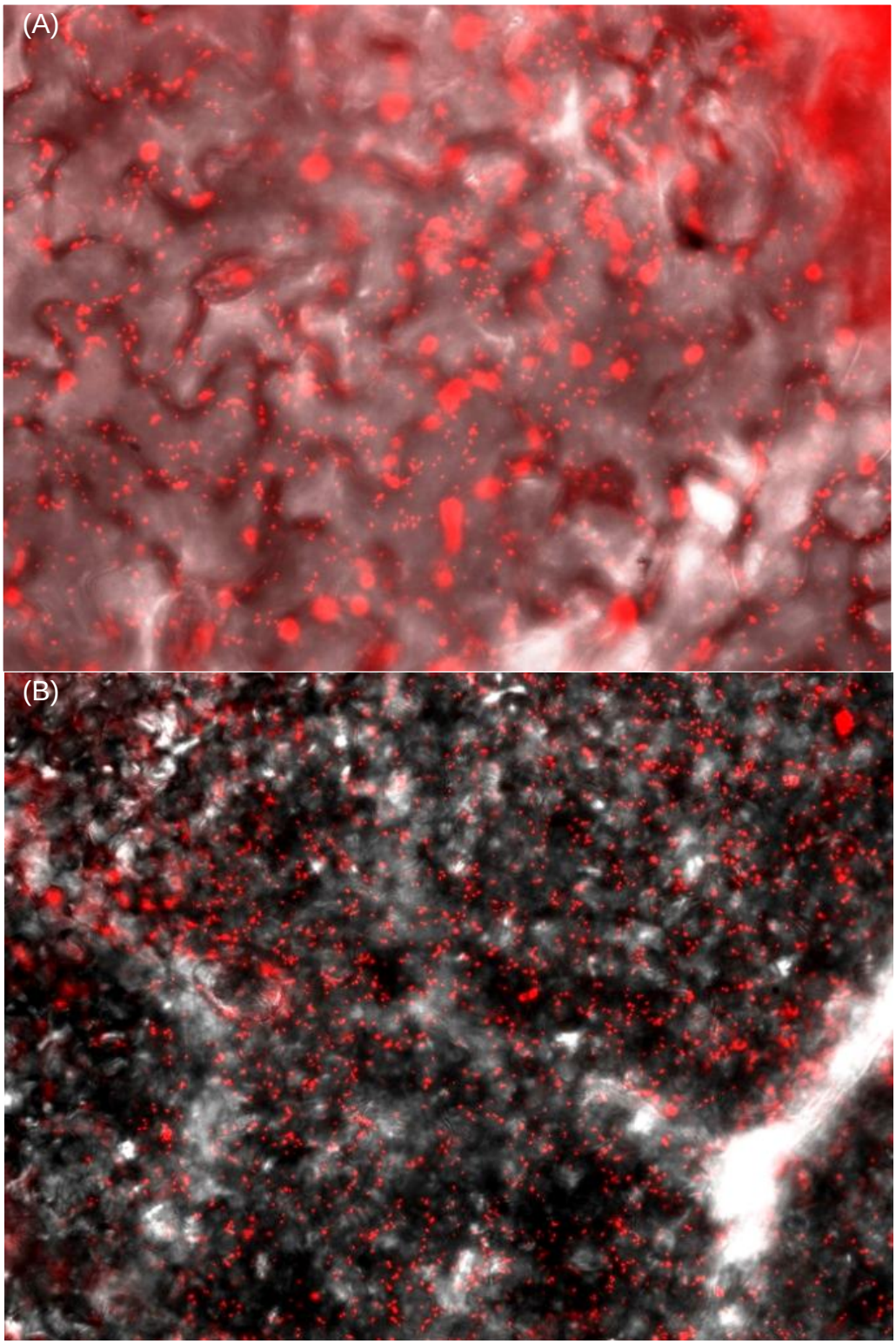


Figure 9 - (**A**) and (**B**). Cells of *Pantoea eucalypti* strain 299R (pFRU97) on bean leaves in presence of *C. hederae* observed by epifluorescence microscopy after 24 hours incubation. Portions of fluorescence bacterial cells remained in the liquid phase of inoculated drops and overlaid settled bacterial cells on leaf surface during the preparation of leaf samples for microscopy causing that no clear distribution pattern could be observed.

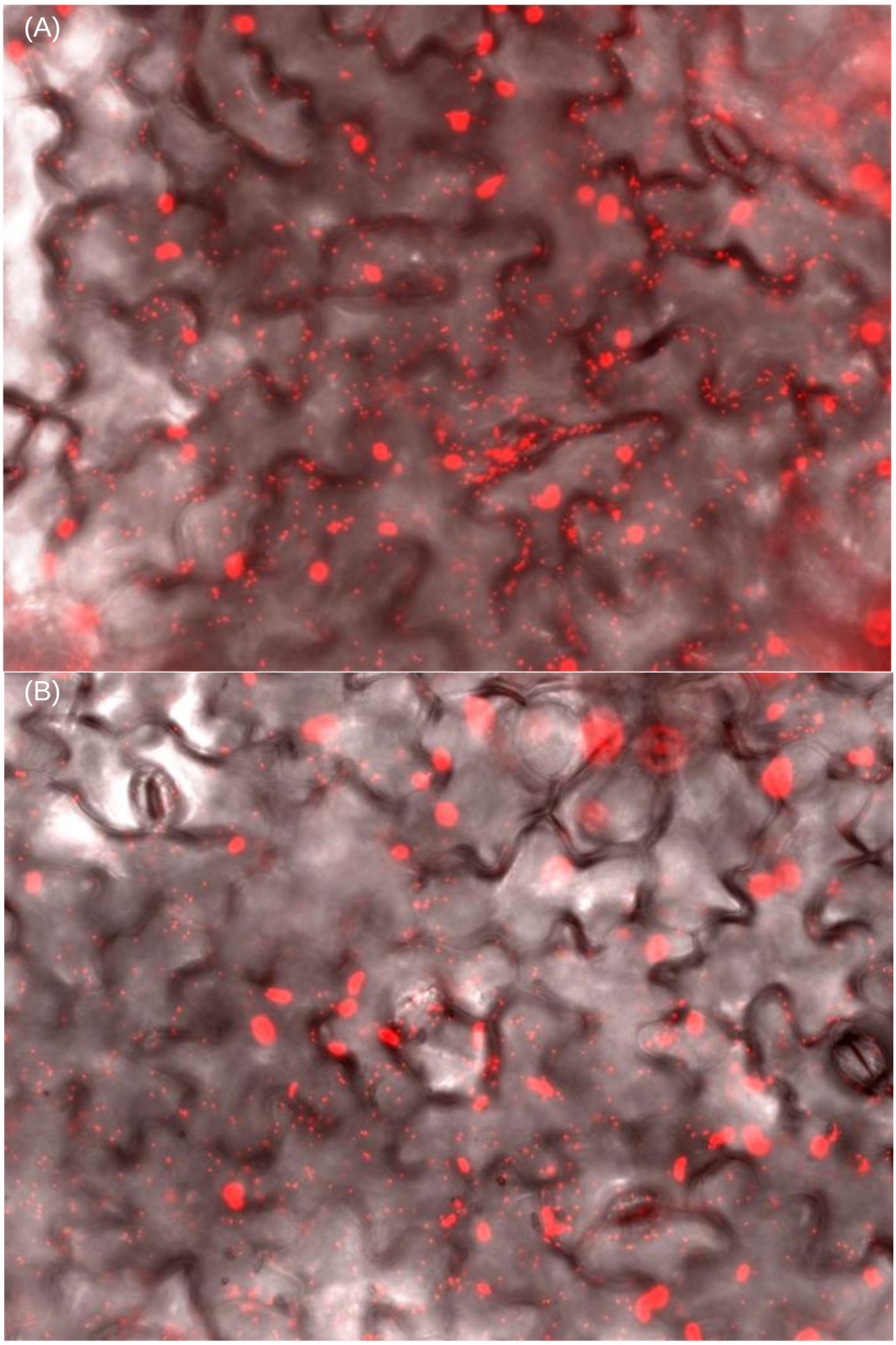


Figure 10 - (**A**) and (**B**). Cells of *Pantoea eucalypti* strain 299R (pFRU97) on bean leaves in presence of *C. hederae* observed by epifluorescence microscopy after 48 hours incubation when inoculated drops were fully desiccated. Portions of fluorescence bacterial cells remained in the liquid phase of inoculated drops and overlaid settled cells on the leaf surface during the slow desiccation and no clear distribution pattern could be observed.

Conclusion

We could prove that all cercozoan strains were successfully feeding on *P. eucalypti* single cells, while capsule-embedded bacterial clusters of *Pantoea eucalypti* were not consumed. This shows that large colonies of agglomerated bacterial cells provide a grazing resistance to predation of surface feeding Cercozoa.

C. hederae, *C. plasmodialis* and *P. producta* remained active and were feeding on *P. eucalypti* cells on the bean leaf surface in water films, but encysted when these evaporated. Our data confirm that the activity of leaf-associated protists is closely connected to the availability of water films on leaves. Water films on leaf surfaces are formed at regular intervals (i.e. morning dew) hence providing regular suitable conditions for the activity of leaf-associated protists on leaves, leading to periodic interactions with phyllosphere bacteria. Moreover, glandular trichomes probably offer better conditions for leaf-associated protists due to their ability to retain water droplets for a longer period (Brewer et al. 1991). We could further confirm that investigated cercomonads were re-cultivable from leaf-washing and in addition, excysted even in the phyllosphere if rewetted. However, due to these biological processes and methodological difficulties it was not possible to investigate the grazing effects of cercozoans on the spatial and temporal dynamics of phyllosphere bacteria using epifluorescence microscopy.

Chapter 4

Rhogostomidae (Cercozoa) from Soils, Roots and Plant Leaves (*Arabidopsis thaliana*): Description of *Rhogostoma epiphylla* sp. nov. and *R. cylindrica* sp. nov.

Chapter 4 - Rhogostomidae (Cercozoa) from soils, roots and plant leaves (*Arabidopsis thaliana*): Description of *Rhogostoma epiphylla* sp. nov. and *R. cylindrica* sp. nov.

