Soil Protists

Diversity, Distribution and Ecological Functioning

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Abstract

Soil protists occupy key nodes in soil food webs due to their high abundance, fast turnover and functional importance as bacterial grazers. However, methodological drawbacks obscure the knowledge of soil protists, so that many taxa remain unknown. The structure of natural protist communities and taxa-specific ecological functions are also largely unknown. This thesis aims to increase the knowledge on soil protists using a variety of approaches.

In the first part, naked amoebae being presumably the most neglected protist morphogroup were cultivated from several distinct geographical locations across Europe and from high altitude sites in Tibet. During the course of this study, 16 new species and seven new genera were discovered, representing two eukaryotic supergroups (Amoebozoa and Excavata), three classes (Discosea, Variosea and Heterolobosea), and 12 genera (*Cochliopodium*, *Stenamoeba*, *Acanthamoeba*, *Ischnamoeba* n.g., *Darbyshirella* n.g., *Heliamoeba* n.g., *Arboramoeba* n.g., *Angulamoeba* n.g., *Telaepolella*, *Schizoplasmodiopsis*, *Allovahlkampfia* and *Pagea* n.g.). This vast number of taxonomic descriptions unveils the tremendous lack of knowledge especially on soil naked amoebae.

The second part aims at deciphering the diversity and community structure of soil protists using four different techniques. First, a modified cultivation technique enabled the quantification of individual morphogroups, allowing determination often up to genus level. It was shown that soil moisture not only impacts the total abundance, but also affects the community composition of soil protists. Second, established cultures of morphologically indistinguishable *Acanthamoeba* spp. were sequenced, enabling differentiation to strain-level. Highly diverse *Acanthamoeba* communities were detected that differed between geographically remote soils. Third, a high-throughput amplicon sequencing approach targeting the protist phylum Cercozoa illustrated that cercozoan communities strongly differed between geographically distant soils and that community composition depended on the type of land management. Finally, a metatranscriptomic approach unveiled the entire active protist community in different soil and litter samples, uncovering a previously unknown diversity. Each of these methods confirmed that soils harbour an enormous diversity of protists and all methods detected differences in soil protist communities depending on geographic origin or treatment. Therefore it is important to understand the respective advantages and disadvantages associated with each of those methods. Further,

available skills, equipment and financial resources need to be considered before applying a method to study soil protists.

The third part of this thesis aims to elucidate rarely considered ecological functions performed by soil protists. Generally, soil protists are considered as bacterivores, but diverse protists facultatively consumed fungi. The small amoeba *Cryptodifflugia operculata* revealed another feeding mode – trapping and consuming nematodes. Metatranscriptomic data revealed high relative abundances of both functional groups in all terrestrial soil samples indicating wider ecological functions carried out by soil protists as has been suggested before.

Zusammenfassung

Bodenprotisten nehmen aufgrund ihrer hohen Abundanzen und Reproduktionsraten sowie ihrer Rolle als Hauptkonsumenten von Bakterien eine Schlüsselposition in Nahrungsnetzen ein. Diese funktionelle Bedeutung weist eine deutliche Diskrepanz zur Berücksichtigung in ökologischen Studien und dem bestehenden Detailwissen über Protisten auf. Methoden der Isolierung und quantitativen Analyse der Protistengemeinschaft in Böden detektieren nur einen Bruchteil der Organismen. Die genaue Artenzusammensetzung und individuelle ökologische Bedeutungen bleiben somit im Verborgenen. Diese Arbeit zielt darauf, die Gemeinschaften von Bodenprotisten und deren Funktion durch eine breite Methodennutzung genauer zu charakterisieren.

Im ersten Teil dieser Arbeit liegt der Fokus auf der Kultivierung und Beschreibung bisher unbekannter Amoeben, die aus deutschen, italienischen, tibetanischen und niederländischen Böden isoliert wurden. Dieser Kultivierungsansatz ermöglichte die Beschreibung von 16 neuen Arten sowie sieben neuen Gattungen, die sich in die eukaryotischen Supergruppen Amoebozoa und Excavata, Klassen Discosea, Variosea und Heterolobosea, und 12 Gattungen (*Cochliopodium*, *Stenamoeba*, *Acanthamoeba*, *Ischnamoeba* n.g., *Darbyshirella* n.g., *Heliamoeba* n.g., *Arboramoeba* n.g., *Angulamoeba* n.g., *Telaepolella*, *Schizoplasmodiopsis*, *Allovahlkampfia* und *Pagea* n.g.) aufteilen. Trotz deren hohen Abundanz und Diversität beweist diese hohe Anzahl an Neubeschreibungen die lückenhafte (Arten-) Kenntnis über Bodenamoeben.

Im zweiten Teil der Arbeit liegt der Schwerpunkt auf der Untersuchung der Diversität und dem Vergleich ganzer Gemeinschaften von Bodenprotisten. Es wurden vier grundsätzlich verschiedene Analysewege genutzt. Eine modifizierte Kultivierungsmethode erlaubte die Bestimmung und Quantifizierung von Morphogruppen, oft bis auf Gattungsebene. In Abhängigkeit von der Bodenfeuchte variierte die Gesamtabundanz sowie die anteilige Zusammensetzung der Protistengemeinschaft. Des Weiteren wurden morphologisch nahezu identische Amoeben der Gattung *Acanthamoeba* molekular bis auf (Unter-) Artebene bestimmt, die im Vorfeld kultiviert wurden. Acanthamoeben zeigten eine hohe Diversität innerhalb und zwischen geographisch entlegenen Böden. Zur Bestimmung des Protistenphylums Cercozoa diente eine primerbasierte, high-throughput Amplikonsequenzierung. Die Cercozoengemeinschaft, deren hohe Diversität in allen Böden

bestätigt wurde, variierte je nach Landnutzung und Geographie. Eine Analyse der aktiven Protistengemeinschaft erfolgte durch einen Metatranskriptomansatz. Neben der detaillierten Bestimmung aller Protistengruppen der Proben, die sich untereinander stark unterschieden, fanden sich in terrestrischer Umgebung bislang unbekannte Protistengruppen. Jede dieser Methoden zeigt einen unterschiedlichen Ausschnitt der Protistendiversität und geht mit Vor- und Nachteilen einher, die vor Nutzung erörtert werden müssen, da alle eine Option zur Bestimmung der Protistengemeinschaft bleiben.

Im dritten Teil werden funktionelle Nischen der Bodenprotisten in Nahrungsnetzen gezeigt. Eine Vielzahl von Protisten konsumierten neben Bakterien auch Pilze und sind daher eher als omnivor einzustufen. Bei der kleinen Schalenamoebe *Cryptodifflugia operculata* ging der Fraß von deutlich größeren Nematoden mit einer erhöhten Reproduktionsrate einher. Metatranskriptomdatensätze zeigen hohe relative Abundanzen für sowohl omnivore als auch nematophage Protisten. Dies legt nahe, dass Protisten über die bakterivore Rolle hinaus ein weites funktionelles Spektrum in Böden einnehmen.

General Introduction

Soil Protists

Heterotrophic protists are among the most abundant soil organisms with estimated numbers usually ranging between 10^3 and 10^4 per gram soil (Bamforth 1980, Clarholm 1985, Finlay et al. 2000, Domonell et al. 2013). The majority of soil heterotrophic protists (hereafter simplified as "protists") is suggested to be small (< 200 µm), unicellular, reproduce asexually and feed on bacteria, but exceptions to all of these features are the rule rather than the exception (Levine et al. 1980, Darbyshire 1994, Adl and Gupta 2006, Esteban et al. 2006). Soil protists are classically divided into the four most abundant morphological groups, i.e. naked amoebae, testate amoebae, flagellates and ciliates. The taxonomical classification of protists has, however, undergone rapid and fundamental changes in the last decades due to technical developments, such as electron microscopy and most fundamentally by molecular tools (Levine et al. 1980, Cavalier-Smith 1993, Cavalier-Smith 1998, Adl et al. 2005, Adl et al. 2012). Heterotrophic, autotrophic and mixotrophic protists turned out to be highly paraphyletic, being intermingled throughout the eukaryotic tree of life, and represent the vast majority of eukaryotic organisms at least in terms of diversity (Cavalier-Smith 1998, Adl et al. 2005, Adl et al. 2012). Therefore, the term "protozoa" deriving from their nutritional requirements, is diminishing from scientific use (Adl et al. 2014). Flagellates, naked and testate amoebae are composed of paraphyletic species that branch in distant places in eukaryotic phylogenies, while only ciliates form a monophyletic group (Cavalier-Smith 1998, 2003, Adl et al. 2005, Adl et al. 2012). The dominant soil flagellate groups, i.e. small amoeboid cercozoans, free-swimming "*Spumella*"-like and "*Bodo*"-like flagellates (Finlay et al. 2000, Ekelund et al. 2001, Domonell et al. 2013) comprise taxonomically unrelated groups, such as "*Bodo*" in the supergroup Euglenozoa, and both Cercozoa (former supergroup Rhizaria) and "*Spumella*" (former supergroup Stramenopiles) being in the huge supergroup SAR (= Stramenopiles, Alveolates and Rhizaria (Burki et al. 2007, Adl et al. 2012)). Ciliates are members of Alveolata in the remaining SAR clade. Testate amoebae are found in both supergroups Amoebozoa and SAR (Rhizaria) (Nikolaev et al. 2005). The majority of naked amoebae belong to Amoebozoa, while most of the remaining naked amoebae are members of the supergroup Excavata (class

Heterolobosea) and SAR (Rhizaria) (Adl et al. 2005, Adl et al. 2012). Knowledge on most other protists in soils is sparse, but there is evidence that some typically aquatic organisms inhabit soils, such as Foraminifera (Meisterfeld et al. 2001, Lejzerowicz et al. 2010), Heliozoa (Stout 1984), Dinoflagellata (Bates et al. 2013) and Choanoflagellata (Ekelund et al. 2001, Tikhonenkov et al. 2012). Additionally, parasitic Apicomplexa seem to be widespread members of soil protist communities (Bates et al. 2013).

Protist species numbers are difficult to estimate because the species concept differs between protist groups and an enormous cryptic diversity within morphologically similar species has been detected by molecular techniques (Boenigk et al. 2005). The use of sequence data is largely based on the small subunit of the ribosomal DNA (SSU), and this sequence data is useful to supplement morphological information on distinct species or strains (Pawlowski et al. 2012). As a consequence, sequence information for (soil) protists is rapidly accumulating and molecular tools now allow soil surveys without intensive cultivation efforts. Environmental cloning and sequencing and the recent advent of high-throughput sequencing (HTS) techniques therefore provided and keep on providing new insights on the community composition of soil protists, resulting in the discovery of entirely new groups or groups unknown from soils (Fell et al. 2006, Lara et al. 2007a, Lejzerowicz et al. 2010, Bates et al. 2013). Nevertheless, knowledge on soil protists on both, individual and community level is still in its infancy, with new taxa being discovered in basically every soil survey (Urich et al. 2008, Lejzerowicz et al. 2010, Bates et al. 2013, Heger et al. 2013). Classical cultivation-based studies are, however, still essential to increase the reference database in order to assign morphological and eventually functional information to the sequence data.

Naked Amoebae

Testate amoebae, hereafter referred to as “testates” are fairly well investigated due to comparably large body sizes and stable morphological characters (Foissner 1999b, 2006, Wilkinson and Mitchell 2010, Lahr et al. 2013), while knowledge on their non-testate, naked counterparts, hereafter simplified as “amoebae”, remains sparse. Amoebae are particularly difficult to identify due to their small size, transparent cell body and the lack of stable morphological characters. Expert knowledge is thus indispensable for their reliable

identification (Foissner 1999b, Smirnov et al. 2008). Amoebae cannot be extracted from soils, and their isolation requires establishment of tedious enrichment cultures (Ekelund and Rønn 1994, Foissner 1999b, Smirnov 2003). Consequently, direct enumeration of amoebae is impossible in soils and largely relies on diverse modifications of the most probable number technique (MPN) (Darbyshire et al. 1974, Rønn et al. 1995). MPN is based on a serial dilution of a soil suspension, but protist detection is hampered by high loads of particles remaining especially in low dilutions, which restrict reliable identification of protists. Therefore, MPN studies usually lump soil protists into very rough morphogroups without further taxonomic differentiation, such that paraphyletic amoebae are commonly treated as a single group (Zwart et al. 1994, Scherber et al. 2010, Gabilondo and Bécares 2014). The liquid aliquot method (LAM) uses highly diluted aliquots of the soil suspension and allows a much more detailed deeper determination of amoebae (Butler and Rogerson 1995, Finlay et al. 2000, Domonell et al. 2013), but the drawback are its high work- and time-intensity and reliance on profound taxonomic expertise (Smirnov et al. 2008, De Jonckheere et al. 2012). Recent LAM studies grouped soil amoebae according to morphogroups (Butler and Rogerson 1995, Smirnov and Brown 2004, Domonell et al. 2013), but most morphogroups turned out to contain distinct paraphyletic taxa (Amaral-Zettler et al. 2000, Lahr et al. 2011, Smirnov et al. 2011b). The taxonomic resolution of LAM studies thus differs considerably from the latest taxonomy. This again raises the issue that detailed information on the abundance and community structure of soil amoebae is largely missing.