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Abstract

Cercozoa are a highly diverse protist phylum in soils and in the phyllosphere of plants. Many families are still poorly described and the vast majority of species are still unknown. Although testate amoebae are among the better-studied protists, only little quantitative information exists on the morphology, phylogeny and ecology of cercozoan Rhogostomidae. We cultured four different strains of *Rhogostoma* spp. isolated from *Arabidopsis* leaves, agricultural soil and rhizosphere soil of *Ocimum basilicum* and *Nicotiana* sp. We describe *Rhogostoma epiphylla* sp. nov. and *R. cylindrica* sp. nov. and present their morphology, studied their food spectra in food range experiments and obtained two SSU rDNA gene sequences resulting in an updated thecofilosean phylogeny. Short generation times, desiccation resistance and the ability to prey on a wide range of algae and yeasts from the phyllosphere were seen as crucial traits for the phyllosphere colonization by *Rhogostoma*. In contrast, the soil-dwelling *R. cylindrica* did not feed on eukaryotes in our experiment.

Introduction

The phylum Cercozoa (Cavalier-Smith and Chao 2003) is highly diverse in morphology and ecology, comprising bacterivorous flagellates, algivorous and fungivorous amoebae as well as endophytic biotrophs and plant parasites (Bass et al. 2009a; Bass et al. 2009b; Dumack et al. 2016b; Howe et al. 2011; Howe et al. 2009; Neuhauser et al. 2014). Cercozoa are a dominant protist phylum in terrestrial systems (Urich et al. 2008) and as environmental DNA surveys of terrestrial, limnic and marine systems have shown, only part of the diversity of Cercozoa taxa has been described to date (Bass and Cavalier-Smith 2004). Recently a high diversity of cercozoan taxa was reported from the plant phyllosphere (Ploch et al. 2016). Grazing experiments indicate that leaf-associated Cercozoa can have a considerable impact on the composition and function of bacterial phyllosphere communities (Flues et al. 2017).

The phyllosphere comprises the leaf surface of plants, whereas roots embody the rhizosphere (Vorholt 2012). Bacteria are by far the most numerous colonizers of leaves (Lindow and Brandl 2003). However, microbial communities of leaves are taxonomically more diverse and include also filamentous fungi, yeasts, algae and protists (Lindow and Brandl 2003). Phyllosphere protists are characterized by diurnal life cycles with short active periods at nighttime when dew accumulates on plant leaves or in the event of rain (Mueller and Mueller 1970). Although the occurrence of protist taxa on plant leaves has long been recognized (Bamforth 1973) and a recent molecular study indicated the occurrence of cercozoan Rhogostomidae in the phyllosphere of Brassicaceae (Ploch et al. 2016), little is known on the ecology and function of Rhogostomidae.

The genus *Rhogostoma* has been erected by Belar (1921) to accommodate thecate amoebae with a cleft-like opening, such as *R. schuessleri* and *R. minus*. Howe et al. (2011) assigned the genus *Rhogostoma* (together with the closely related genus *Capsellina*) to the Thecofilosea (Cercozoa) and described a further species, *R. micra*. The class Thecofilosea was established by Cavalier-Smith and Chao (2003) to comprise flagellates and amoebae with an organic theca. Although testate amoebae in general have been of considerable interest to protistologists and ecologists since their discovery, only little is known about thecofiloseans. Only in recent years a series of studies has been conducted which sheds light on their diversity. Two amoeboid groups (i.e. the Tectofilosida and Rhogostomidae (in the Cryomonadida)) were characterized, both with highly similar morphology although being only distantly related. Four families have been proposed in the Tectofilosida; the Fiscullidae, Chlamydophryidae, Rhizaspididae and Pseudodifflugiidae, all of which are known algi- or fungivores, with no evidence for bacteria ingestion (Dumack et al. 2017b; Dumack et al. 2017c; Dumack et al. 2016c; Wylezich et al. 2002). In contrast, the genus *Rhogostoma*,

assigned (together with *Sacciforma* and *Capsellina*) to the Rhogostomidae, is well known to feed on bacteria and evidence for eukaryote ingestion has recently been given for algae but is still missing for fungal cells (Dumack et al. 2017c; Seppey et al. 2017).

Little is known about the impact of protists on the microbial food webs in the phyllosphere and we have only a vague idea of these complex interactions, particularly with respect to fundamental questions such as which microorganisms are present, what they do there and whether they may function as plant disease suppressors. One first prerequisite to increase our knowledge on these interactions is to unravel the identity and feeding habits of leaf-associated protists and to determine their potential prey spectra.

To shed light on terrestrial Rhogostomidae spp., we isolated four different strains of *Rhogostoma* spp. from *Arabidopsis* leaves, agricultural soils and rhizosphere soils of *Ocimum basilicum* and *Nicotiana* sp. For each of the two morphospecies we provide a detailed morphological description as well as an SSU rDNA sequence and in simple food range experiments we further investigated the potential ingestion of other eukaryotes and gave indications on how the Rhogostomidae also prey on other (co-isolated) members of the phyllosphere microbiome and could therefore exert predation pressure on fungi and algae.

Material and Methods

Sampling and identification

Bulk and rhizosphere soil as well as leaf samples were collected (Table 1). Leaves were submerged in Waris-H (Mcfadden and Melkonian 1986) and incubated for up to three days prior to analyses. The soil samples were pre-pared by diluting 1 g of bulk or rhizosphere soil in 300 ml of 0.15% Wheat Grass (WG)-medium, which was shaken for 20 min at 30 rpm to detach protists from soil particles. The WG was made by adding dried wheat grass powder (Weizengras, Sanatur GmbH, D-78224 Singen) to Prescott and James (PJ) medium (Prescott and James 1955). For incubation, the suspension was diluted by a factor of 4 and 20 µl of the suspension were incubated in 180 µl WG-medium in a 96-wellplate (Sarstedt, Germany). The samples were incubated for up to three weeks and screened weekly for *Rhogostoma*-like cells with an inverted microscope (Nikon Eclipse TS100; Ph1; 40–400× magnification).

Isolation and cultivation

Cells were picked with a glass micro pipette and transferred into a new well of the 96-well plate, containing 180 µl WG-Medium. Cells were sub-cultured approximately every two months.

Microscopical observations

Pictures and videos were taken with a Nikon digital sight DS-U2 camera (program: NIS-Elements V4.13.04) with a Nikon Eclipse 90i upright microscope (up to 600× magnification, DIC) and a Nikon TE2000-E inverted microscope (up to 400× magnification, phase contrast).

Sequencing of cultured amoebae

For sequencing, 15 µl of a clonal culture were transferred into PCR-tubes. The tubes were frozen at −20°C for storage. Then, a total volume of 35 µl of PCR mixture was added. The mixture contained 5 µl of 0.1 µM forward and 5 µl of 0.1 µM reverse primers, 5 µl of 200 µM dNTPs, 5 µl of Thermo Scientific Dream Taq Green Buffer, 0.3 µl of Dream Taq polymerase (Thermo Fisher Scientific, Dreieich, Germany) and 14.7 µl of ultrapure water. The SSU rDNA sequences were obtained in two successive steps. First, the whole SSU rDNA was amplified with the general eukaryotic primers, EukA and EukB (Medlin et al. 1988). In the second step, semi-nested re-amplifications were performed with primers specifically designed for cercozoans (Fiore-Donno et al. 2017), with the same settings as above and the primer pairs EukA + S963R Cerco (Fiore-Donno et al. 2017) (*R. cylindrica*) or EukA + 1256R (Bass and Cavalier-Smith 2004) (*R. epiphylla*) targeting the 5´ part of the SSU rDNA and S616F

Cercomix (Fiore-Donno et al. 2017) (*R. cylindrica*) + EukB or 25F + EukB (Bass and Cavalier-Smith 2004) (*R. epiphylla*) for the 3´ part of the gene. One microlitre of the first PCR product was used as template. The PCR products were purified by adding 0.15 µl Exonuclease, 0.9 µl FastAP and 1.95 µl water to 8 µl of the second PCR product. Then heated for 30 min at 37°C, and subsequently for 20 min at 85°C. The Big dye Terminator Cycle sequencing Kit (Thermo Fisher Scientific, Dreieich, Germany) and an ABI PRISM automatic sequencer were used for sequencing.

Table 1 - Strains of *Rhogostoma* spp. and corresponding data.

Species	Strain	SSU rDNA accession	Sequence length	Sampling spots	Coordinates	Isolation date	Habitat
Rhogostoma cylindrica	KD1020	KY905096	1635	Belgium	50.985905, 3.786282	April 2016	Bulk soil, sandy loam
	KD1021	-	-	Germany, Cologne	50.925688, 6.936097	February 2017	Root washings of *Ocimum basilicum*, grown in the bio-center greenhouses
	KD1022	-	-	Germany, Cologne	50.925688, 6.936097	February 2017	Root washings of *Nicotiana* sp., grown in the bio-center greenhouses
Rhogostoma epiphylla	KD1019	KY905095	1653	Germany, Cologne	50.956402, 6.859931	May 2015	Leaf surface of *Arabidopsis* sp.; the plant grew next to the Max Planck Institute for Plant Breeding Research

Phylogenetic analyses

The partial sequences were manually checked for sequencing errors before being assembled into one sequence contig. For phylogenetic analyses sequences were blasted (blastn2.3.0) against the NCBI GenBank database (last date of accession: 14.06.2016) and closely related sequences were added to an already existing alignment (Dumack et al. 2017b) in SeaView (V4.5.3, Gouy et al. (2010)). Downloaded sequences were manually aligned, using 1725 sites, which were to 51.59% invariant. The best fitting model GTR + I + G was chosen according to the analyses of Dumack et al. (2017b). With this model, phylogenetic trees were calculated in PhyML 3.1 (Guindon and Gascuel 2003) and MrBayes (settings: mcmc ngen = 1 M, sample freq = 100, print freq = 100, diagn freq = 1000; (Altekar et al. 2004; Ronquist and Huelsenbeck 2003)). Additionally a genetical distance

matrix was calculated in PAUP (V4.0a152; (Swofford 2002)). The sequences were submitted in the NCBI database under the accession numbers: KY905095 andKY905096.

Food range experiment

Four fungal cultures were grown on Potato Glucose Agar (PGA) according to the manufacturer's instructions (Sigma-Aldrich). The three algal strains were grown in Waris-H at room temperature and day light. Yeasts and algae were transferred to Waris-H before use. The organisms were separately distributed in 5 cm diameter Petri dishes. Feeding success of *Rhogostoma epiphylla* (strain KD1019) and *Rhogostoma cylindrica* (strains KD1020 and KD1021) was determined by observations of ingestion, and cultures were checked for sustained growth and morphological changes of protist cells after 1, 4 and 12 days respectively with an inverted microscope.

Results

Sampling and culturing

We isolated four different strains of *Rhogostoma* spp. from Germany and Belgium. One strain (KD1019), which was obtained from *Arabidopsis* leaves, matched superficially the morphology reported for *Rhogostoma schuessleri*. Three similar strains with a distinct morphology were isolated from bulk soil of an agricultural field (KD1020) and rhizosphere soils of *Ocimum basilicum* (KD1021) and *Nicotiana* sp.(KD1022); strains KD1019 and KD1020 are described as *R. epiphylla* sp. nov. and *Rhogostoma cylindrica* sp. nov., respectively. During the isolation process, *Rhogostoma epiphylla* was observed feeding on co-isolated phyllosphere algae, which then were cultured separately.

Microscopical observations

Strain KD1019 - *Rhogostoma epiphylla* sp. nov.

The amoebae bear a thin and hyaline test, carried in an upright position. The theca of healthy cells is apical-basal (basal corresponds to the surface-attached part of the test with the aperture) compressed with a mean diameter of 19 µm (range of 15–27 µm) and a length/width ratio of about 1.2 (Fig. 1). The surface of the theca is usually smooth without any prominent folds or attached xenosomes (Fig. 1).

The spherical nucleus with a large spherical nucleolus usually located in its center is 6.6 ± 1 µm in size (Fig. 1). The nucleus is located at, but not necessarily central to the apical end of the cell (i.e. opposite to the aperture). Granules and food vacuoles are dispersed all over the cell body (Fig. 1F). Several contractile vacuoles can be observed close to the aperture (Fig. 1B). The aperture, being slit-like, divides the cell in two more or less (as the nucleus lies sometimes in one of the two sides) symmetric parts (Fig. 1B). The cell is therefore (more or less) bilateral.

The amoebae move with thin and often branched filopodia with up to 30 µm length. Lamellipodia are formed frequently (Fig. 2). Starving cells retract their filopodia and float passively. Cell division is longitudinal.

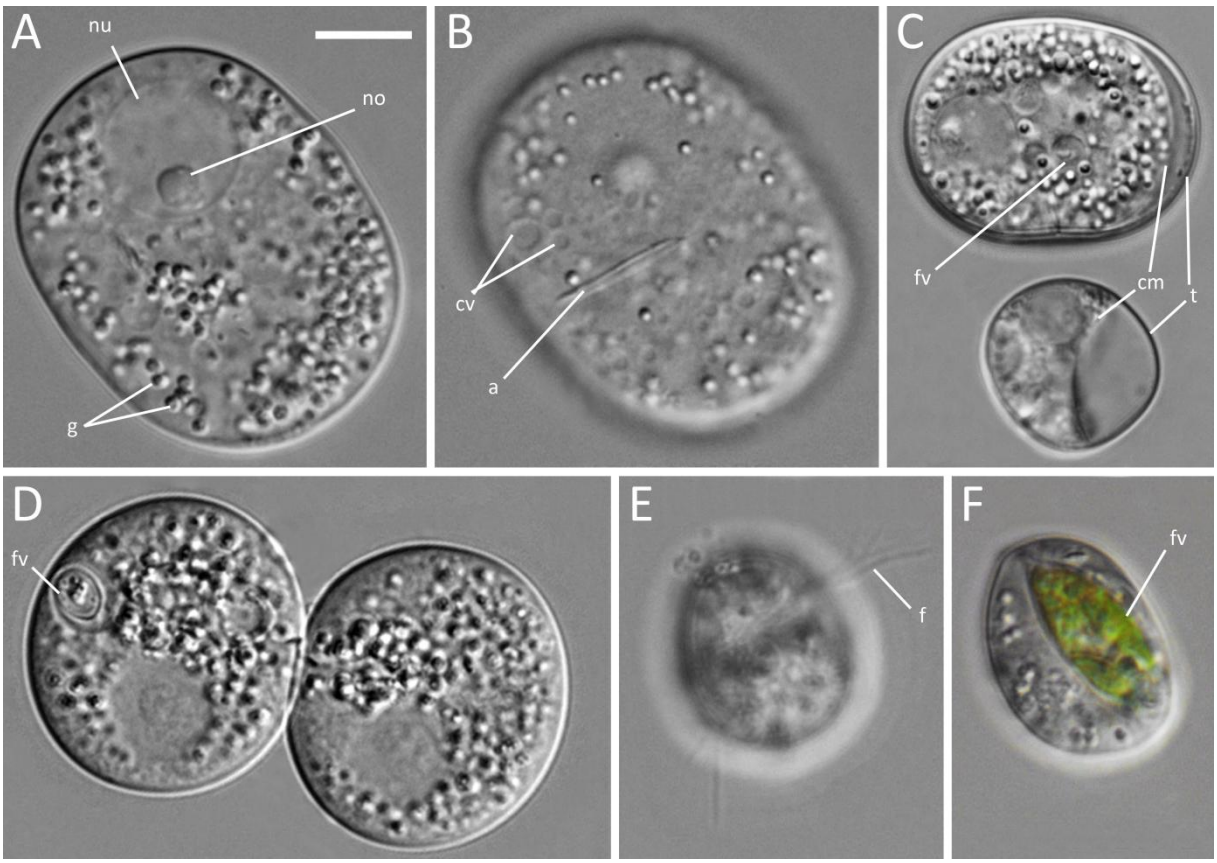

Figure 1 - Overview of *R. epiphylla* (KD1019): (A + B) same cell in two different focus layers; (C) starving cells, note under starving conditions the cell membrane separates from the theca; (D) dividing cells, one with ingested yeast cell; (E) focus on basal end with filopodia; (F) cell with ingested alga, *Characium* sp. Measure bar indicates 10 µm. Abbreviations: a = aperture; cm = cell membrane; cv = contractile vacuole; f = filopodia; fv = food vacuole; g = granules; no = nucleolus; nu = nucleus; t = theca.

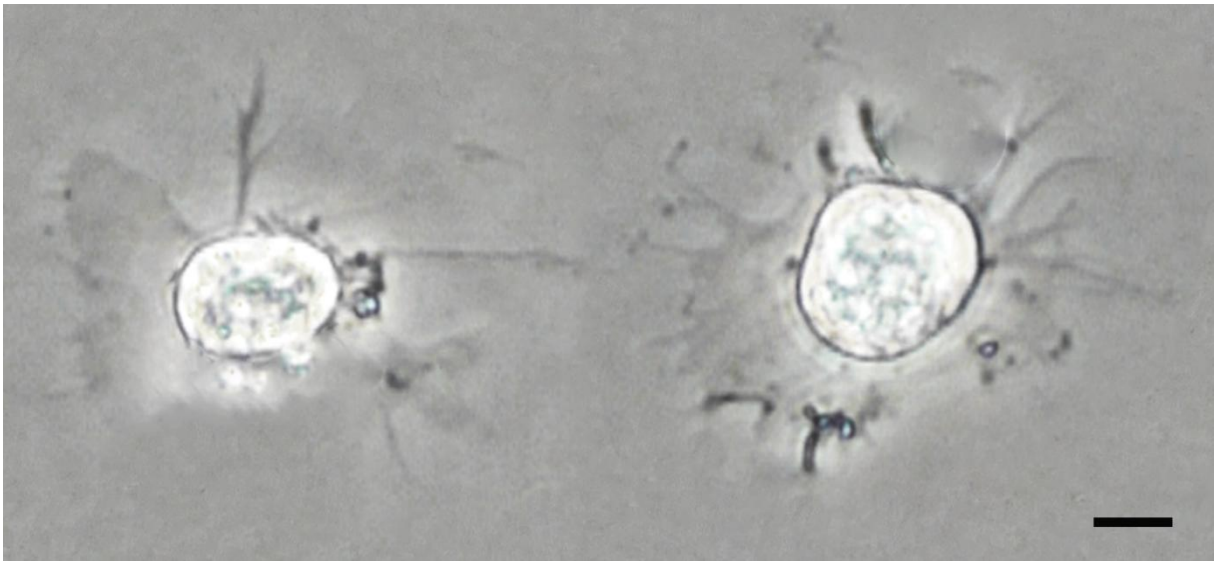

Figure 2 - Inverted images of two *Rhogostoma epiphylla* (KD1019) individuals with broad lamellipodia. Measure bar indicates 10 µm.

Strain KD1020 - *Rhogostoma cylindrica* sp. nov.

The amoebae bear a thin and hyaline test, carried in an upright position. The theca is cylindrical and 10.6 ± 0.5 µm in length and 6.1 ± 0.9 µm in width (Fig. 3A, n = 8). The surface of the theca is smooth without any prominent folds or attached xenosomes (Fig. 3).

The nucleus (2.5 ± 0.5 µm; Fig. 3A, E) is located opposite to the aperture at the apical end of the cell and spherical in shape, with a fine marbled structure. It bears in its center a large and spherical nucleolus. The nucleus is embedded in hyaline plasma which contains large granules. Food vacuoles filled with bacteria are located central to the cell body (Fig. 3F, see also Fig. 4). The cell is bilateral and divided by a slit-like aperture in two symmetric parts (Fig. 3A). Mostly two, rarely three contractile vacuoles can be observed close to the aperture (Fig. 3B, C). Contractile vacuoles completely fill the gap between the slit-like aperture and the lateral side of the theca before systole (see Fig. 3B, C). Contractile vacuoles usually beat alternating where one starts to increase in size while the other is in systole (Supplementary Video 1, also seen in Supplementary Videos 2 + 3).

The amoebae move with thin often branched filopodia with a measured length of maximal 13 µm (Fig. 4, Supplementary Video 3). Lamellipodia are formed frequently (Supplementary Video 2). Starving cells retract their filopodia and float passively. Cell division is longitudinal.