Molecular tools promised to fill that information gap by avoiding many problems involved with cultivation-based methods especially when studying amoebae. However, when applying "general eukaryotic primers" on soil DNA that target a wide range of protists, amoebae, especially the supergroup Amoebozoa, turned out to be largely underrepresented (Berney et al. 2004, Baldwin et al. 2013, Bates et al. 2013, Risse-Buhl et al. 2013). Reasons are inferior SSU amplification due to above-average length of the SSU and common mismatches in primer regions (Berney et al. 2004, Epstein and López-García 2008). Specific amoebozoan-wide primers do not exist, and more specific primers targeting groups within Amoebozoa have not yet been applied in soil surveys. Such focused studies on other protists have, however, revealed a much deeper resolution of the protist diversity (Bass and Cavalier-Smith 2004, Lara et al. 2007b, Lejzerowicz et al. 2010, Vannini et al. 2013). Most of all, a reliable

comprehensive reference database to extrapolate more meaningful information from soil molecular surveys is urgently needed.

Diversity, distribution and community structure

The limited knowledge on the diversity of soil protists, and especially amoebae, has resulted in an ongoing debate about the diversity and distribution of these organisms (Finlay 1998, Foissner 1998, Finlay 2002, Foissner 2006). Recent molecular information targeting some protist groups, however, strongly suggests that heterogeneous spatially distinct soil communities exist even within morphologically indistinguishable protist groups (Bass et al. 2007, Boenigk et al. 2007, Heger et al. 2013). For amoebae, one study found different sequences within the morphologically indistinguishable amoeba species *Vannella simplex* that differed between sites of isolation (Smirnov et al. 2002). Sequence data are rapidly accumulating in the genus *Acanthamoeba*. A plethora of sequenced *Acanthamoeba* spp. indicate an enormous hidden diversity within morphologically indistinguishable species in this genus with unique sequences being discovered in basically all studies (Gast et al. 1996, Stothard et al. 1998, Qvarnstrom et al. 2013, Risler et al. 2013). The morphological species concept of *Acanthamoeba* spp. has thus been replaced by sequence types to cope with the enormous sequence diversity in *Acanthamoeba* (Page 1988, Gast et al. 1996, Stothard et al. 1998, Risler et al. 2013). Several of these sequence types include, however, different distinct species that have formerly been described based on morphological features.

Some studies evaluated the community composition of soil protists with a morphotype approach and found that soil protist communities are largely influenced by precipitation, soil moisture and organic matter content of soils (Anderson 2000, Bass and Bischoff 2001, Anderson 2002). Information about the community composition of soil protists acquired with molecular tools are still sparse, but moisture content has also been identified as the key driver affecting the protist community in a large-scale 454 study (Bates et al. 2013). In this study the authors detected an increasing change of the protist community with distance, supporting the notion that deep molecular studies will fundamentally revolutionize the knowledge on soil protist communities. Also plants and even distinct plant species might strongly affect the soil protist community (Turner et al. 2013).

Nevertheless, molecular tools are not without limitations. Amplification efficiency is strongly dependent on primer choice with amoebae are being underrepresented (Berney et al. 2004, Epstein and López-García 2008). DNA of dead or inactive organisms can be transcribed (Pawlowski et al. 2011), while fundamental differences in SSU copy numbers between protists exclude quantitative comparisons (Gong et al. 2013). PCR errors further inflate protist-specific sequence numbers (Medinger et al. 2010, Behnke et al. 2011). Therefore, evaluating the community composition of soil protist with molecular techniques based on DNA are strongly hampered. Using RNA in metatranscriptomic approaches combined with reverse-transcribing RNA with random hexamers is a promising alternative and avoids most problems associated with DNA and primers. Few studies demonstrating the suitability of this approach have successfully recovered diverse protist communities from soils revealing more plausible protist community compositions than comparable DNA based studies (Urich et al. 2008, Tveit et al. 2012, Turner et al. 2013). As expected, amoebae represented a significant proportion of protist sequences in those studies. By capturing the active protist community, metatranscriptomic approaches might therefore even outperform cultivation-based techniques, that have been suggested to predominantly enumerate inactive, encysted protists that might be less important in soil functioning (Foissner 1987, Berthold and Palzenberger 1995). Depending on the question being addressed, all methods seem to find their eligibility in soil surveys, but (dis)advantages of the respective methods have to be considered.

Ecological importance

Protists and especially amoebae occupy key positions in soil systems. They are considered to represent the major bacterial grazers, thereby linking nutrient flow to higher trophic soil organisms (Hunt et al. 1987, De Ruiter et al. 1995, Crotty et al. 2011). They also liberate nutrients from the consumed bacterial and stimulate plant growth (Clarholm 1985, Bonkowski 2004). However, most information on the impact of protist grazing on bacterial communities has been obtained in studies with just one or few model protists. Since the diversity of protists in morphology and phylogeny is enormous (Cavalier-Smith 1993, Cavalier-Smith 1998, Adl et al. 2012), it seems inevitable that at least certain protists differ in their ecological functions. Glücksman et al. (2010) investigated the impact of cercozoan flagellates and found that the bacterial community was changed in a species-specific

manner. Most strikingly, they found substantial feeding differences even between closely related species. Similarly, different feeding impacts on the bacterial community were shown for related ciliates (Weisse et al. 2001), while amoebae-specific feeding differences have not yet been studied. More natural conditions investigating interactive effects of different protists and potential feeding differences are even more rarely approached. Recent studies showed that an increased diversity of the protist community enhanced bacteria community diversity by reducing bacterial competition in favour of subdominant species, while total bacterial abundance was reduced (Saleem et al. 2012, Saleem et al. 2013).

Protists are generally treated as bacterivores, but other functional feeding groups and respective ecological functions have rarely been addressed. Cryptic information, however, strongly suggests that trophic interactions among protists are common, as successful cultivation of various protists is only possible in presence of other protists. For instance, *Paradermamoeba levis* has been cultivated on small vannellid amoebae (Smirnov et al. 2007), *Deuteramoeba algonquinensis* needed small amoebae or flagellates for growth (Mrva 2010) and *Thecamoeba* spp. has been co-cultivated on other amoebae or ciliates (Page 1977). Not surprisingly, also algae serve as prey for a variety of amoebae (Smirnov and Brown 2004, Mrva 2010, Smirnov et al. 2011a, Hess et al. 2012, Berney et al. 2013). Several protist taxa are further known to feed on fungi, for example flagellates (Hekman et al. 1992, Ekelund 1998, Flavin et al. 2000), ciliates (Petz et al. 1985, Petz et al. 1986, Foissner 1999a) and a variety of amoebae (Old and Oros 1980, Chakraborty and Old 1982, Chakraborty et al. 1983, Mrva 2010). Among soil fauna, nematodes have been shown to be affected by protists. Small flagellates have been shown to kill, but not feed on nematodes (Bjørnlund and Rønn 2008), while larger testate amoebae (Yeates and Foissner 1995), ciliates (Doncaster and Hooper 1961) and amoebae (Sayre 1973) directly feed and grow on nematodes. In addition to direct feeding, non-trophic interactions, both positive and negative have been reported between protists and fungi (Rønn et al. 2002a, Vohník et al. 2011, Koller et al. 2013), nematodes (Neidig et al. 2010, Bjørnlund et al. 2012) and earthworms (Bamforth 1988, Bonkowski and Schaefer 1997, Winding et al. 1997, Tiunov et al. 2001). Detailed knowledge on all of these interactions remains, however, on a very crude taxonomic level. It seems that we only grasped the tip of the iceberg on deciphering the importance and the multitude of ecological functions carried out by soil protists.

Further, some soil protists pose direct risks to human health. Among the facultative pathogens are amoebae of the genus *Acanthamoeba*, which are ubiquitous and very abundant in soils (Page 1988). Several *Acanthamoeba* spp. cause the eye infection Amoebic Keratitis (Schuster 2002, Siddiqui and Ahmed Khan 2012), and also fatal Granulomatous Amoebic Encephalitis (GAE) (Schuster 2002, Khan 2006, Qvarnstrom et al. 2013). Similiarly, GAE can be caused by *Naegleria fowleri* and *Balamuthia mandrillaris* (Visvesvara et al. 1993, Schuster 2002, De Jonckheere 2004). While *N. fowleri* has so far only been reported from hot water environments (De Jonckheere 2004), soils are likely to represent environmental niches for *B. mandrillaris* (Dunnebacke et al. 2004, Ahmad et al. 2011). In addition to these directly hazardous protists, endosymbiontic pathogenic bacteria are common in amoebae, such as *Mycobacterium*, *Legionella*, *Salmonella* and *Listeria* (Anand et al. 1983, Abu Kwaik 1996, Greub and Raoult 2004, Lamoth and Greub 2010). Protists are therefore considered to be "Trojan horses" for pathogenic bacteria to evade the human immune defence (Greub and Raoult 2004, Horn and Wagner 2004). Taken together, soil protists are highly diverse members of the soil food web and their ecological importance is commonly being accepted, but detailed knowledge on a wide range of species, the community compositions and taxon-specific ecological functions of soil protists is largely missing.

Aims

The main objective of this thesis was to increase the knowledge on abundant taxa, community structures and functions of soil protists. Therefore, a variety of distinct approaches were applied that are summarised in three major parts.

Part 1

This part, aimed at increasing the knowledge on soil protists focusing on amoebae, through extraction and cultivation from geographically distant soils across Europe and Tibet. These cultures form the basis for the description of new species and genera from four distinct clades of amoebae, summarized in four chapters;

Chapter 1 – Two *Stenamoeba* species, *S. sardiniensis* n. sp. and *S. berchidia* n. sp. isolated from the same soil on Sardinia, Italy are described, revealing morphological characters that help in taxonomic re-classification of the class Discosea.

Chapter 2 – *Cochliopodium plurinucleolum* n. sp. also extracted from Sardinian soil is described and the genus *Cochliopodium* spp. is revised.

Chapter 3 – Six new genera of the class Variosea cultivated from diverse soils (and freshwater) are erected, enabling a deep re-structuring of Variosea.

Chapter 4 – The new heterolobosean genus *Pagea* and six new *Allovahlkampfia* spp. are described cultivated from a range of soils from Tibet, Sardinia and the Netherlands.

Part 2

This part aimed at amending existing information on the diversity, abundance and community composition of soil protists. Four different methods are applied to target distinct questions on soil protist communities, each depicted in a single chapter;

Chapter 5 – The abundance and diversity of soil protists is investigated microscopically using a modified enrichment cultivation technique, to analyse the impact of changes in soil moisture conditions on the protist community.

Chapter 6 – Cultures of *Acanthamoeba* established in the first part of this thesis (Chapters 1 - 4) are subject to more focused molecular analyses followed by phylogenetic analyses to investigate potential cryptic diversity of *Acanthamoeba* spp.

Chapter 7 – The protist phylum Cercozoa is targeted in a HTS approach applying specific primers to compare cercozoan community compositions in a range of geographically distant soils and between different levels of soil treatment in order to evaluate main drivers determining cercozoan soil communities.

Chapter 8 – The entire soil protist community is analysed using a metatranscriptomic approach to investigate and compare active protist communities between soils and to explore protist clades uncommon for soils.

Part 3

The last part aimed at deciphering ecological functions performed by soil protists focusing on feeding interactions other than with bacteria. Using predominantly cultures obtained in

Part 1, interactions between protists and other soil eukaryotes are being investigated, to evaluate whether the current classification of protists as mainly bacterivorous holds true.

Chapter 9 – A wide range of cultivated soil protists were tested for facultative fungal-feeding potential. Further, the presence and relative abundance of known obligate fungal feeding protists is determined using datamining approaches, e.g. from sequences obtained in Chapter 8.

Chapter 10 – The interaction of a small testate amoebae, *Cryptodifflugia operculata* with soil nematodes are investigated and the presence and potential importance of this interaction in soil investigated in terrestrial ecosystems examined using the metatranscriptomic data obtained in Chapter 8.

Part 1

Cultivation and descriptions of new protist species and genera

Part 1 – Chapter 1

Two new species of the genus *Stenamoeba* (Discosea, Longamoebia): Cytoplasmic MTOC is present in one more amoebae lineage

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Abstract

Two new species of the recently described genus *Stenamoeba*, named *S. berchidia* and *S. sardiniensis* were isolated from a single soil sample on Sardinia, Italy. Both share morphological features characteristic to *Stenamoeba* and form in phylogenetic analyses together with other *Stenamoeba* spp. a highly supported clade within the family Thecamoebidae. The ultrastructural investigation of *Stenamoeba sardiniensis* revealed the presence of cytoplasmic microtubule-organizing centers (MTOCs), located close to one of several dictyosomes found inside the cell. This is the first report of cytoplasmic MTOCs among Thecamoebidae. The presence of MTOCs is now shown in five of nine orders comprising the class Discosea and potentially could be a phylogenetic marker in this group. We re-isolated *Stenamoeba limacina* from German soils. This strain shows a similar morphology and an almost complete SSU rDNA sequence identity with the type strain of *S. limacina* originating from gills of fishes, collected in Czech Republic.