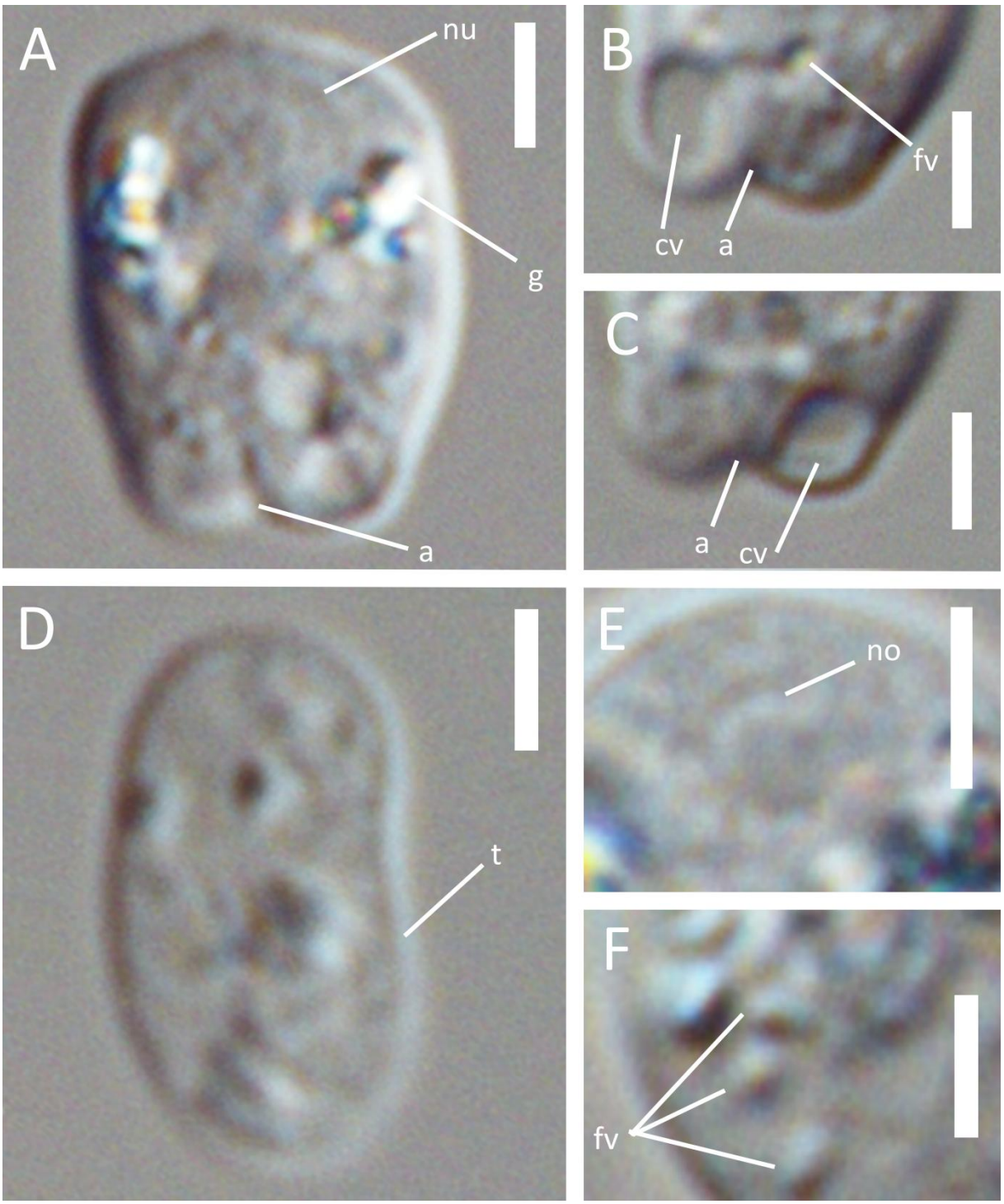

Figure 3 - Overview of *R. cylindrica* (strain KD1020): (A) overview, front view; (B + C) same individual, pictures were taken only a few seconds apart from each other, showing one contractile vacuole growing while the other is in systole; (D) lateral view of a dead cell, note the slimness compared to front view and the empty theca that remains when individuals die; (E) nucleus with nucleolus; (F) bacteria in food vacuoles. Measure bar indicates 2.5 µm. Abbreviations: a = aperture; cv = contractile vacuole; fv = food vacuole; g = granules; no = nucleolus; nu = nucleus; t = theca.

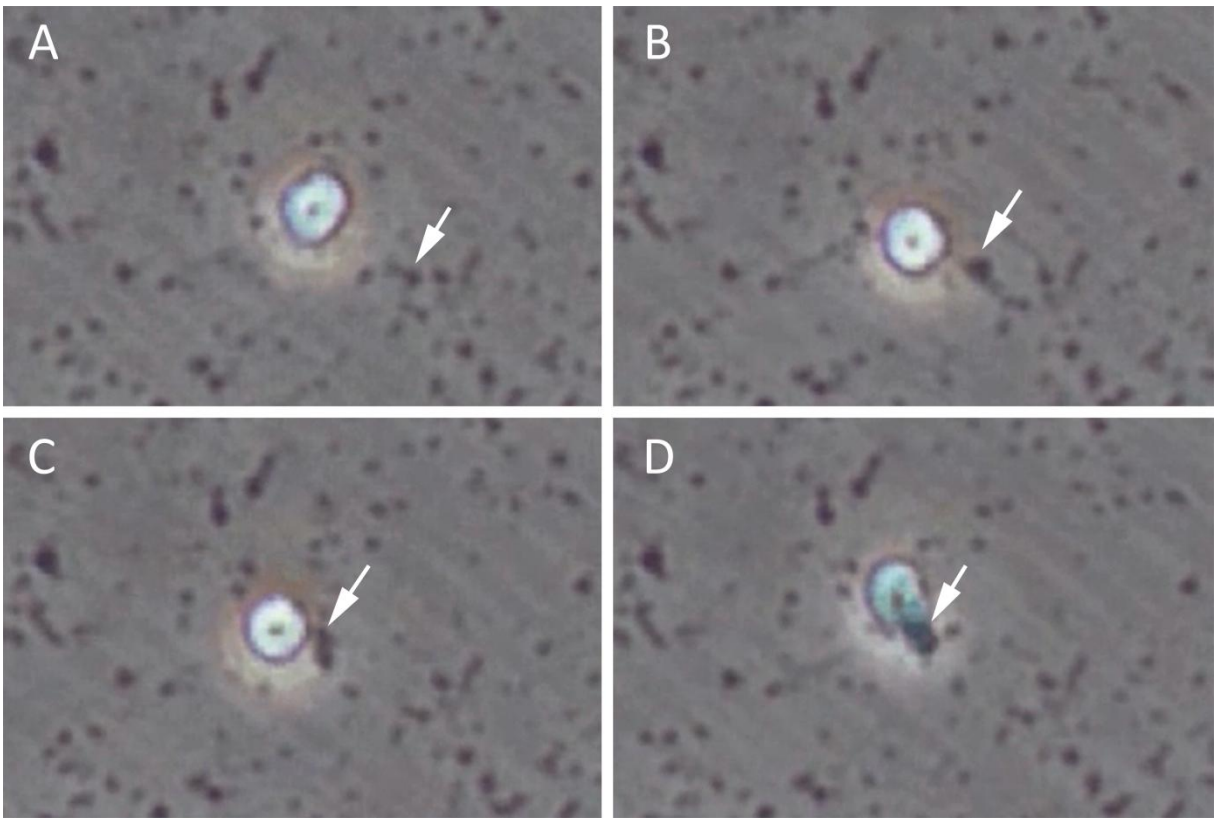

Figure 4 - Inverted image of a *Rhogostoma cylindrica* (strain KD1020) cell feeding on a bacteria cluster (arrow). (A) cell approaches bacteria; (B) filopodia reach to bacteria; (C) bacteria are dragged to the aperture; (D) bacteria are engulfed. Measure bar indicates 10 µm.

Strains KD1021 and KD1022

Strains KD1021 and KD1022 were similar in cell morphology to KD1020, this includes cell architecture, shape and size. Based on this, these strains were considered as the same species.

Food range experiment

For food ingestion *R. cylindrica* and *R. epiphylla* move their prey with their filopodia to the front of the aperture, similar to other thecofilosean amoebae (Fig. 4). Both tested strains of *R. cylindrica* (KD1020 and KD1021) showed similar results and are therefore presented as one (Table 2). *Rhogostoma cylindrica* and *R. epiphylla* consumed co-isolated bacteria in the culture medium (Table 2; Supplementary Video 2). *R. epiphylla* additionally consumed a range of algae (Fig. 1F) and unicellular fungi (Fig. 1D). As already shown for other thecofilosean species, the prey ingestion depended on the size of the prey relative to the cell body of *Rhogostoma*. Although *R. cylindrica* did not feed on eukaryotes in our experiment (Table 2), we observed that *R. cylindrica* attempted to ingest yeasts (Supplementary Video 3) and algal cells.

Table 2 - Results of the feeding experiments. ✓ food source has been consumed; - no ingestion could be observed; [co] refers to *R. epiphylla* co-isolated organisms.

Functional group	Phylum	Species	*R. epiphylla* (strain KD1019)	*R. cylindrical* (strains KD1020 and KD1021)
Yeasts	Ascomycota	*Saccharomyces cerevisiae*	✓	-
	Basidiomycota	*Sporobolomyces ruberrimus*	✓	-
		Cryptococcus laurentii	✓	-
Spores	Ascomycota	*Fusarium culmorum*	-	-
Algae	Chlorophyta	*Characium* sp.[co]	✓	-
		Chlorella sp.	✓	-
	-	unidentified coccal green alga[co]	✓	-
Bacteria	-	not further determined[co]	✓	✓

Sequencing of cultured amoebae and phylogeny

Two near full length SSU rDNA sequences were obtained. The sequenced parts ranged from 1635 (*Rhogostoma epiphylla*) to 1653 (*Rhogostoma cylindrica*) nucleotides. No introns were found. A maximum likelihood tree (Fig. 5) was inferred for the cercozoan subphylum Filosa, which included Thecofilosea, Sarcomonadea and Imbricatea as some of its subtaxa, and selected sequences of Endomyxa as the outgroup. Similar to previous analyses using SSU rDNA sequence comparisons, some basal branches within the Cercozoa were not supported (Bass et al. 2009a; Howe et al. 2011). The class Thecofilosea is composed of the Cryomonadida and Tectofilosida (thecate amoebae highlighted in color (Fig. 5)). The family Rhogostomidae was maximally supported and composed of the three genera *Sacciforma*, *Capsellina* and *Rhogostoma*, the latter accommodating *R. cylindrica* sp. nov. and *R. epiphylla* sp. nov.

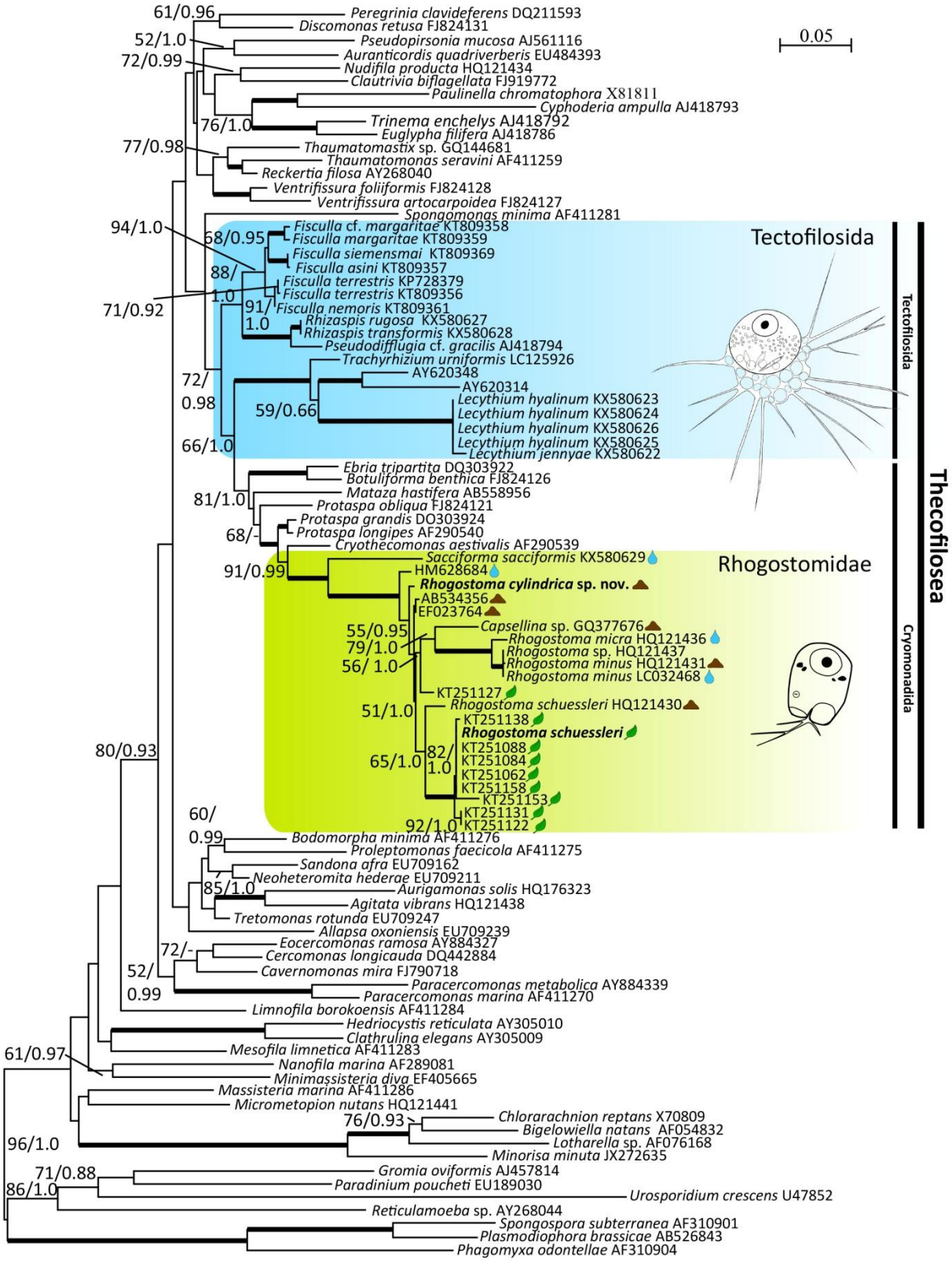

Figure 5 - SSU rDNA phylogeny of Filosa focusing on the Thecofilosea with selected Endomyxa as outgroup. Shown is the maximum likelihood tree obtained by the PhyML GTR + I + G analyses including 92 sequences and using 1725 aligned sites. The support levels of the PhyML and the Bayesian analysis are shown on the respective branches (ML/BI) if ML support was over 50%. Bold lines: ML support ≥95%. Support under 50% or 0.85 are omitted. Thecofilosean amoebae are highlighted; and amoebae sequenced in this study are in bold. Sequences or isolates of the Rhogostomidae derived from the phyllosphere, soil or aquatic systems are identified by distinct symbols. Drawings are not to scale.

Diagnoses

***Rhogostoma cylindrica* sp. nov. FLUES ET DUMACK**

Diagnosis: Test: Cylindrical shape, length 9.9–11.3 µm, and width 4.8–7.5 µm. Nucleus: spherical, about 2–3 µm. Nucleolus: one, round; central to nucleus. Aperture: cleft-like. Cells show a clear zonation (apical to basal): (I) zone of nucleus embedded in few granules, (II) food vacuoles, (III) aperture and contractile vacuoles, (IV) filopodia or lamellipodia. Locomotion: actively creeping, filopodia rarely extending to 30 µm. Prey: mainly bacteria, potentially also very small eukaryotes. Cell division: longitudinal, binary.

Illustrations of type generating strain: Figs. 3 and 4; Supplementary Video files: 1, 2; this material constitutes the name-bearing type of the species.

Type generating strain: KD1020, other here reported strains (KD1021 and KD1022) indistinguishable by morphology.

Sequence of type generating strain (SSUrDNA): KY905096

Etymology: cylindrica [Latin] derived from cylindrus meaning cylinder, referring to the shape of the cell body.

***Rhogostoma epiphylla* sp. nov. FLUES, HERMANNS ET DUMACK**

Diagnosis: Test: roundish, diameter of 19 µm (range of15–27 µm), length/width ratio about 1.2. Nucleus: spherical, about 6.6 ± 1 µm. Nucleolus: one, round; central to nucleus. Aperture: cleft-like. Easy to confuse with the slightly smaller (in mean test diameter) *R. schuessleri*, Belar 1921. Locomotion: actively creeping, filopodia rarely extending to 30 µm. Prey: mainly bacteria also algae and unicellular fungi. Cell division: longitudinal, binary.

Illustrations of type generating strain: Figs. 1 and 2; this material constitutes the name-bearing type of the species.

Type generating strain: KD1019

Sequence of type generating strain (SSUrDNA): KY905095

Etymology: epiphylla [Greek] derived from *epi-* (meaning upon, near to, in addition) and *phyllon* or *phylla* (meaning leaf), referring to the association between *Rhogostoma epiphylla* and plant leaves.

Table 3 - Summary of morphological traits described for all currently known *Rhogostoma* spp.

Reported characteristics	***R. micra***	***Rhogostoma. minus***		***Rhogostoma schuessleri***		***R. epiphylla***	***R. cylindrica***
		Belar 1921	Howe et al. 2011	Belar 1921	Howe et al. 2011		
Test diameter*	7.5 µm (6.5-10 µm)	10-12µm	7.3µm (6.5-8.5µm)	12-15µm	15µm	19 µm (15-27 µm)	4.8-7.5 µm
Test height*	-	-	-	-	-	-	9.9-11.3µm
Test shape	spherical; slightly basal/apical compressed	spherical; slightly basal/apical compressed	spherical; slightly basal/apical compressed	spherical; slightly basal/apical compressed	spherical; slightly basal/apical compressed	spherical; slightly basal/apical compressed	elongated, cylindrical
Nucleus	spherical	spherical	spherical	spherical	spherical	spherical	spherical
Nucleolus	spherical	spherical	spherical	spherical	spherical	spherical	spherical
Habitat type	Freshwater	Freshwater	Freshwater	Freshwater	Soil	Phyllo-sphere	soil/ rhizosphere

*measurements refer to healthy growing cultures

Table 4 - Genetical distance table for currently sequenced and name-associated Rhogostomidae sp.

	Species	**SSU rDNA accession**	1	2	3	4	5	6	7
1	*R. micra*	HQ121436	-						
2	*R. 'schuessleri'*	HQ121430	0.03737	-					
3	*R. minus*	HQ121431	0.0108	0.03662	-				
4	*R. epiphylla*	KY905095	0.0388	0.01891	0.03905	-			
5	*R. cylindrica*	KY905096	0.03312	0.01258	0.03333	0.01901	-		
6	*'Capsellina* sp.'	GQ377676	0.0415	0.02848	0.03987	0.03094	0.02585	-	
7	*Sacciforma sacciformis*	KX580629	0.06754	0.06306	0.06871	0.06133	0.05947	0.06377	-

Discussion

Rhogostomidae phylogeny and taxonomy

There are only three *Rhogostoma* species known yet, namely *R. schuessleri* (type species), *R. minus* and *R. micra*. Our isolate of *R. epiphylla* shows a high similarity of cell morphology to *R. schuessleri* but an increased variability in size (Table 3). Belar (1921) described a variation of12–15 µm in the cell size of *R. schuessleri*; Howe et al. (2011) could not find a clear morphological difference of their isolate HQ121430, thus concluding that their isolate resembles *R. schuessleri*. However, their isolate originated from a terrestrial sample whereas Belar's *R. schuessleri i*nhabited freshwaters. Molecular surveys as well as phylogenetic approaches of Thecofilosea support mostly close relationships of freshwater to soil inhabitants but there is no evidence of the same species sharing both habitat types (Bass and Cavalier-Smith 2004; Cavalier-Smith and Chao 2003; Dumack et al. 2017c). Furthermore, our isolate of *R. epiphylla*, although showing a high morphological resemblance to *R. schuessleri* (Table 3), exhibits a high genetical distance to *R. schuessleri* HQ121430 (Table 4) and inhabits a very distinct habitat, backed up by culture-based and genetical surveys (Ploch et al. 2016). We are therefore not convinced that the *Rhogostoma* sp. of which sequence HQ121430 originated from resembles a genuine *R. schuessleri* and call it *R.* cf. *schuessleri* henceforth and argue that *R. epiphylla* resembles a cryptic species of the genus *Rhogostoma* inhabiting the phyllosphere. In contrast to all other described *Rhogostoma* spp., *R. cylindrica* sp. nov. shows, next to genetic differences, a ventral elongation and an overall smaller cell size, justifying the description of *R. cylindrica* as a new species.