Introduction

Naked lobose amoebae (gymnamoebae) belong to the phylum Amoebozoa Luhe 1913, which comprises a number of phylogenetic lineages, covering all groups of lobose amoeboid protists (Cavalier-Smith 1998; Smirnov et al. 2011a). Among them, gymnamoebae are distributed among three groups currently recognized in the rank of classes - Tubulinea, Discosea and Variosea (Smirnov et al. 2011a). While Tubulinea are well outlined and proven to be monophyletic, and the same is probably true for Variosea, the monophyly of Discosea is more ambiguous, since they appear paraphyletic in most analyses (Cavalier-Smith et al. 2004, Smirnov et al. 2005, Tekle et al. 2008, Kudryavtsev et al. 2009a, Pawlowski and Burki 2009, Lahr et al. 2011). Discosea usually segregate into a number of relatively independent lineages with unstable position; a remarkable exception is the stable coupling of the orders Vannellida and Dactylopodida. However, Dermamoebida, Thecamoebida and Centramoebida showed weak statistical support in recent analyses (e.g. Kudryavtsev and Pawlowski 2013), but a surprisingly stable tendency to form a clade, recognized as the subclass Longamoebia in Smirnov et al. (2011b). Morphologically Thecamoebida, Centramoebida and Dermamoebida are rather different and until now there is no evident character that could be considered as a synapomorphy of this clade.

The genus *Stenamoeba* (order Thecamoebida, family Thecamoebidae) was established in 2007 to accommodate the former *Platyamoeba stenopodia* Page 1969 after the genus *Platyamoeba* was abandoned based on molecular data by Smirnov et al. (2007). It comprises oblong, linguiform amoebae with a large anterior area of hyaloplasm, usually occupying half or more of the cell. During locomotion several longitudinal ridges similar to those in *Thecamoeba* occasionally appear, but they never remain stable such as in *Thecamoeba*. A single nucleus is usually positioned at the border separating hyaloplasm and granuloplasm. Amoebae with *Stenamoeba*-like morphology are common in various environments, but *Stenamoeba* was monotypic until *S. limacina* and *S. amazonica* isolated from organs of freshwater fish hosts added (Dyková et al. 2010) Both of these species display characteristic morphological features unique to *Stenamoeba*, supported by molecular data revealing a solid monophyly of the genus *Stenamoeba* (Dyková et al. 2010b). This was the first indication that the genus *Stenamoeba* may be rather species-rich, but many new species may have not been recognized earlier due to morphological similarities with *S. stenopodia* and the

shortage of discriminating characters. Recent add-ons of sequences, probably belonging to unnamed members of the genus *Stenamoeba* to the GenBank database further supported this idea.

In the present paper we describe two more species of *Stenamoeba* and show the presence of cytoplasmic microtubule-organizing centers (MTOCs) in one of the newly described isolates. We also report a new strain of *Stenamoeba limacina* isolated from soil of the Hainich-Dün region (Germany) that shares profound morphological and almost complete sequence identity with the type strain described by Dyková et al. (2010b).

Materials and Methods

Sampling sites

Samples were taken from the upper 20 cm of mineral soil of an intensively managed grassland plot at Berchidda-Monti long term observatory, managed by the University of Sassari, on the island of Sardinia (Italy), Berchidda district, 40°46'N, 9°10'E (Lagomarsino et al. 2012), the upper 20 cm of an ex-arable field in the central part of the Netherlands (52°06'N, 6°00'E), and the upper 10 cm of a beech forest in the Hainich Dün region (Germany), 50°56' - 51°22'N, 10°10' - 10°46'E. Sampling was performed in the course of two different projects, EcoFINDERS (http://ecofinders.dmu.dk/) in 2011 (Sardinian site), and Biodiversity Exploratories (Fischer et al. 2010), http:/www.biodiversity-exploratories.de /1/home/, in the year 2008 (German site).

Isolation and cultivation

Soil samples were incubated in 90 mm Petri dishes with 0.15 % wheat grass (WG) medium, made by adding vacuum-dried wheat grass powder (Weizengras, Sanatur, Singen, Germany) to PJ medium (Prescott and James 1955) to the weight concentration of 0.15 %. The medium was autoclaved and sterile filtered through 0.45 µm Whatman filter paper. Parallel samples were inoculated with the same medium and agarised by adding non-nutrient agar (AppliChem, Darmstadt, Germany) at a final concentration of 1.5 % (WG agar). The agar was autoclaved (122 ^{o}C, 20') prior to use. Enrichment cultures were maintained for 10 - 14 days at room temperature and ambient light. To establish clonal cultures, amoebae were picked manually with a tapered glass pipette using an inverted phase-contrast microscope, and

transferred to 60 mm Petri dishes with WG medium. Amoebae were subcloned once or twice until free from other eukaryotes, and fed on the accompanying non-identified bacteria. Observations and measurements of live cultures in Petri dishes were made using Leica DMI3000 inverted microscope equipped with PhaCo (Phase contrast) and IMC (Integrated modulation contrast) optics. Observations and photographs of amoebae moving across the glass surfaces were done using either a Leica DM2500 microscope equipped with PhaCo and DIC (Differential Interference Contrast) optics or a Nikon Eclipse 90i equipped with PhaCo and DIC optics.

Electron microscopy

For transmission electron microscopy (TEM) amoebae were fixed at 4 °C with a 2.5 % solution of glutaraldehyde in a 0.05 M sodium cacodylate buffer (pH 7.4) for 40' followed by postfixation with 1 % osmium tetroxide prepared with the same buffer for 60'. Cells were washed with buffer (3 x 5') between fixation steps. Fixation was initiated in Petri dishes by a quick replacement of culture medium with glutaraldehyde. During buffer washes cells were scraped away from the Petri dish bottom and collected by gentle centrifugation. After osmium tetroxide treatment, cells were washed with buffer (2 x 5') followed by a gradual decrease in buffer concentration down to glass-distilled water. Amoebae were finally embedded in 2 % agar prepared with glass-distilled water; blocks of agar (ca 1 mm^3) containing cells were subsequently cut out, dehydrated in a graded ethanol series followed by 100 % acetone, infiltrated and embedded in Epon 812 epoxy resin (Fluka). Silver to light gold sections were cut with a diamond knife on a Leica Ultracut 6 ultramicrotome and double-stained with 2 % uranylacetate prepared with 70 % ethanol and Reynolds' lead citrate. Sections were observed using a JEOL JEM1400 transmission electron microscope operated at 80 kV.

DNA Extraction

DNA was extracted with guanidine isothiocyanate buffer (Maniatis et al. 1982). To collect cells, 60 mm Petri dishes containing amoebae were washed 2 - 3 times with fresh medium. The medium was always completely discarded form the dish. Subsequently, 100 µl guanidine isothiocyanate buffer was added to the dish and distributed across its entire bottom. Cells were scraped off using a disposable cell scraper, and the buffer with floating cells was

collected with a Pasteur pipette and transferred into an Eppendorf tube before being precipitated with isopropanol and ethanol according to above cited protocol.

PCR

SSU rDNA gene fragments were amplified using universal eukaryotic primers (Table 1). The cycling conditions included a 5' initial denaturation at 95 °C followed by 30 cycles (each comprising 95 °C for 30'', 50 °C for 60'', and 72 °C for 120''), and a final elongation step at 72 °C for 5'. Subsequently, 8 µl of the PCR product were purified by adding 0.15 µl Endonuclease I (20 U * μl^{-1}, Fermentas GmbH, D-68789 St. Leon-Rot), 0.9 µl Shrimp Alkaline Phosphatase (1 U * μl^{-1}, Fermentas GmbH) and 1.95 µl water to a final volume of 11 µl. This mixture was incubated for 30' at 37 °C, and for another 20' at 85 °C. Afterwards (partial) PCR products were sequenced using the Big Dye Terminator Cycle sequencing kit and an ABI PRISM automatic sequencer. Primers used for sequencing are listed in Table 1.

Table 1. Primers used for amplification, lengths of fragments and accession numbers at GenBank as well as the Culture Collection of Algae and Protozoa (CCAP). Primer sequences are as follows: RibA (5' - acc tgg ttg atc ctg cca gt - 3'), RibB (5' - tga tcc atc tgc agg ttc acc tac - 3') S12.2 (5' - gat cag ata ccg tcg tag tc - 3') S20R (5' - gac ggg cgg tgt gta caa - 3') (Cavalier-Smith and Chao 1995, Pawlowski 2000)

Species	Primer pair	Sequence length	GenBank accession number
S. berchidia	RibA-RibB	2124 bp	KF547921
S. sardinensis	S12.2-S20R	772 bp	KF547922
S. limacina	RibA-S20R	698 and 963 bp	KF547923 and KF547924

Phylogenetic analysis

For phylogenetic analysis newly obtained sequences were aligned with an extensive alignment covering all major groups of eukaryotes using Seaview 4 (Gouy et al. 2010); the alignment was manually polished. To increase sampling of *Stenamoeba*-related sequences, sequences obtained in the present study and all named *Stenamoeba* sequences downloaded from GenBank were used as a query for BLASTn search using default search parameters in GenBank; top 10 hits were downloaded; mounted in the same alignment and analyzed using PhyML (1,148 sites, GTR + Γ model with 4 rate categories, optimized proportion of invariable sites). Sequences that robustly grouped with *Stenamoeba* species were added to the analysis; the rest were removed.

The phylogenetic analysis to obtain maximum-likelihood (ML) tree was performed using a subsample of the alignment containing all Stenamoeba-related sequences and a

representative amoebozoan sample. RaxML (Stamatakis 2006) was used with the following parameters: 1,605 sites; GTR+Γ model, 25 rate categories (Lanave et al. 1984), optimized proportion of invariable sites. The tree showing the best likelihood value was bootstrapped with 100 replicates. Bayesian analysis was performed using MrBayes 3.2 (Huelsenbeck and Ronquist 2001) with the GTR+Γ model, 8 rate categories and the covarion model. The analyses were performed as two separate runs of four chains each with default parameters, until they ceased to converge (final average standard deviation of the split frequencies less than 0.01); this required about 1 million generations; the first 30 % of generations were discarded as burnin. Calculations were performed using the facilities of Bioportal of the University of Oslo (Kumar et al. 2009).

GenBank numbers of newly obtained sequences are represented in the Table 1; type cultures of *Stenamoebae sardinensis* and *S. berchidia* as well as a strain of *S. limacina* isolate 61 are deposited with CCAP under the accession numbers CCAP 2571/1 – CCAP 2571/3.

Results

Morphological observation

Stenamoeba berchidia n. sp.

Light microscopy (Figure 1 - 4): Active, moving trophozoites were oblong, or linguiform (Figure 1). The hyaloplasm was very pronounced, covering up to 2/3 of the cell. *S. berchidia* was broader than *S. sardiniensis*, occasionally exhibiting a nearly fan-shaped appearance. The granular part of the cell was narrower than the hyaline part, often pointed at the posterior end. No distinct uroidal structure was observed. Pronounced longitudinal surface ridges were formed during active locomotion (Figure 2, black arrow) and transverse waves running over the hyaloplasm were observed occasionally. A single contractile vacuole was always located in the posterior part of the cell.

Average length of the locomotive form was 18.9 µm (range 12 - 24 µm), and the average breadth 8.6 µm (range 6 - 12 µm). The average length / breadth ratio was 2.3 (range 1.4 - 3.7).

Non-directionally moving cells adopted variable shapes, often forming dactylopodia-like projections or assuming an irregular crescent-like shape (Figure 3). As *S. berchidia* was very active, amoebae virtually never formed a stationary phase during observations. Floating amoebae produced several small pseudopods, not reaching far out of the central cell mass; cells floated not readily and only for a short time under our conditions of observation, so these forms might not have represented developed floating forms.