The characterization of *R. cylindrica* gives further insight into morphological adaptations of the Rhogostomidae. According to the taxonomical concept proposed by Meisterfeld (2002), being revisited by Howe et al. (2011) and Dumack et al. (2017c) the (now called) Rhogostomidae are composed of three genera, *Sacciforma*, *Rhogostoma* and *Capsellina*. For *Capsellina* genetical evidence confirming this taxonomical concept is still obscure, since the only available sequence of a '*Capsellina* sp.' (GQ377676) is questionable. Its phylogenetic placement intermingles with *Rhogosotoma* spp., there is no morphological documentation of the sequenced strain and the sequence itself seems to be of low quality (many obscure base insertions, presence of undefined base reads and multiple point mutations and deletions in highly conserved regions). Nevertheless, the genus *Capsellina* has been repeatedly associated in close relation to *Rhogostoma* on morphological basis and was therefore included as a close relative in the taxonomical concepts of (Dumack et al. 2017c; Howe et al. 2011; Meisterfeld 2002).

According to our phylogenetic analyses, following assumptions about the morphological adaptation of the Rhogostomidae can be made: *S. sacciformis* (originally described as *Plagiophrys sacciformis* (Hertwig and Lesser 1874)) shows a laterally compressed and longitudinally elongated cell body. *R. cylindrica* is basal to all other characterized *Rhogostoma* spp. and in contrast to those, but similar to *S. sacciformis*, it shows a longitudinal elongation, although it is not laterally compressed. According to these observations the Rhogostomidae underwent evolution with ventral thickening and longitudinal contraction, resulting in the roundish shape of all other *Rhogostoma* and *Capsellina* species. Possibly, this roundish shape evolved when colonizing terrestrial habitats, minimizing the surface to volume ratio and thus reducing evaporation losses.

Rhogostoma in soils and on plant leaves

Rhogostoma spp. have been isolated from soils, sediments and freshwaters (Belar 1921; Howe et al. 2011). Ploch et al. (2016) identified OTUs of *Trinema* and *Rhogostoma* from leaves of Brassicaceae. They were surprised to find testate amoebae on plant leaves and suggested that these could have been contaminations from the surrounding soil. However, we could successfully isolate *R. epiphylla* from leaves of *Arabidopsis thaliana*, which was in its SSU rDNA sequence up to 100% identical to some of the phyllosphere derived clone sequences reported by Ploch et al. (2016). These results give strong evidence that *Rhogostoma*, in particular the species *R. epiphylla*, is a true phyllosphere colonizer. The phyllosphere is a harsh environment where desiccation resistance and fast reproduction are considered as the most important traits of leaf-associated protists. However, *Rhogostoma* spp. are not known to form cysts (Belar 1921; Howe et al. 2011; Mylnikova and Mylnikov 2012), but Belar (1921) described resting stages of *R. schuessleri* with retracted filopodia and a closed aperture without a cyst wall. These resting stages could withstand continuous desiccation up to three months and could be reactivated within 12 h when moistened (Belar 1921). Similar resting stages were observed in our isolate of *R. epiphylla* (Fig. 1C). It is suggested for testate amoebae that a smaller aperture is advantageous in drier environments (Bobrov and Mazei 2004). Schönborn (1992) showed that the size of apertures in testate amoebae is a functional trait that is adapted to drought after a few generations. With fast doubling times of ≤5 h, corresponding to a growth rate of ≥0.14 h^{-1} (Belar 1921), the reported growth rate of *R. schuessleri* is concordant with those reported for colpodeans in the phyllosphere (Bamforth 1973; Mueller and Mueller 1970).

The *Rhogostoma* isolates enabled us to obtain a first insight into their morphology and autecology, with respect to their feeding range. Both *Rhogostoma* species consumed bacteria, but the prey spectrum of *R. epiphylla* included a wide range of eukaryotic prey taxa. A variety of algae, some of which were co-isolated from the phyllosphere, and yeasts were

ingested and digested by *Rhogostoma epiphylla* and led to sustained reproduction. This broad food spectrum and preying on large unicellular organisms is certainly of advantage during the short activity periods in the phyllosphere. This trait differentiated the leaf inhabiting *R. epiphylla* from the rhizosphere isolates of *R. cylindrica*, and can be seen next to desiccation resistance and fast reproduction rates as a functional adaption to life on leaves.

Supporting Information

Supplementary Video 1 - 3 Cell overview and movement of *Rhogostoma cylindrica*
http://www.sciencedirect.com/science/article/pii/S0932473917300779?via%3Dihub

General Discussion

The studies described in this thesis made significant contributions to fill the gaps of fundamental knowledge on plant-associated protists by investigating the diversity and functional roles of Cercozoa in the rhizosphere and phyllosphere of plants. It has been shown that specific leaf-associated taxa among the Cercomonadida and Thecofilosea exist and they can exhibit important functions as grazers on bacterial communities and other members of the phyllosphere microbiome. Knowledge on the diversity of leaf-associated protists and in particular on their functional roles was scarce. Those studies investigating the occurrence of phyllosphere protists largely focused on their role as human pathogens or vectors of bacterial pathogens on vegetables (Ciurea-Van Saanen 1981; Gourabathini et al. 2008; Napolitano and Collettieggolt 1984; Napolitano 1982; Rude et al. 1983; Vaerewijck et al. 2014; Vaerewijck et al. 2011), while recent studies report their diversity by cultivation-independent molecular surveys that prevent assessing their ecological functions (Ploch et al. 2016; Sapp et al. 2018).

Within this thesis, several plant-associated Cercozoa were isolated, cultivated and described using sequence information and morphology. Using this information a total of five new species have been formally described and ten new previously unknown cercomonad genotypes were reported (Chapter 1 and 4). Leaf-associated Cercozoa were further analyzed according to their feeding habits to increase the knowledge on their functions. Chapter 1 revealed that plant-associated cercomonad community composition differed between the rhizosphere and phyllosphere but is not influenced by plant species identity. To get more exhaustive knowledge on the grazing impact of leaf-associated cercomonads on phyllosphere bacterial communities, chapter 2 of this thesis aimed to describe the grazing-induced shifts in bacterial community composition, function and interaction patterns. This study emphasizes the specific feeding preferences of leaf-associated cercomonads and that their grazing structures the bacterial community composition and function, leading to changes in the bacterial interaction patterns. Chapter 3 aimed to reveal the spatio-temporal interactions between leaf-associated cercomonads and phyllosphere bacteria on plant leaves and provides useful insights to their dynamics on leaves. The last chapter focused at increasing the knowledge on plant-associated cercozoan testate amoebae and reveals that leaf-associated and rhizosphere-associated Cercozoa can differ in their prey spectra.

Diversity of leaf-associated Cercozoa

The description of two new species and eight previously unknown genotypes obtained from the phyllosphere (Chapter 1 and 4) confirms the prevalent opinion that Cercozoa diversity is far from being completely revealed (Bass et al. 2009b; Brabender et al. 2012) and indicates that phyllosphere Cercozoa are highly undersampled. In contrast to the indentified Cercozoa isolated from the rhizosphere, only marginal numbers of cercozoan taxa from the phyllosphere could be affiliated to described species or published sequences. Interestingly several of those which could be affiliated were in their SSU rDNA sequence up to 100% identical to some of the phyllosphere derived clone sequences reported by Ploch et al. (2016). This corroborates the assumption that cercozoan taxa which are preferentially associated with the phyllosphere exist. Also the detection of novel described species *Neocercomonas epiphylla,* which was represented with nine strains exclusively isolated from the phyllosphere across all sampled plant species (Chapter 1); and *Rhogostoma epiphylla* (Chapter 4), which was also detected several times on leaves of Brassicaceae (Ploch et al. 2016), further confirms this assumption. The detection of *Rhogostoma epiphylla* by Ploch et al. (2016) and our study, with 100% identical sequences, additionally give strong evidence that also some cercozoan testate amoebae can be true phyllosphere colonizers.

As indicated by the results within chapter 1, we prove that plant-associated cercomonad communities show a clear deterministic assembly in the above- and belowground compartments of plants. Only few cercomonad taxa were shared in both compartments. These results are fully in line with the findings reported by Sapp et al. (2018). This recent study, based on *Arabidopsis thaliana*, reported detected cercozoan taxa belonged mainly to the Glissomonadida and Cercomonadida and their communities were strongly differentiated by plant compartments with only few taxa shared in both. While we could observe a clear assembly pattern to plant compartments for the clade A cercomonad community, this association was not so clear for the *Paracercomonas* community of clade B. We could not observe differences in the *Paracercomonas* community composition along the rhizosphere or phyllosphere, although several *Paracercomonas* taxa were exclusively isolated from the phyllosphere. These results correspond to the findings that *Paracercomonas* taxa appear to be generalists (Sapp et al. 2018). However, Sapp et al. (2018) also reported the presence of OTU 2, a relative of *P. minima*, in 100% of the leaf samples. This shows an amazing consensus with our results, since *Paracercomonas* sp. WA10, a close relative of *P. minima*, was the only *Paracercomonas* taxon consistently and exclusively isolated from phyllosphere samples. This gives evidence that true phyllosphere colonizers might also exist among the genus *Paracercomonas*. Especially cercomonad taxa from clade A1b2 represent high affiliation to leaf surfaces as indicated by chapter 1 and Ploch et al. (2016). Nevertheless,

leaf-associated cercomonads are not limited to clade A1b2, since taxa from several clades were detected on or isolated from the phyllosphere (Bass et al. 2009b; Ploch et al. 2016; Sapp et al. 2018).