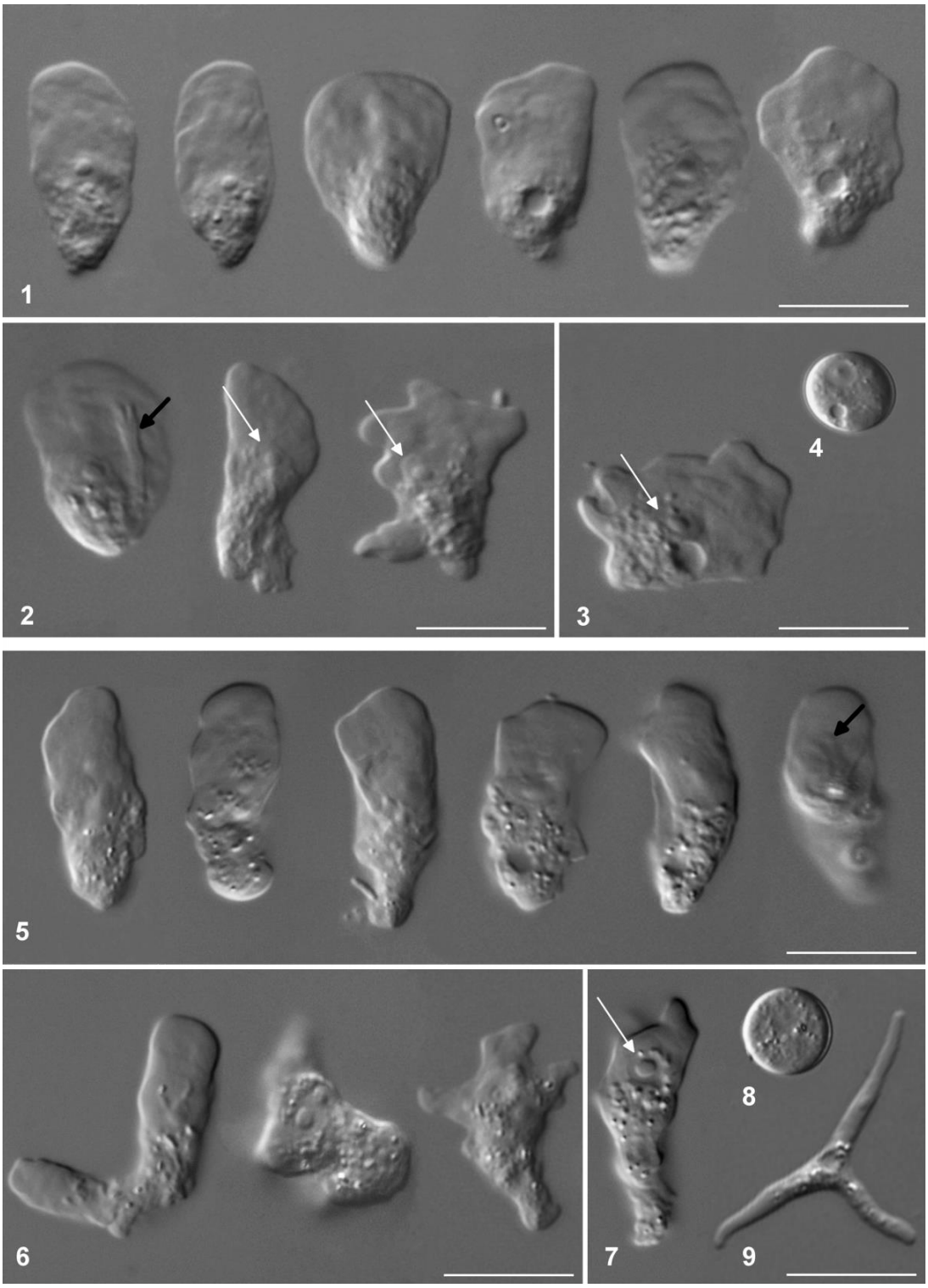
A single, vesicular nucleus was always located close to the border between the hyaloplasm and the granuloplasm (Figures 2 and 3, white arrows). The average diameter of the nucleus was 1.7 µm (range 1.3 - 2.2 µm); the average diameter of the nucleolus was 0.75 µm (range 0.5 - 0.9 µm). No inclusions such as crystals were observed. Amoebae produced spherical, double-walled cysts of ca 10 µm (Figure 4) in diameter in old cultures.

***Stenamoeba sardiniensis* n. sp.**

Light microscopy (Figures 5 - 9): Trophozoites in active locomotion were oblong or linguiform, with a distinct frontal area of the hyaloplasm, occupying about 1/2 of the cell (Figure 5). The granular part of the cell was in general narrower and thicker than the hyaline part, almost pointed at the posterior end in some individuals. No distinct uroidal structures could be observed. At times small longitudinal surface ridges occurred on the dorsal surface of moving cells (Figure 5, black arrow), but they never stayed stable for a long time. The single contractile vacuole was always located in the posterior part of the cell.

Average length of the locomotive form was 17.0 µm (range 13 - 24 µm), and the average breadth 6.1 µm (range 5 - 7 µm). The average length / breadth ratio was 2.8 (range 2.2 - 3.9).

During non-directional movement, cells sometimes produced very short dactylopodia-like cytoplasmic projections, or became narrow and stretched, or even temporarily branched; the latter was especially characteristic when amoebae tried to adhere both to the object slide and the coverslip (Figure 6). Stationary amoebae usually adopted an irregular shape with no distinctive characteristics or most often floated just above the cover slip surface, without adopting a pronounced floating form. Notable was also the extremely low ability of trophozoites to attach to the glass slides, making it difficult to observe the typical locomotive morphology. Fully developed floating forms produced up to three thin, blunt and radiating pseudopods of different length (Figure 9).

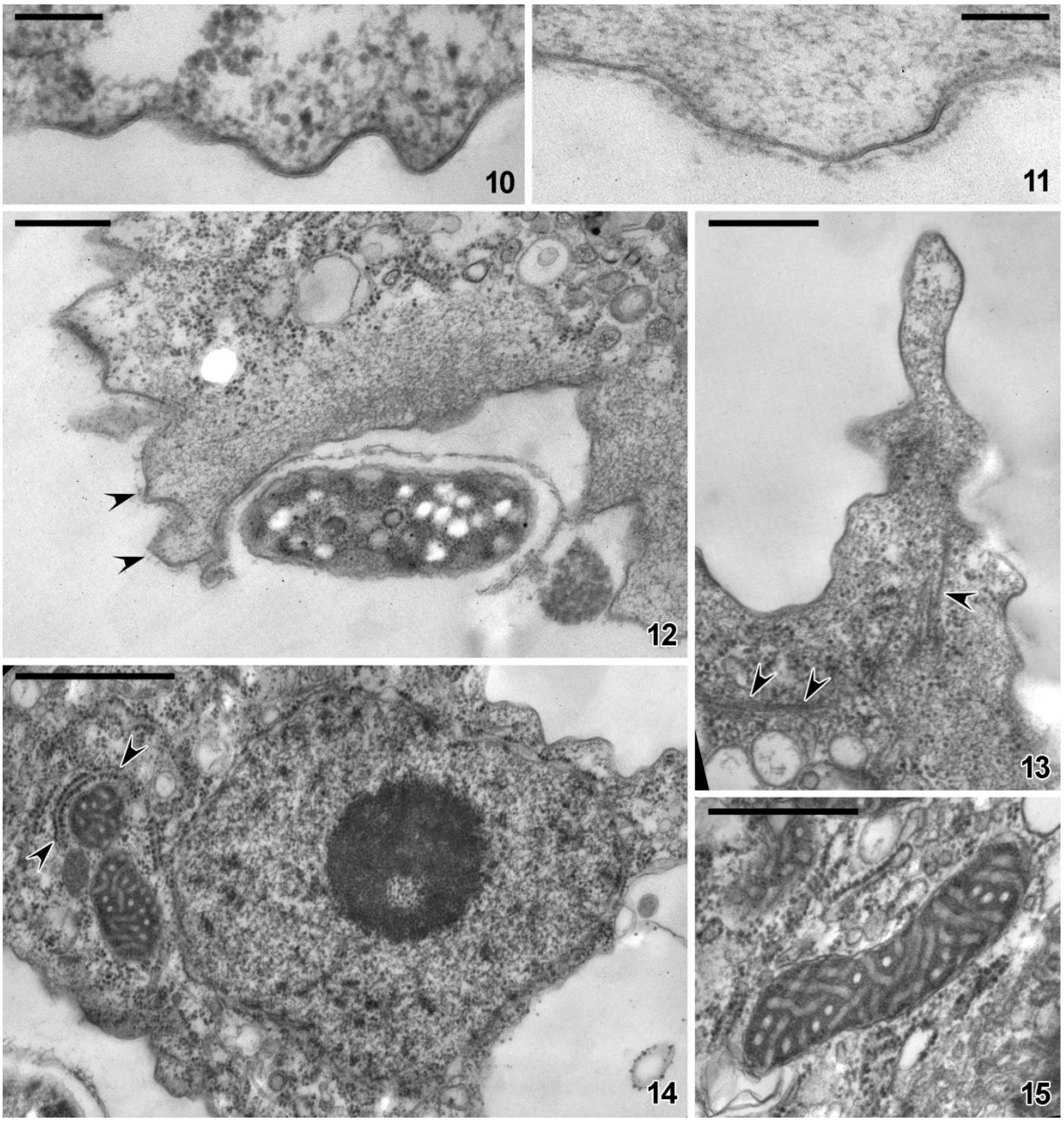


Figures 1-9. Light microscopic images of newly described *Stenamoeba* species; Scale bars: 10 µm
1-4. *Stenamoeba berchidia*. **1**. Locomotive form, ranging from oblong and linguiform to almost fan-shaped. **2**. Pronounced longitudinal surface ridges, occasionally formed in few cells (black arrow); single nucleus located at the border between granuloplasm and hyaloplasm (white arrows). **3**. Stationary amoebae of irregular shape. White arrow shows nucleus. **4.** Mature cyst.
5-9. *Stenamoeba sardiniensis*. **5.** Locomotive form, demonstrating characteristic oblong, linguiform shape. some cells show short-lived small longitudinal surface ridges (black arrow); **6**. Cells in non-directed movement. **7.** The single nucleus located at the border between granuloplasm and hyaloplasm (white arrow). **8**. Mature cyst. **9.** Floating form.

The cells observed contained a single vesicular nucleus, always located at the border of hyaloplasm and granuloplasm (Figure 7, white arrow). The average diameter of the nucleus was 1.7 µm (range 1.5 - 2.1 µm), the average diameter of the nucleolus 0.65 µm (range 0.5 - 0.9 µm). Cells contained no crystals or any other remarkable inclusions. Amoebae in old cultures formed smooth, spherical, double-walled cysts ca 10 µm in diameter (Figure 8).

Electron microscopy

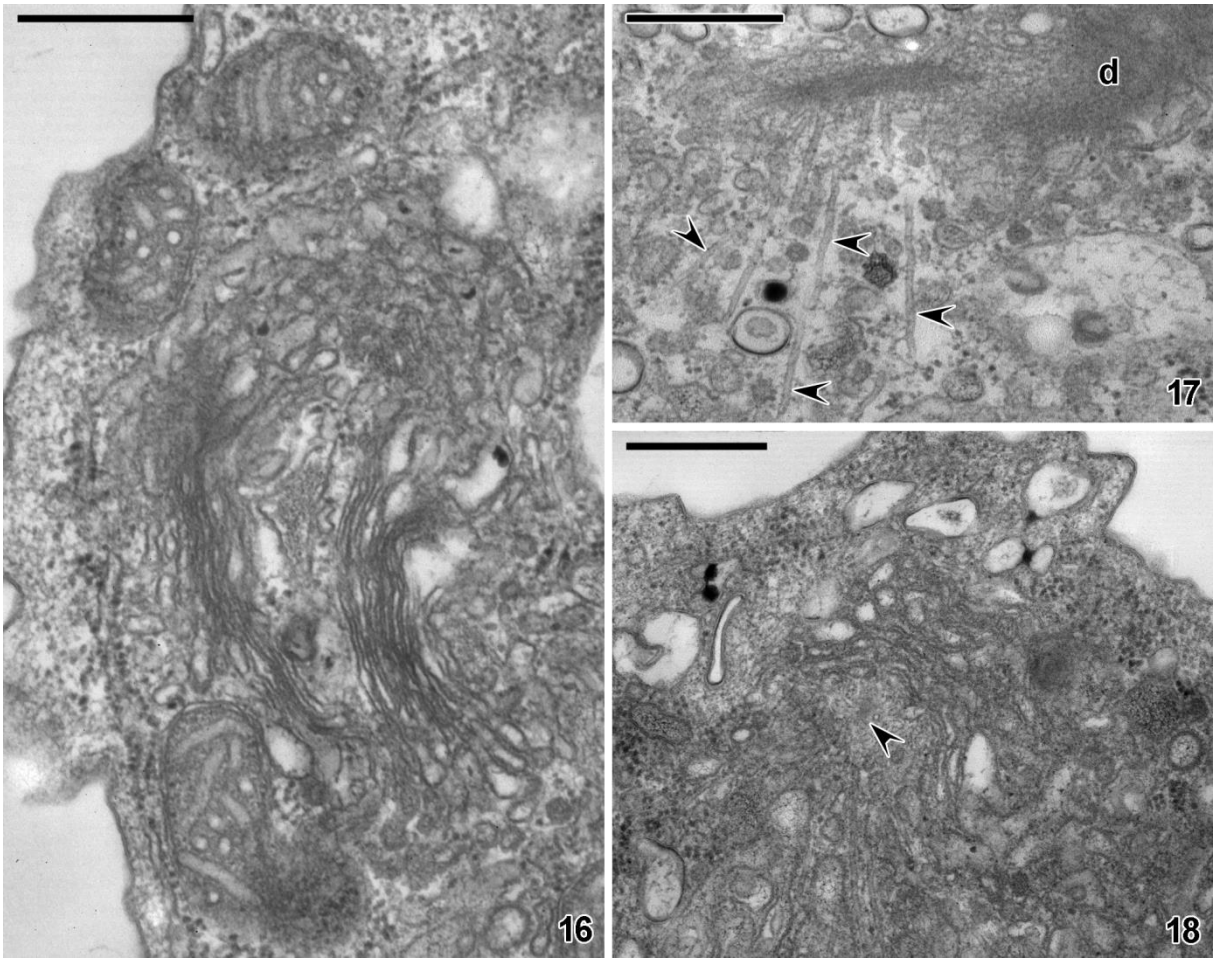
The majority of observed cells were covered with the plasma membrane without any visible glycocalyx on its surface (Figure 10). However, in a few cases a layer of fuzzy material 20 - 30 nm thick was observed in a limited part of the cell surface area that looked like a food cup under formation (Figures 11 - 12). The peripheral part of the cytoplasm contained filamentous material that looked like a microfilament network, and some of these filaments were seen to form bundles extending into the cytoplasmic projections (Figure 13). The nucleus was irregularly rounded in sections, and contained a rounded, central electron-dense nucleolus that sometimes contained more transparent areas visible in sections, and numerous patches of electron-dense heterochromatin scattered in the nucleoplasm (Figure 14). Mitochondria in sections were rounded or oval, containing an electron-dense matrix and tubular cristae. Associations of mitochondria with cisternae of rough endoplasmic reticulum were regularly observed (Figure 15).



Figures 10-15. *Stenamoeba sardiniensis*. General ultrastructure. Scale bar: 0.2 µm in 10-11, 1 µm in 14, 0.5 µm in others.
10-11. Plasma membrane and cell coat (note a layer of fuzzy material over the plasma membrane in 11). **12**. Formation of a food cup for ingestion of a bacterium. Arrowheads indicate glycocalyx-like fuzzy layer over the plasma membrane. **13**. Cytoplasmic projection with bundles of microfilaments (arrowheads). **14**. Nucleus, mitochondria and cisternae of rough endoreticulum (arrowheads). **15**. Mitochondrion and associated cisternae of rough endoreticulum.

Large dictyosomes consisting of ca 10 flattened cisternae were scattered in the cytoplasm (Figure 16). In all cells numerous microtubules were observed around the dictyosomes expanding into the cytoplasm. In few sections an electron-dense body was seen close to the dictyosomes with numerous microtubules radiating from it (Figures 17 - 18). Therefore, it was interpreted as a microtubule-organizing center (MTOC). This MTOC was most probably cylindrical as it looked in sections either as a rounded body ca 100 nm in diameter, or had an

elongated shape ca 600 nm in length. No internal structure of a MTOC could be seen in sections. The very low frequency of occurrence of MTOCs in sections suggests that each cell had a single MTOC that was always located close to the dictyosomes. Not all dictyosomes in the cytoplasm were, however, associated with the MTOC.



Figures 16-18. *Stenamoeba sardiniensis*. Dictyosomes and MTOC. Scale bar = 0.5 µm.
16. Part of the cytoplasm showing dictyosomes, mitochondria and cisternae of rough endoreticulum. **17**. Microtubule-organizing center adjacent to a dictyosome (**d**) in a longitudinal section; arrowheads indicate microtubules. **18**. Dictyosome and a putative microtubule-organizing center in a cross-section (arrowhead).

Stenamoeba limacina strain 61

Light microscopy (Figure 19)

Locomotive morphology and other features of trophozoites were very similar to those presented in the original description of *S. limacina* (Dyková et al. 2010b). Following this description, we also noted the poor adhesion of amoebae to the glass surface, which made light microscopic observations very difficult. Amoebae detached from substratum started to move only after several hours. Average length of trophozoites was 19.9 µm (range

15 - 29 µm), breadth 6.9 µm (range 4 - 11 µm) and length / breadth ration (L / B) 3.0 (range 1.6 - 4.9). The average diameter of the nucleus was 2.7 µm (range 2.0 - 3.5 µm), the average diameter of the nucleolus 1.3 µm (range 0.9 - 1.7 µm). Cells contained no crystals or any other remarkable inclusions. We have not seen cysts in our cultures.

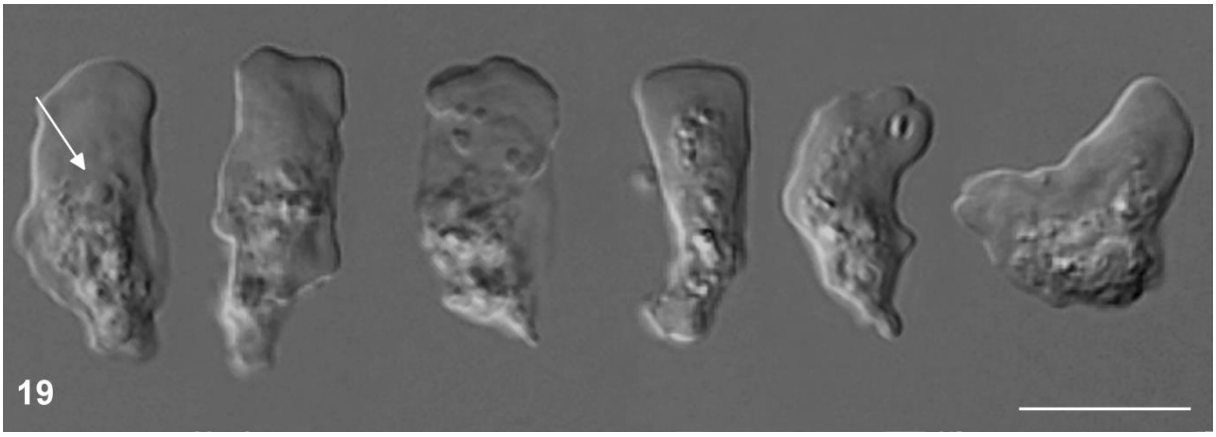


Figure 19. Light microscopic images of *Stenamoeba limacina* strain 61 in locomotion. The single nucleus is located at the border between granuloplasm and hyaloplasm (white arrow). Scale bar = 10 µm.

Molecular phylogeny

The resulting phylogenetic tree (Figure 20) revealed three large clades, corresponding to the classes Discosea, Variosea and Tubulinea; the class Discosea divided into two subclasses - Flabellinia and Longamoebia with high PP but negligible bootstrap support. The same pattern occurred across the entire tree with high posterior probabilities for nearly all branches, and with often much lower bootstrap values. The present analysis revealed a well-supported clade unifying the genera *Thecamoeba*, *Sappinia* and *Stenamoeba*, which corresponded to the order Thecamoebida *sensu* Smirnov et al. (2011b) and a clade unifying the orders Dermamoebida, Centramoebida as well as environmental sequences of a so-called LKM74 clade. All three *Stenamoeba* sequences investigated in the present study robustly grouped within the clade corresponding to the genus *Stenamoeba*. Among these, *S. limacina* strain 61 in two sequenced fragments, 698 bp in the 5' and 963 bp in the 3' region of the SSU rDNA gene (including a fragment of the V4 region) showed nearly complete sequence identity (1 bp difference at position 601 of *S. limacina*) with the sequence of *S. limacina* studied by (Dyková et al. 2010b), which is congruent with their morphological similarity. Two new species, named here *S. sardiniensis* and *S. berchidia* showed distinct sequence differences from all yet known *Stenamoeba* strains. Although these species form short branches within the *Stenamoeba* clade (Figure 20), this should not

be interpreted as evidence of close sequence similarity, because sequences belonging to distinct species and isolates mostly differ in variable regions not included in the phylogenetic analysis, while the sequence differences within the set of sites used for the Amoebozoa-wide analysis were minimal.

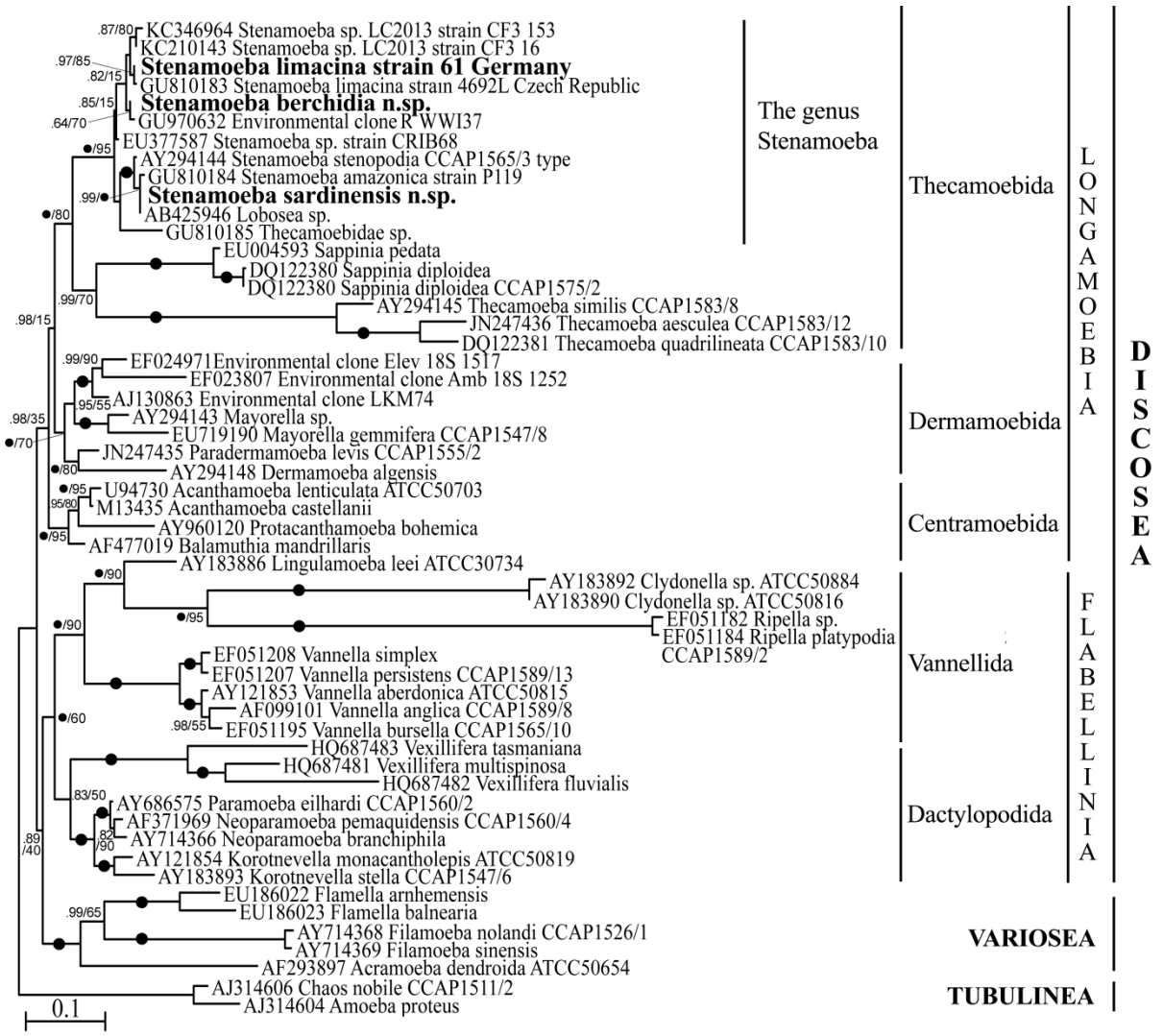


Figure 20. Phylogenetic tree based on the SSU-rDNA gene. Strains described in this study are in bold. Support values at each node indicated as RAxML / PP value. Black dots on nodes indicate 1.00 / 100 support level; smaller black dots show 1.00 PP values. Indications in nodes without strong support (< 60 % (RAxML) and < 0.60 (BI) omitted). All branches are drawn to scale.

Manual analysis of the alignment revealed that the V4 region of the SSU rDNA gene sequence in *S. berchidia* possessed a unique 56 bp long signature sequence ranging from position 686 to 742 (AGGGAGAGGGGCGGGGAGGGGCGACCCTCCTCGTTTTCCTTCTCCCTTC GTGCGGC). The V7 region in *S. berchidia* also included a unique signature motif, i.e. GTTCCTTTC (position 1849 - 1857). The SSU rDNA gene sequence of *S. sardiniensis* has a very distinct V7 region with characteristic motifs at position 595 - 622

(TTTATCGAACACCGTCTCTTCTTCCTTC) followed by a G-rich area in position 623 - 641 (GCGGGAGGGGGGGGCGGCG), differentiating it strongly from all other *Stenamoeba* strains.

Discussion

Identification of *Stenamoeba limacina* strain 61 and justification to establish two new species - *Stenamoeba berchidia* n.sp. and *Stenamoeba sardiniensis* n.sp.

Stenamoeba limacina strain 61. The *S. limacina* strain 61 in morphology and size is very similar to *Stenamoeba limacina* Dyková et al., 2010. The limax-like locomotive form reported for *S. limacina* was less pronounced in our strain as the posterior end seemed to narrow more strongly as originally described for *S. limacina.* Our strain was slightly larger than the type one (length 15 - 28 µm against 15.4 - 19.2 µm reported for the type strain) but maintained similar length to breadth ratio. Trophozoites overloaded with phagocytosed bacteria noted by Dyková et al. (2010b) were not observed in our strain, which probably depends on the culture conditions. We have not seen cysts in our cultures, but this also may be a physiological property of a particular strain and cannot serve as a distinctive character. Based on the sequence similarity we have to conclude that these differences represent intraspecific polymorphisms. We provide a slightly revised diagnosis of *S. limacina* to incorporate our data on this species and to remove some characters repeated in the diagnosis of the higher taxon (the genus *Stenamoeba*) (Smirnov et al. 2007).