In chapter 1, we could not demonstrate that plant functional group identity affects the cercomonad species composition as known for bacteria (Laforest-Lapointe et al. 2016a; 2016b), suggesting that no plant species are preferentially colonized by protists. This is also in line with the results of previous studies which detected similar cercomonad communities on leaves of *A*. *thaliana*, *Cardamine hirsute*, *C*. *pratensis* and *Draba verna* (Ploch et al. 2016; Sapp et al. 2018). However, it is likely that not every plant species has a suitable leaf surface for protist colonization. Some plant species such as *Colocasia esculenta* (taro) might prevent leaf colonization by protists due to their hydrophobic water repellent leaf structure (Neinhuis and Barthlott 1997) as it has been shown for bacteria and fungi (Barthlott and Neinhuis 1997; Lips and Jessup 1979; Ma et al. 2011). Furthermore, it is also likely that protist communities on leaves are influenced by the biodiversity of their prey as shown by the results of chapter 2 and 4 as well as by the study of Sapp et al. (2018). In addition, it is known that bacterial communities on leaves undergo seasonal succession (Redford and Fierer 2009) and differences in plant traits shape their associated microbial communities (Lambais et al. 2017). While evergreen plants possess leaves throughout the year (Niinemets 2007), deciduous perennial and annual plants develop new leaves within every growth season. This raises the question how these leaves get colonized by leaf-associated protists. Protist cysts are constantly distributed by passive transport in the air (Rogerson and Detwiler 1999) and this mechanism is suggested to be the main source for the colonization of leaves (Mueller and Mueller 1970). Thus, protists which show a preferential association to the phyllosphere might have narrow competitiveness and find their ecological niche only on leaves.

Nonetheless, during the course of investigations within this study, we also observed a vast diversity of other cercozoan lineages in phyllosphere samples, in particular from the order Glissomonadida. The occurrence of several taxa from the Glissomonadida, Cercomonadida and Thecofilosea across all sampled plants, supports finding of previous studies and corroborates the assumption that leaf-associated Cercozoa constitute an integral part of the phyllosphere microbiome (Ploch et al. 2016; Sapp et al. 2018).

Functional roles of leaf-associated Cercozoa

Detailed knowledge on the feeding preferences of bacterivorous leaf-associated cercomonads and their direct and indirect effects on the composition and function of phyllosphere bacterial communities was obtained in chapter 2. For the first time we report

detailed insights how protist predation modifies the taxonomic and functional composition of bacterial communities using a metagenomics approach. Shotgun metagenomic sequencing provides information both about which organisms are present and what metabolic processes are possible in the community (Segata et al. 2013) and is thereby an excellent tool to examine the structure and function of protist-grazed bacterial communities at very fine levels. This is an important improvement for protist research, since detailed knowledge how protist predation modifies the taxonomic and functional composition of bacterial communities is scarce. Previous studies on protist grazing identified the bacterial communities only at low taxonomic resolution, while most information on altered bacterial functions and interactions has been obtained in studies with just one or few model bacteria (Glücksman et al. 2010; Jousset and Bonkowski 2010; Jousset et al. 2009; Jousset et al. 2008; Kreuzer et al. 2006; Mazzola et al. 2009; Rosenberg et al. 2009; Saleem et al. 2013). Furthermore, chapter 4 revealed that the functional roles of leaf-associated Cercozoa are not limited to bacterivory and confirmed their algivorous and fungivorous feeding habits.

Grazing structures bacterial community composition and interaction

As indicated by the results obtained within chapter 2, we prove that leaf-associated cercomonads significantly structure bacterial community composition, leading to an altered interaction pattern among them. We further show that shifts in bacterial community composition could be linked to phenotypic protist traits. Concordant with findings of Glücksman et al. (2010) multiplication rates of bacterivorous leaf-associated cercomonads were most important in determining bacterial community composition. Our data indicate that losses in bacterial numbers of one taxon were compensated by other genera. Bacterial gains and losses were not random and showed regular patterns, indicating clearly deterministic patterns of bacterial community assembly when subjected to predation. Grazing shifted the community from Alpha- and Betaproteobacteria as well as Sphingobacteriia (phylum Bacteroidetes) towards Gammaproteobacteria and Opitutae. Concordant with other studies, Alpha- and Betaproteobacteria are less resistant to protist grazing (Boenigk et al. 2004; Murase et al. 2006; Rosenberg et al. 2009), highlighting the importance of top-down processes for structuring bacterial communities. Combinations of data from several studies on bacterial microbiomes on leaves defined the first catalogs of phyllosphere-associated generalist bacterial phyla present in different plant species, thus highlighting the involvement of Bacteroidetes, Actinobacteria, and Proteobacteria (Bringel and Couee 2015; Delmotte et al. 2009; Kim et al. 2012; Lopez-Velasco et al. 2011; Rastogi et al. 2012; Redford et al. 2010). This bacterial core microbiome on different plant species, hence offer optimal conditions for grazing by leaf-associated cercomonads and can enable them to persist in the phyllosphere. Furthermore, according to the current understanding specific associated

bacterial communities also shape the composition of Cercozoa (Sapp et al. 2018), indicates that microbial communities on leaves are likely determined by top-down and bottom-up processes. Moreover, it is also likely that these observed bacterial core microbiomes represent already influenced communities, since the occurrence and grazing effects of their predators have never been considered in those studies.

In presence of leaf-associated cercomonads bacterial interaction networks clearly changed and showed less complex correlations between bacterial taxa with a higher proportion of positive correlations. This suggests that predation reduce the influence of strong competitors, leading to weaker interaction strength in grazed communities with an increased competitive advantage of formerly subordinate species in response to predation (Bell et al. 2010; Jousset et al. 2008). Hence, as recently revealed for leaf-associated Oomycota *Albugo* (Agler et al. 2016), it is likely that leaf-associated cercomonads also act as important hub taxa on leaves and shape the plant microbiome in the phyllosphere by influencing large interaction networks of microbial communities. But this has to be evaluated in further studies by direct investigation on the leaf surface. However, our data confirm significant shifts in abundance and co-occurrence of bacterial taxa that contain important leaf pathogens and strains beneficial for plant growth such as *Pseudomonas*, *Burkholderia*, *Herbaspirillum* and *Stenotrophomonas* (Compant et al. 2008; Hirano and Upper 2000; Lugtenberg et al. 2013; Mahenthiralingam et al. 2008; Ryan et al. 2009; Schmid et al. 2006) and therefore it is likely that protist grazers may have indirect effects on plant performance by shifting the plant microbiome.

Grazing alters bacterial community function

The results of chapter 2 demonstrate that leaf-associated cercomonads can have significant impact on the physiological function of bacterial communities. Protist predation influenced 14 metabolic core functions from which membrane transport were in particular up- or downregulated. Within membrane transport an overrepresentation of type VIII secretion systems (T8SS) was seen in the non-protist controls. The downregulation of T8SS in the protist treatments might indicate a reduced surface colonization in response to predation and can be expected to reduce predation risk by surface feeding protists. Moreover, we found a high abundance of sequences affiliated with type VI secretion systems (T6SS) in the protist treatments and studies suggest a role of T6SS in the bacterial defence against protists and competing bacteria (Coulthurst 2013; Hood et al. 2010; Schwarz et al. 2010). This overrepresentation of T6SS in the protist treatments suggests that the grazed bacterial communities upregulated their T6SS to defend themselves against protist predation by the injection of antimicrobial toxins. Nevertheless, only few transcriptional changes of the grazed

bacterial communities could be so clearly interpreted, but our results confirm that the physiological function of natural bacterial communities and their ecosystem functions might not be understood if protist grazers are not considered (Trap et al. 2016).

Leaf-associated Cercozoa are more than bacterial feeders

In contrast to the functions of bacterivore leaf-associated cercomonads, chapter 4 reveals that phyllosphere Cercozoa are far more than just bacterial feeders and prey also on other members of the leaf-microbiome. The microbial communities of leaves are diverse and among others, also include a variety of yeasts and algae (Lindow and Brandl 2003; Morris and Kinkel 2002). As indicated by the results of chapter 4, leaf-associated *Rhogostoma epiphylla* consumes bacteria, but also wide range of eukaryotic prey taxa including yeasts and algae, some of which were co-isolated from the phyllosphere. This broad food spectrum is certainly of advantage in the phyllosphere with potential important repercussions to the leaf-microbiome and its interactions within. This gives evidence that interaction patterns on leaves are by far more complex as expected and our data (Chapter 2 and 4) indicate that the phyllosphere of plants contains complex food webs with different trophic levels where top-down and bottom-up processes are likely to occur.

Dynamics between Cercozoa and bacteria on leaves

We aimed to reveal the spatio-temporal dynamics between leaf-associated cercomonad Cercozoa and phyllosphere bacteria on bean leaves using epifluorescence microscopy (Chapter 3). As indicated by the results, we prove that all three cercozoan strains are successfully feeding on *P. eucalypti* single cells, while agglomerated bacterial cells provide grazing resistance to predation. This study also exhibits that *C. hederae*, *C. plasmodialis* and *P. producta* are actively feeding on bacterial cells in the phyllosphere when water films exist, but encyst when these evaporate. We further confirm that these excyst again, if the leaf surface is rehydrated.

However, due to the biological processes of en- and excystment and methodological difficulties in epifluorescence microscopy on wet leaf surfaces, it was not possible to investigate the grazing effects of cercozoans on the spatial and temporal dynamics of phyllosphere bacteria in chapter 3. Therefore, the use of epifluorescence microscopy is not an ideal method to answer these questions, but spatio-temporal dynamics between leaf-associated protists and phyllosphere bacteria can be resolved using different techniques like metagenomic approaches in further studies.

Nevertheless, the results of chapter 3 give evidence that leaf-associated cercomonads resemble the same diurnal lifestyle as reported for *Colpoda cucullus*, which is a ciliate especially adapted to the life in the phyllosphere (Mueller and Mueller 1970). This detailed study on epiphytic protozoa describes that phyllosphere protists are characterized by diurnal life cycles usually with short active periods at nighttime when dew accumulates on plant leaves, or in the event of rain (Mueller and Mueller 1970). Concordant with these results we prove that leaf-associated cercomonads are closely connected to the availability of water films on leaves and that they are well adapted to the transient moisture conditions of plant surfaces. Since water films are regularly formed on leaves (i.e. morning dew), the phyllosphere provides suitable conditions for the activity of leaf-associated protists and periodic interactions with their prey are likely to occur. Moreover, these interactions are also depend on the phyllosphere characteristics (see above) and the ability of the leaf surface to retain water films for a longer period (Brewer et al. 1991).