The type strain of *S. limacina* was isolated from kidney tissue of the freshwater fish *Gobio gobio* Linnaeus (Cypriniformes), which was caught in the Lužnice River (Czech Republic, South Bohemia), whereas our strain was isolated from a mineral soil of a beech forest in the Hainich-Dün region (Germany, Thuringia). This is a remarkable (and quite rare) case of reliable amoebae species re-isolation from two very different and relatively distant locations (ca 550 km linear distance), confirmed both at the morphological and molecular level. The finding of *S. limacina* free-living in the environment supports the idea on its amphizoic nature (Dyková et al. (2010b) and show that this species is not necessarily a freshwater fish-associated one, but can live in soil as well.

Stenamoeba berchidia: Cells of *S. berchidia* were on average slightly longer (18.9 µm) and explicitly broader (5.9 - 11.6 µm) than those of *S. sardiniensis* and *S. limacina*. Sometimes

S. berchidia demonstrated an almost fan-shaped appearance, similar to the shape of *S. amazonica*, but only for short periods. A remarkable character distinguishing *S. berchidia* from all other *Stenamoeba* species is the strong tendency to attach and move on coverslips; trophozoites of *S. berchidia* were always very active, and nearly never stationary. Wave-like surface ripples passing from the anterior to the posterior edge of the cell during locomotion are characteristic for *S. berchidia*. Similar ripples were only documented for species of the genus *Clydonella,* but waves in this genus move in the opposite direction from the posterior to the anterior edge of moving cells (Sawyer 1975).

Stenamoeba sardiniensis: Cells of *S. sardiniensis* shared several characteristics with *S. limacina* such as shape, ratio of hyaloplasm to granuloplasm, low attachment to coverslips, size and length / breadth ratio. However, cyst morphology allows a differentiation as *S. sardiniensis* forms double walled cysts, while cysts in *S. limacina*, if present, appear single-walled. Further, the average cyst diameter of ca 10 µm is larger in *S. sardiniensis.* However, the most reliable way to differentiate these two species remains the SSU sequence, detailed below.

Cytoplasmic MTOC as a phylogenetic marker among lobose amoebae

In contrast with many other groups of amoeboid protists, naked lobose amoebae move predominantly with their acto-myosin cytoskeleton; cytoplasmic microtubules are rare and never form cortical networks or organized bundles (Grebecki 1994). However, a number of amoebae species are known to possess cytoplasmic microtubule-organizing centers (MTOCs) showing a characteristic pattern of microtubules, radiating from an amorphous or lamellar central mass (e.g. Bowers and Korn 1968). Normally MTOCs in naked amoebae are associated with dictyosomes, but in lobose amoebae dictyosomes were never shown to be specifically associated with the nucleus unlike in most other organisms; moreover, in amoebae MTOC may be associated with only one of numerous dictyosomes, as it as shown in the present study. Among lobose amoebae, cytoplasmic MTOCs are known in Centramoebida (Bowers and Korn 1968); members of the genera *Stygamoeba* (Smirnov 1996), *Gocevia* (Pussard et al. 1977), *Endostelium* (Bennett 1986), *Cochliopodium* (Kudryavtsev 2004); *Thecamoeba* and *Pellita* (Kudryavtsev et al., unpublished). The present study adds the genus *Stenamoeba* to this list. All these genera belong to different phylogenetic lineages comprising the class Discosea and represent five of nine orders,

currently included in this class. Therefore, the presence of MTOC theoretically could be a synapomorphic character, unifying several lineages of Discosea.

Among the known discosean lineages, cytoplasmic MTOCs were never shown in the clade comprising Vannellida and Dactylopodida despite these groups were the subject of close attention and various TEM studies (e.g. Page and Blakey 1979; Page 1980; Dyková et al. 2000; Dyková et al. 2003). Therefore, it seems that MTOCs are absent in this clade. Among other discosean lineages, MTOCs are not yet reported in the orders Trichosida and Dermamoebida. However, EM studies of both orders never specifically aimed at recovering cytoplasmic structures. Among various TEM data available for *Trichosphaerium* (Angell 1975, 1976, Rogerson et al. 1998), only Schuster (1976) provided detailed TEM data. His study did not reveal MTOCs, but a single study of a single species might miss to identify these delicate structures and thus provide no conclusive answer to such a minute detail in the ultrastructure. For example, a rather detailed study of the ultrastructure of the genus *Stenamoeba* (Dyková et al. 2010) revealed no MTOCs (also we cannot reject the hypothesis that it might be absent in some species, such as *S. limacina* or *S. amazonica*). Another group not showing MTOCs is Dermamoebida, but, similarly, there are few studies dedicated to the general ultrastructure of their cells – *Mayorella pussardi* was studied by Hollande et al. (1981), and although no MTOCs were detected, the authors noted a microtubular layer "surrounding the nucleus", which was never recovered in any other amoebae species. Other TEM studies of Dermamoebida were either aimed to the cell coat structure (Page 1983, Page 1988, Smirnov and Goodkov 2004, Smirnov et al. 2007) or to the general overview of the ultrastructure (Cann 1981; Smirnov et al. 2011b). Cytoplasmic MTOCs are tiny structures, often visible only in a few TEM sections of many examined ones and requiring good fixation quality to be preserved. They can easily be missed if a study not specifically aims at recovering cytoplasmic structures, and from this point of view both mentioned lineages require more detailed study to prove or disprove the presence of cytoplasmic MTOCs.

The above analysis shows that the presence on MTOCs potentially may be a shared character of Discosea with the exception of the clade containing Vannellida and Dactylopodida. The later clade is also unified by the presence of unique surface structures - pentagonal glycostyles that led Cavalier-Smith et al. (2004) to establish a taxon Glycostilida. Later we have abandoned this group as a taxon (Smirnov et al. 2005; Smirnov et al. 2011a),

but the present data indicate that this might have been premature. Distribution of MTOCs along with the accumulation of molecular data and increasing quality of phylogenetic analysis support the hypothesis that Discosea might split in two groups, differing in composition from the present Flabellinia and Longamoebia (Smirnov et al. 2011a). Those would be the previously mentioned "glycostilida" (Vannellida and Dactylopodida) and a group combining the remaining lineages within Discosea. This suggestion is weakly supported by some molecular data, where Stygamoebida and Himatismenida tend to group with other members of the subclass Longamoebia, thus reducing numbers of independent discosean lineages (e.g. Kudryavtsev and Pawlowski 2013) and shaping a sort of larger clade within Discosea. Support for this remains, however, weak and more data and analyses are required to prove or reject this hypothesis.

MTOCs are also known in amoebae of the genera *Corallomyxa* and *Stereomyxa* (Benwitz and Grell 1971a, b, Grell and Benwitz 1978), classified by Page (1987) in a separate suborder Leptoramosina within the order Leptomyxida. Leptomyxida are members of Tubulinea, however in modern systematics both *Stereomyxa* and *Corallomyxa* are placed as Lobosa incertae sedis (Smirnov et al. 2011b). The reason for this is that none of those strains were studied using molecular methods, and no reliable re-isolations of these amoebae are recorded. A strain, resembling *Corallomyxa* and named *C. tenera* (Tekle et al. 2007) was shown to belong to the rhizarian genus *Filoreta* (Bass et al. 2009a). These two genera share a very specific morphology and there is no agreement on the phylogenetic position of both. Page (1983) lists the genus *Stygamoeba* in the family Stereomyxidae together with *Stereomyxa* and *Corallomyxa* based on certain morphological characters, especially for the species *Stygamoeba polymorpha* (Sawyer 1975). Smirnov (1995) showed the *Stygamoeba* differs from *Stereomyxa* and *Corallomyxa* by the shape of mitochondrial cristae, but the value of this character remains uncertain since all phylogenetic relatives of *Stygamoeba* share tubular cristae. However, in the system by Page (1987) *Stygamoeba* was missing, while *Stereomyxa* placed in Leptomyxida (together with *Gephyramoeba*), and *Corallomyxa* formed a separate order Loboreticulatida (Page 1987 p. 206). All this indicates that the position of *Corallomyxa* and *Stereomyxa* is uncertain, and unless the opposite is proven we can assume that cytoplasmic MTOCs are not present in Tubulinea.

Finally, MTOCs are present in all lineages of Conosa (Cavalier-Smith 1998). This suggests that the presence of cytoplasmic MTOCs is a plesiomorphic feature of Amoebozoa, while its absence in certain lineages such as Tubulinea and "glycostilida" probably is a secondary loss.

***Stenamoeba* - a widely distributed and probably species-rich genus**

Stenamoeba stenopodia was long believed to represent a single species of this genus, because even before this genus was erected, no other platyamoebian with similar morphology was described. To a certain extent, the marine species *Lingulamoeba leei* Sawyer 1975 somehow resembled *S. stenopodia*, but molecular studies clearly place *L. leei* in a separate lineage (Peglar et al. 2003, Kudryavtsev et al. 2005, Smirnov et al. 2005). More recently, *Stenamoeba stenopodia*-like species were recorded at Valamo Island (North-West Russia) by Smirnov and Goodkov (1999) and meanwhile detected in soil samples from many different locations, including various sites in Russia, Switzerland, Denmark and Canada (Smirnov, unpublished observations), as well as The Netherlands, Germany and high altitude soils from Tibet (Geisen, unpublished observations). *Stenamoeba limacina* isolated from gills of fishes (Dyková et al. 2010b) and the present isolation of this species from a soil habitat, as well as a number of unnamed environmental sequences and sequences from strains identified at the morphological level as *Stenamoeba* spp. (see Figure 20), suggest that this amoeba genus may be another widely distributed taxon consisting of no less than 10 different species hard to identify by light microscopic characters. Molecular markers will be helpful to discriminate species and estimate the true diversity within this amoeba genus.

Diagnoses

***Stenamoeba berchidia* n.sp.**

Diagnosis: Length in locomotion 12 - 24 µm (average 18.9 µm), breadth 6 - 12 µm (average 8.6 µm), L / B ratio 1.4 - 3.7 (average 2.3). Pronounced frontal hyaloplasm covering up to 2/3 of the cell. Sometimes nearly fan-shaped appearance in addition to the typical oblong or linguiform locomotive form. Posterior part of the cell narrower than anterior hyaloplasm, often pointed. Pronounced longitudinal surface ridges formed during active locomotion, sometimes transverse waves running over the hyaloplasm. Single nucleus usually located at the border of hyaloplasm and granuloplasm with average diameter of 1.7 µm (range

1.3 - 2.2 µm), nucleolus 0.75 µm (range 0.5 - 0.9 µm). Smooth, spherical double-walled cysts ca 10 µm in diameter.

Food: bacterivorous; habitat: soil; ethymology: the species name refers to the geographic area of the origin (Berchidda); type location: soil, Sardinia region (Italy), Berchidda district, 40°46'N, 9°10'E; type material: type culture *Stenamoeba berchidia* strain 22 deposited with CCAP, accession number CCAP 2571/2.

Differential diagnosis: Morphologically typical for the genus *Stenamoeba*, with size dimensions similar to *S. limacina*. High activity even on cover slips, the absence of a pronounced floating form and the large hyaline area (up to 2/3 of the cell) are unique to *S. berchidia*. A new feature in the genus *Stenamoeba* present in *S. berchidia* are transverse waves running over the hyaloplasm as has been observed in the genus *Clydonella* (Sawyer, 1975a). Distinct SSU rDNA gene sequence and nucleotide pattern in region V4 of the SSU rDNA gene sequence starting in positions 686 - 742 and a short V7 region differentiate this species from any other known *Stenamoeba*.

***Stenamoeba sardiniensis* n. sp.**

Diagnosis: Length in locomotion 13 - 24 µm (average 17.0 µm), breadth 5 - 7 µm (average 6.1 µm), L / B ratio 2.2 - 3.9 (average 2.8). Posterior granular part of the cell is thicker than the frontal hyaline part, narrowing to the pointed end. Longitudinal surface ridges on the dorsal surface are occasional and briefly present on the moving cell. Floating form with up to three thin, blunt and radiating pseudopods of different length. Single nucleus located at the border of hyaloplasm and granuloplasm with an average diameter of 1.7 µm (range 1.5 - 2.1 µm), nucleolus 0.65 µm (range 0.5 - 0.9 µm). Smooth, spherical double-walled cysts ca 10 µm in diameter.

Food: bacterivorous; habitat: soil; ethymology: the species name refers to the geographic area of the origin (Sardinia); type location: Sardinia region (Italy), Berchidda district, 40°46'N, 9°10'E; type material: type culture *Stenamoeba sardiniensis* strain 17 deposited with CCAP, accession number CCAP 2571/1.

Differential diagnosis: Locomotive form typical for members of the genus *Stenamoeba*. Size and length / breath ratio are only within the values reported for *S. limacina*. Also the tendency to detach from the substratum is a shared feature of *S. sardiniensis* and *S. limacina*

but cysts differentiate both; *S. sardiniensis* has larger (10 µm), double walled cysts, while those of *S. limacina* are smaller (5.6 µm) and single walled. A unique SSU rDNA gene sequence with a distinct V7 region is characteristic for *S. sardiniensis*.

***Stenamoeba limacina* Dyková, Kostka et Pecková 2010, emend.**

Flattened amoeba of elongated limax-like or linguiform shape with anterior part round or truncate; anterior hyaline area covering up to 1/2 of the cell, posterior part granular; single contractile vacuole; length in locomotion 15 - 28 µm (average 19.9 µm), breadth 4 - 11 µm (average 6.9 µm), L / B ratio 1.6 - 4.9 (average 3.0); transitory folds or wrinkles on the surface of trophozoites, floating form with blunt radiating pseudopodia; cysts spherical, single walled. *Stenamoeba limacina* deposited with CCAP (accession number CCAP 2571/3).

Part 1 – Chapter 2

New insights into the phylogeny of the genus *Cochliopodium* (Amoebozoa, Himatismenida) with the description of *Cochliopodium plurinucleolum* n. sp.

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Abstract

Amoebae of the genus *Cochliopodium* are characterized by a tectum that is a layer of scales covering the dorsal surface of the cell. A combination of scale structure, morphological features and, nowadays, molecular information allows species discrimination. Here we describe a soil species *Cochliopodium plurinucleolum* n. sp. that besides strong genetic divergence from all currently described species of *Cochliopodium* differs morphologically by the presence of several peripheral nucleoli in the nucleus. Further, we unambiguously show that the Golgi attachment associated with a dictyosome in *Cochliopodium* is a cytoplasmic microtubule organizing center (MTOC). Last, we provide detailed morphological and molecular information on the sister clade of *C. plurinucleolum*, containing *C. minus*, *C. minutoidum*, *C. pentatrifurcatum* and *C. megatetrastylus*. These species share nearly identical sequences of both, small subunit ribosomal RNA and Cox1 genes, and nearly identical structure of the scales. Scales of *C. pentatrifurcatum* differ, however, strongly from scales of the others while sequences of *C. pentatrifurcatum* and *C. minus* are nearly identical. These discrepancies urge for future sampling efforts to disentangle species characteristics within *Cochliopdium* and to investigate morphological and molecular patterns that allow reliable species differentiation.

Introduction

The genus *Cochliopodium* Hertwig et Lesser, 1874, comprises lens-shaped amoebae covered by a flexible layer of carbohydrate scales (tectum) located only on the dorsal surface of the cell (Bark 1973, Kudryavtsev 2004, Kudryavtsev et al. 2005, Kudryavtsev 2006). When the cell is viewed from the top during locomotion, it shows a thick central granuloplasmic mass surrounded by a broad peripheral sheet of hyaloplasm. It is widely accepted that scales comprising the tectum of *Cochliopodium* are species-specific, so that details of scale structure allow unambiguous identification of morphospecies (Bark 1973, Kudryavtsev 1999, 2004, 2005, 2006). However, it was demonstrated previously that there is a group of morphospecies in *Cochliopodium* that can be distinguished based on light microscopic data, while sharing nearly identical scales. Namely, these are *C. barki*, *C. minutoidum* and several strains morphologically resembling *C. minus* (Kudryavtsev et al. 2004, Kudryavtsev 2006). The distinction of these morphospecies may be facilitated by using gene sequence data (Kudryavtsev et al. 2005, Kudryavtsev et al. 2011, Anderson and Tekle 2013, Tekle et al. 2013). Therefore it seems unavoidable to decipher how morphological and ultrastructural differences are related to the genetic divergence between strains. Yet, the number of sequenced species is relatively small compared to the total number of around 20 morphologically defined *Cochliopodium* spp., and this is the main obstacle for understanding the evolutionary relationships within the genus and borders between morphospecies.

In the present paper we revise the phylogenetic relationships in a clade of *Cochliopodium* comprising closely related species *C. megatetrastylus*, *C. minus*, *C. minutoidum* and *C. pentatrifurcatum* (hereinafter referred to as *minus*-clade) and describe a new soil species *C. plurinucleolum* whose scale structure is very similar to the members of this clade. However, molecular trees show only distant relationships between this species and members of the *minus*-clade.

Materials and Methods

Establishing cultures

Soil from the upper 20 cm of an intensively managed grassland plot at the Berchidda-Monti long term observatory, managed by the University of Sassari, Italy, 40°46'N, 9°10'E (Bagella et al. 2013) was collected after 2 mm sieving and transferred in thermo isolated containers to the lab. For inoculation, 50 g of soil mixed with 50 ml of sterile distilled water were shaken for 20' and allowed to settle for 15'. Ten enrichment cultures were established by transferring 5 µl of the soil suspension in 90 mm Petri dishes filled with soil extract solution (Page 1988). Each dish was carefully examined twice (at days 10 - 14 and 24 - 28) with an inverted Nikon Diaphot phase contrast microscope under 100 x and 400 x magnifications. To establish clonal cultures, amoebae of different morphotypes were individually transferred with a glass pipette to new 60 mm Petri dishes filled with Prescott-James (PJ) medium (Page and Siemensma 1991), enriched with 0.15 % wheat grass (WG) (Weizengras, Sanatur, Singen, Germany). Two strains of *C. plurinucleolum* n. sp. designated 8 and 86 were isolated and shown to be identical in gene sequence data and light microscopic characters; therefore, only one (strain 8) was further described in detail. A freshwater strain of *Cochliopodium minus* CPE was donated by Dr. Rolf Michel (Department of Parasitology, Central Institute of the Federal Armed Forces Medical Services, Koblenz, Germany). It was isolated from *Elodea canadensis* purchased from a local pet shop in Neuwied, Germany, and cultured initially on 1.5 % non-nutrient agar prepared with PJ medium. Later the strain was transferred into 0.025 % Cerophyl infusion (roughly equivalent to WG) prepared with PJ. *C. minus* CCAP 1537/1A was obtained from CCAP on NN agar supplemented with *Escherichia coli* and further cultured on Cerophyl infusion.

Light and electron microscopic observations

Several hundred cells of each clonal culture of *C. plurinucleolum* were placed on glass cover slips and observed using a Leica DM2500 microscope with an attached Nikon DS-Fi 1 digital 5-megapixel microscope camera; 30 cells were measured. *C. minus* CPE was observed using a Carl Zeiss Axiovert 200 inverted microscope. Both microscopes were equipped with phase contrast and differential interference contrast optics (DIC). For transmission electron microscopy (TEM), amoebae were briefly (up to 5') prefixed at 4 °C with 0.5 % (w/v) osmium

tetroxide followed by fixation with 2.5 % (v/v) glutaraldehyde for 30 – 40’ and postfixation with 1 % (w/v) osmium tetroxide for 1 hour; all fixatives were prepared with 0.05 M cacodylate buffer, pH 7.4. Cells were washed with the same buffer (3 x 5’) between fixation steps and before dehydration. Dehydration was conducted in a graded ethanol series, followed by 100 % acetone and embedding in medium hardness epoxy embedding medium (Fluka) or Araldite M (Serva Electrophoresis). Silver to light gold ultrathin sections were cut with Reichert Ultracut E or Leica UC 6 ultramicrotomes, double stained with 2 % (w/v) uranyl acetate in 70 % ethanol for 20 – 30’ and Reynolds’ lead citrate for 10’ and observed with Philips EM208 and Jeol JEM 1400 electron microscopes. Whole mounts of the cells for TEM observations were prepared by rinsing amoebae with glass-distilled water and placing the cell suspension on formvar-coated copper grids. Cells were allowed to settle and fixed for 5’ with osmium vapor. Grids were then air-dried and shadowed with chromium at an angle of ca 15 ° using a Jeol JEE-420D vacuum evaporator. Grids were observed with TEM as described above.

Molecular phylogenetic studies

Genomic DNA was isolated from fresh cell cultures using a guanidine isothiocyanate method (Sambrook et al. 1989). In short, liquid medium was replaced with 100 µl of guanidine isothiocyanate after rinsing the dishes twice with sterile WG. Amoebae were scraped off the plate using a disposable cell scraper, and transferred into 2 ml centrifuge tubes. Subsequent stages were performed according to the cited protocol.

The gene encoding the small subunit (SSU) ribosomal RNA (rRNA) was fully amplified from strains 8 and 86 using the universal eukaryotic primers RibA (5' - ACC TGG TTG ATC CTG CCA GT - 3') and RibB (5’ - TGA TCC ATC TGC AGG TTC ACC TAC - 3’) (Cavalier-Smith and Chao 1995, Pawlowski 2000). Cycling consisted of initial denaturation at 95 °C for 5’, followed by 35 cycles of denaturation at 95 °C for 30’’, annealing at 50 °C for 45’’ and elongation at 72 °C for 90’’ with a final elongation at 72 °C for 5’. 8 µl of the PCR products were enzymatically purified by adding 0.15 µl Endonuclease I (20 U * μl^{-1}, Fermentas GmbH, St. Leon-Rot, Germany), 0.9 µl Shrimp Alkaline Phosphatase (1 U * μl^{-1}, Fermentas, Germany) and 1.95 µl H_2O and incubating the mixture for 30’ at 37 °C followed by incubating the samples at 85 °C for 20’ to stop the reaction. Subsequently, purified products were partially sequenced by GATC (Konstanz, Germany) using RibB as a sequencing primer. Lengths of the resulting

sequences was 1073 bp and 1145 bp for strains 8 and 86, respectively. In addition, we amplified, cloned and sequenced the full SSU rRNA gene of *Cochliopodium minus* CPE, *C. minutoidum* CCAP 1537/7 (type strain of *C. minutoidum*) and *C. minus* type strain CCAP 1537/1A. To increase the number of nucleotide positions available for phylogenetic analysis, we also updated previously published incomplete SSU rRNA gene sequences of *C. kieliense* and *C. minus* CCAP 1537/5 (AY785057 and AY785056, respectively in Kudryavtsev et al. 2005) by amplification, cloning and sequencing the full-length SSU rDNA from the same DNA samples that were used in the previous study (op. cit.). PCR primers used and procedures for amplification, cloning and sequencing were as described in Kudryavtsev et al. (2009a).

5' fragment of mitochondrial Cox1 gene was in all cases amplified from the same DNA samples as SSU rDNA. We sequenced this marker for all strains used in this study as well as *C. actinophorum* CCAP 1537/10 and *Cochliopodium* sp. previously identified as *C. minus* (Kudryavtsev et al. 2011). Amplicons of all strains except *Cochliopodium* sp. 8 and 86 were cloned, and 5 - 8 molecular clones from each amplicon were sequenced in both directions. Primers and protocol for amplification, cloning and sequencing were the same as in Nassonova et al. (2010).

Sequences were manually aligned in Seaview 4 (Gouy et al. 2010) using all published sequences within the genus *Cochliopodium* (SSU rRNA: 14 sequences in total with 1,404 unambiguously aligned positions, Cox1: 24 sequences in total with 495 aligned nucleotide positions). *Ovalopodium* and *Parvamoeba* were treated as outgroups in the trees based on SSU rRNA gene; *Vannella* spp. were used as outgroup in Cox1 gene trees. Phylogenetic trees were reconstructed using maximum likelihood algorithm with RaxML (Stamatakis 2006). A GTR+γ+I model of evolution with 25 substitution rate categories was applied for the analysis. All model parameters were estimated from the data. The stability of the clades was assessed using a non-parametric bootstrap with 1,000 pseudoreplicates. Bayesian reconstructions of phylogenetic relationships were performed using Mr Bayes Version 3.2.1 with covarion and autocorrelation models for among-site rates (Huelsenbeck and Ronquist 2001). Two runs of four simultaneous Markov chains were performed for 5,000,000 generations (default heating parameters) and sampled every 100 generations; 25 % of the samples were discarded as a burnin. All analyses were run at the University of Oslo Bioportal computer service (http://www.bioportal.uio.no; Kumar et al. 2009.

Results

Microscopic observations of *Cochliopodium* strains

Cochliopodium plurinucleolum n. sp.

Trophic amoebae were highly variable, showing most frequently oval, fan-shaped, or crescent-shaped locomotive forms (Figure 1A-B). Many amoebae temporarily adopted a triangular, drop-shaped locomotive form with length greater than breadth caused by adhesion of the posterior end to the substratum (Figure 1C). The granuloplasm in above view was entirely surrounded by a thin hyaloplasmic sheet (Figure 1A-C), which never exceeded $1/5^{th}$ of the total body length and was often equally broad at the anterior and lateral parts of the body. The dorsal surface of the hyaloplasmic sheet was often completely covered by the most peripheral scales of the tectum (Figure 1A-B). Sometimes the anterior margin of the hyaloplasm extended beyond the border of the scale layer; it was then smooth or slightly irregular (Figure 1C). Subpseudopodia were never seen. Few short trailing adhesive filaments were visible in the posterior end of the cell during fast, directed locomotion (Figure 1A-C). Amoebae in non-directed movement (Figure 1D) were oval, triangular, or had an irregular shape, often angulate and stretched over the substratum, with the hyaloplasm split into several flattened projections on distinct sites of the cell margin. The stationary form was nearly rounded, sometimes slightly wrinkled or oval without any extensions of the peripheral hyaline area, which was equally broad but retracted in comparison to the locomotive form.