Based on the analysis of functions in chapter 2, which prove that phyllosphere bacterial communities respond to grazing with the downregulation of T8SS to reside in the water column, might be only a limited strategy to reduce predation by phyllosphere protists. Obviously this strategy will reduce predation risk by surface feeding protists such as leaf-associated cercomonads, but will not reduce predation by filter feeders located in the water column such as *Colpoda cucullus*. Concordant with this, Lindow (2006) reports an unpublished experiment where *C. cucullus* reduced numbers of inoculated *Pseudomonas syringae* on bean leaves by two orders of magnitude, showing that leaf-associated protists can have considerable impact on phyllosphere bacteria.

The phyllosphere is a harsh environment where desiccation resistance and fast reproduction are considered as the most important traits of leaf-associated protists (Mueller and Mueller 1970; Ploch et al. 2016). As shown for leaf-associated cercomonad Cercozoa, successful phyllosphere protists are obliged to have the ability to pass quickly between active and latent metabolism and to tolerate moisture fluctuations by developing resistant cysts (Bamforth 1980; Mueller and Mueller 1970). Although the occurrence of Rhogostomidae on leaves is proven by chapter 4 and Ploch et al. (2016), it has to be mentioned that *Rhogostoma* spp. are not known to form cysts (Belar 1921; Howe et al. 2011; Mylnikova and Mylnikov 2012). However, testaceans can withdraw into their shell to supplement encystment ability (Bamforth 1980). Concordant similar resting stages were observed in our isolate of *Rhogostoma epiphylla* (Chapter 4). It has been reported these resting stages can withstand continuous desiccation up to three months and could be reactivated within 12 h when moistened (Belar 1921). This characteristic reinforces the assumption that cercozoan testate amoebae like Rhogostomidae can be true phyllosphere colonizers. Short generation times,

desiccation resistance and the ability to prey on a wide range of algae and yeasts from the phyllosphere are seen as crucial traits for the phyllosphere colonization by *Rhogostoma*.

Diversity and functional roles of Cercozoa in the rhizosphere

Although the studies within this thesis focused towards the investigation of leaf-associated Cercozoa and their functional roles in the phyllosphere, the results also allow interferences to their diversity and functional roles in the rhizosphere of plants.

As shown by the results of chapter 1, the rhizosphere of plants harbors a vast diversity of cercomonads representing a divergent community composition than the aboveground compartments of plants. Studies confirm the taxon *Cercomonas* to be dominant in soils (Bates et al. 2013; Brabender et al. 2012; Domonell et al. 2013) especially in the rhizosphere of plants (Lara et al. 2007; Turner et al. 2013) and as being dominant feeders on rhizosphere bacteria (Lueders et al. 2004). It is further known that plant diversity influences cercozoan richness (Tedersoo et al. 2016). However, as indicated by the results of chapter 1 we could not demonstrate that plant functional group identity affects the cercomonad community composition in the rhizosphere, suggesting that no plant species are preferentially colonized by protists. Nevertheless, it is likely that cercomonad community compositions differ among different rhizosphere systems, since a recent study show strong effects of edaphic factors on the composition of the Cercozoa in the rhizosphere (Sapp et al. 2018).

It can be assumed that the general directions of grazing-induced shifts in bacterial community composition, as revealed by chapter 2, also apply to the effects of cercomonads on rhizosphere communities. However, this must be elucidated in further studies, since Cercozoa control bacterial communities differentially via grazing as shown by chapter 2 and (Glücksman et al. 2010). Nonetheless it is likely that Alpha- and Betaproteobacteria are also less resistant to cercomonad grazing in the rhizosphere as shown for other protists (Rosenberg et al. 2009). Furthermore, *Cercomonas* taxa are not solely bacterivorous and have been shown to graze also on yeasts and spores of the plant pathogenic fungus *Fusarium culmorum* (Geisen et al. 2016). Thus, the genus *Cercomonas* might be of substantial ecological importance in the rhizosphere of plants, since soil protists are suggested to form a dynamic hub in the soil microbiome linking diverse bacterial and fungal populations (Xiong et al. 2018).

As shown by the results of chapter 4 and in contrast to the leaf-associated *Rhogostoma epiphylla*, the soil-dwelling *R. cylindrica* has a narrow food spectrum and preys only on bacteria. Since this species was isolated several times from the rhizosphere of different plant

species, *R*. *cylindrica* might not represent a prominent algivorous or mycophagous taxon within the rhizosphere. However, it is likely that soil dwelling cercozoan testate amoebae such as the Thecofilosea represent important algivorous taxa in soils, since they show a high correlation to phototroph (i.e. algae) abundances in soils (Seppey et al. 2017). It can be assumed that plant-associated Cercozoa in the rhizosphere of plants can have considerable impact on bacterial communities as well as on fungi, yeasts and algae, but details must be investigated in further studies. Hitherto we have only a vague idea of the multitude of ecological functions carried out by soil protists and the description of protist diversity in association with plants has been highlighted as one of the 30 fundamental questions in soil protist research (Geisen et al. 2017).

Leaf-associated protists and their relevance for phytobiome research

Although this thesis made significant contributions to the basic knowledge on leaf-associated protists, little is known about the impact of protists on the microbial food webs in the phyllosphere. The proven ability of phyllosphere protists to shape bacterial community composition, function and interaction (Chapter 2) as well as to additionally prey on a wide range of eukaryotes (Chapter 4), suggests complex interactions within phyllosphere microbiomes. However, detailed knowledge on all these interactions is absent and we still have only a vague idea of these complex interactions, particularly with respect to fundamental questions such as which organisms are present and what they do there.

Considering the phyllosphere hosts microbiomes including bacteria, algae, yeasts, fungi and the highly diverse group of protists, it seems that we only grasped the tip of the iceberg on deciphering the importance and the multitude of ecological functions carried out by leaf-associated protists. Nevertheless, studies demonstrated that the phyllosphere act as a habitat for protists across different plant species (Mueller and Mueller 1970; Napolitano and Collettieggolt 1984; Ploch et al. 2016; Sapp et al. 2018; Vaerewijck et al. 2014; Vaerewijck et al. 2011). Considering the large global surface area represented by leaves, which is twice as large as the global land surface, the extent of their ecological functions in different ecosystems could be enormous. Knowing that moisture on leaves strongly determines the activity of leaf-associated protists, indicates that phyllosphere protists might be less important for plant microbiomes in dry regions, but likely they will be more important in wetter regions such as the tropics with enhanced precipitation and leaf wetness.

However, knowing that bacterial communities from the aboveground surface of plants influence plant performance and epiphytic microbial communities provide a new target for

crop yield optimization (Methe et al. 2017), it is surprising that leaf-associated protists up to now have gained little consideration in epiphytic phytobiome research. It is crucial to consider them, since they can have strong influence on bacterial community composition, function and interaction as well as on other members of the phytobiome. As shown by the results of chapter 2, leaf-associated cercomonads influenced both abundance and co-occurrence of bacterial taxa that contain important leaf pathogens and strains beneficial for plant growth (e.g. *Pseudomonas*, *Burkholderia*, *Herbaspirillum* and *Stenotrophomonas*) and it is likely that protist grazers may have indirect effects on plant performance by shifting the bacterial communities. A comprehensive understanding of the mechanisms that govern selection, activity and interaction of microbial communities on plant leaves might provide new opportunities to increase plant performance and remain an open field for new exciting discoveries in protistology.

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I dedicate this work to my **mom Karin Flues**.

Subpublications and Record of Achievement

Chapter 1[1,2,3] - Diversity of Cercomonad Species in the Phyllosphere and Rhizosphere of Different Plant Species with a Description of *Neocercomonas epiphylla* (Cercozoa, Rhizaria) a Leaf-Associated Protist

Flues, S., Blokker, M., Dumack, K., Bonkowski, M. (in Review) Diversity of Cercomonad Species in the Phyllosphere and Rhizosphere of Different Plant Species with a Description of *Neocercomonas epiphylla* (Cercozoa, Rhizaria) a Leaf-Associated Protist. Journal of Eukaryotic Microbiology - Manuscript ID JEUKMIC-17-5014

Chapter 2[1,4] - Grazing of Leaf-Associated Cercomonads (Protists: Rhizaria: Cercozoa) Structures Bacterial Community Composition and Function

Flues, S., Bass, D., Bonkowski, M. (2017) Grazing of Leaf-Associated Cercomonads (Protists: Rhizaria: Cercozoa) Structures Bacterial Community Composition and Function. Environmental Microbiology **19**(8):3297–3309. doi:10.1111/1462-2920.13824

Chapter 4[1,3,5,6] - Rhogostomidae (Cercozoa) from Soils, Roots and Plant Leaves (*Arabidopsis thaliana*): Description of *Rhogostoma epiphylla* sp. nov. and *R. cylindrica* sp. nov.

Dumack K., **Flues, S.**, Hermanns, K., Bonkowski, M., (2017) Rhogostomidae (Cercozoa) from Soils, Roots and Plant Leaves (*Arabidopsis thaliana*): Description of *Rhogostoma epiphylla* sp. nov. and *R. cylindrica* sp. nov. European Journal of Protistology **60**:76-86. https://doi.org/10.1016/j.ejop.2017.06.001

1 The study was designed and planned together with Prof. Dr. Michael Bonkowski and all results described in this chapter – molecular, phylogenetic, taxonomic, morphologic and statistical – were carried out by the author, if not stated otherwise. The article was written by the author under guidance of Prof. Dr. Michael Bonkowski, if not stated otherwise.

2 Isolation and cultivation was performed with help of Malte Blokker.

3 Morphologic analyses were performed by the co-author Dr. Kenneth Dumack.

4 Laboratory experiments were performed at the Natural History Museum, London, UK under guidance of Dr. David Bass.

5 The article was written or adjusted by the author, by usage of the developed thesis of the supervised student, under guidance of Prof. Dr. Michael Bonkowski.

6 Both first authors were involved in writing of the article and their complementary contributions merit equal credit.

Erklärung gemäß § 4 Absatz 1 Punkt 9 der Prüfungsordnung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Michael Bonkowski betreut worden.

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