A differentiated floating form was rarely developed, and only when the cell was artificially disturbed. Amoebae contracted shortly after detachment from the substratum; the central granuloplasmic area became spherical, and the peripheral hyaloplasmic sheet contracted and produced several hyaline pseudopodia. Those pseudopodia were mostly short, but extended and became longer than the central cell body in amoebae getting closer to the substratum and attaching to it. Amoebae started locomotion immediately after settling down on the substratum.

A single spherical nucleus (Figure 1E) was located in the central part of the granuloplasm close to the dorsal surface of the cell. It had finely granular contents and several spherical pieces of nucleolar material at the periphery (1 - 2 of these pieces were usually visible

simultaneously). The nucleus was inconspicuous and often concealed by the organelles and inclusions in the granuloplasm such as abundant food vacuoles or crystals of varying sizes and shapes. Several asynchronously working contractile vacuoles, capable of fusing together, were present in different areas of the granuloplasm. Cysts (Figure 1F) were always formed in older cultures. The cyst wall consisted of two layers; the outer one incompletely enclosing the cell was made up of the scales comprising the tectum in a trophic amoeba. No cyst pores were observed. Cysts could survive at least several weeks of complete drought in culture. Amoebae were feeding on bacteria and multiplied both in liquid and on agar media.

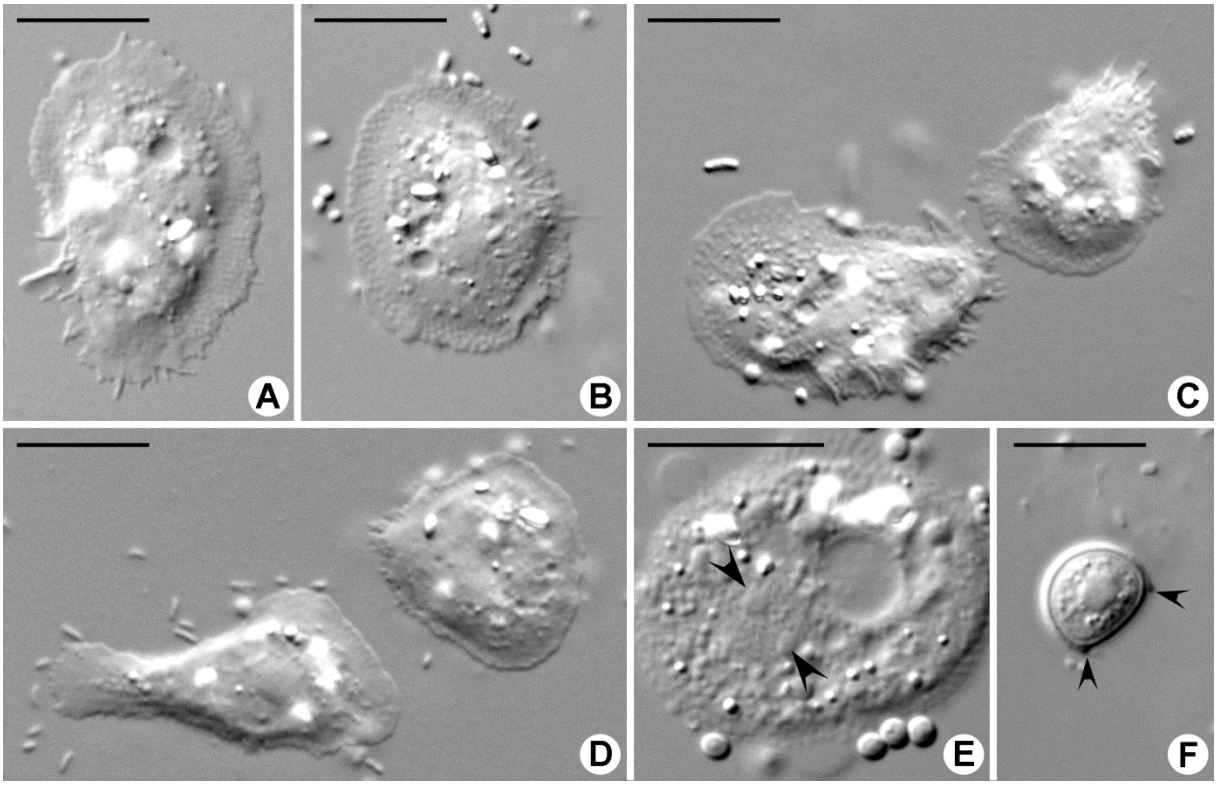

Figure 1. *Cochliopodium plurinucleolum* n. sp., light micrographs, DIC. **A-C.** Locomotive forms on the glass surface. **D.** Amoebae during non-directional movement. **E.** Nucleus in an amoeba partly squeezed with a coverslip (arrowheads indicate nucleoli). **F.** Cyst (arrowheads indicate margins of an outer cyst wall formed by scales). Scale bar = 10 µm in all figures.

Transmission electron microscopy showed that the tectum consisted of scales of uniform structure (Figure 2) located on the dorsal surface of the cell (Figure 3A). Scales had flat, circular base plates with a grid-like structure formed by a square mesh (mesh size 0.015 µm; Figure 2, 3B-D). Four vertical stalks were rising from the center of the base plate and terminated with a funnel-shaped apical part consisting of ca 15 fine radial spokes and a dense outer rim. The material between radial spokes was organized in poorly discernible

concentric rings thus giving an apical part a somewhat spiderweb-like appearance (Figure 3C-D).

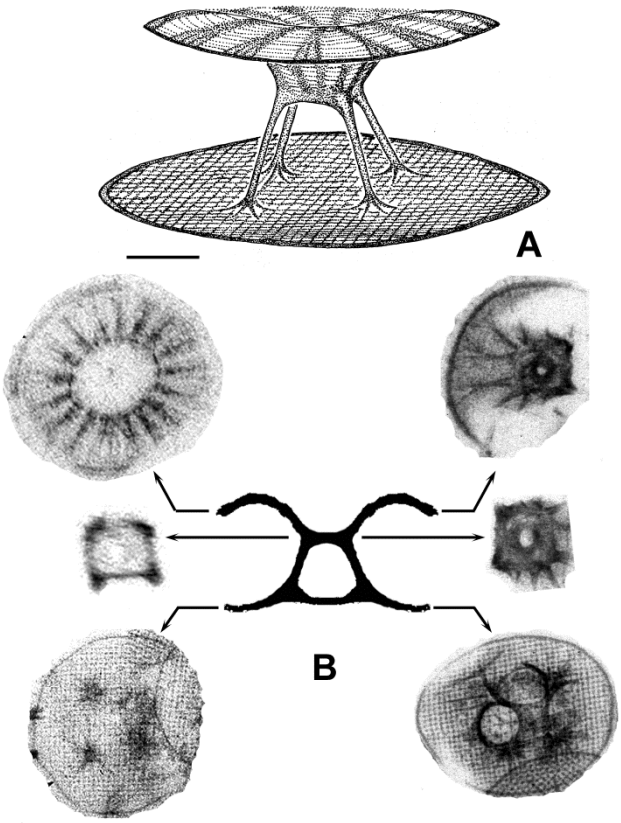

Figure 2. Diagram representing scale reconstruction in *Cochliopodium plurinucleolum* n. sp. and *C. minus* CPE based on the ultrathin sections. **A.** Drawing of a scale, scale bar = 0.1 µm. **B.** Scheme of the scale appearance at different levels of sectioning in *C. minus* CPE (left) and *C. plurinucleolum* n. sp. (right), not to scale.

The nucleus in sections was oval or irregular in shape (Figure 3A, E). It contained numerous dense patches of heterochromatin. In most of the sections one or two rounded nucleolar pieces were visible at the periphery of the nucleus (Figure 3E). A single large dictyosome was located between nucleus and dorsal surface of the cell (Figure 3E). An elongated granular structure, the "Golgi attachment", was located close to the side of a dictyosome opposite to the nucleus, and numerous microtubules often emerged from the Golgi attachment into the cytoplasm (Figure 3F). Mitochondria in sections were rounded or ovoid with tubular cristae (Figure 3G). Cysts in sections were ovoid, with the denser and more poorly preserved cytoplasm than in the trophic amoebae. The cyst wall consisted of an inner layer of medium electron density that was 0.1 - 0.2 µm thick and an outer layer consisting of scales (Figure 3H). The scale layer occupied the part of the cyst surface where the tectum on the surface of a trophic amoeba was located, in accordance to what was seen with light microscopy (Figure 1F, 3H).

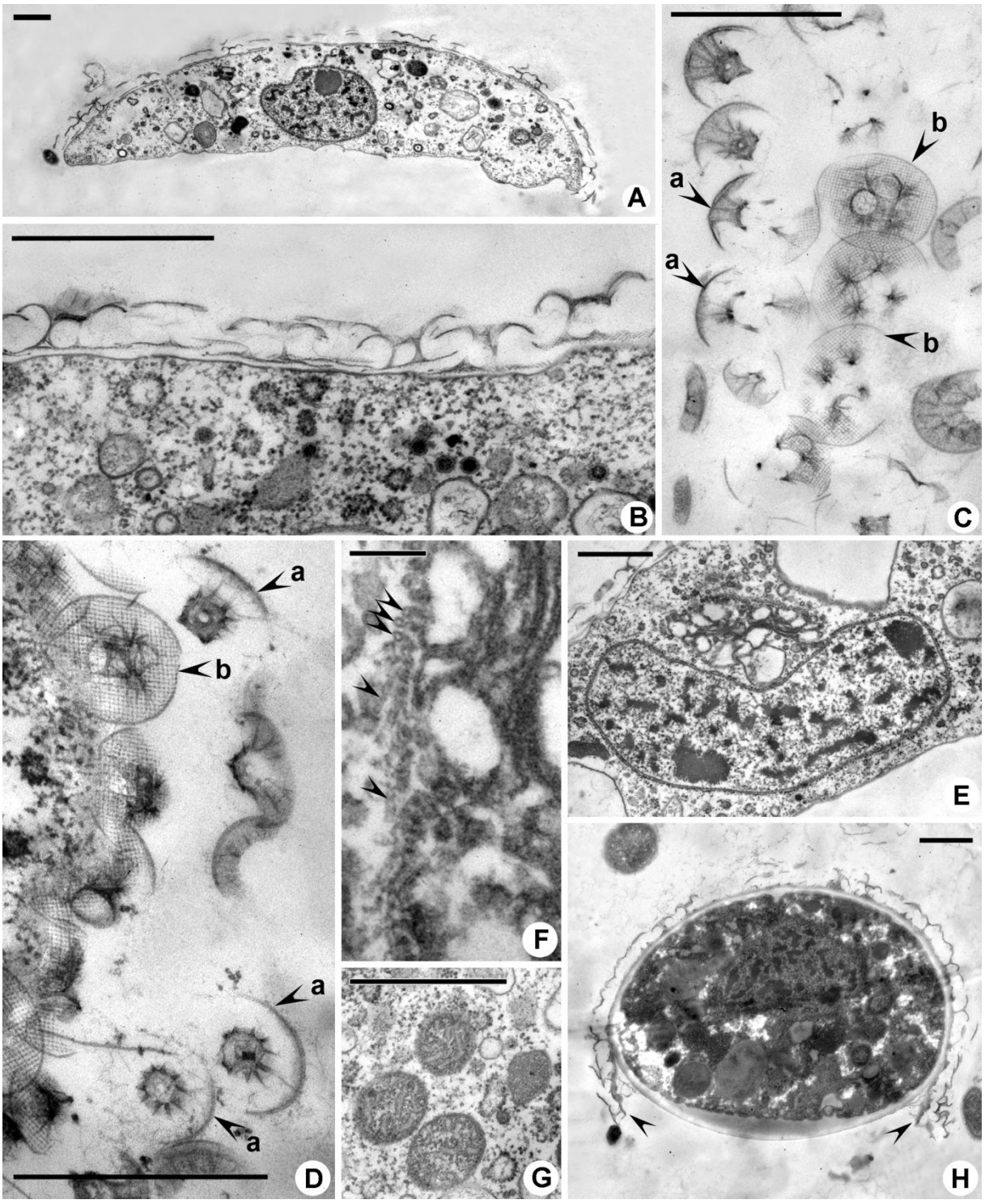

Figure 3. Transmission electron micrographs of *Cochliopodium plurinucleolum* n. sp., strain 8. **A.** Cross-section of the cell adhering to the substratum showing dorsal surface covered with tectum (above) and ventral, naked, surface (below). Nucleus is seen in the center. **B.** Vertical section of scales on the dorsal cell surface. **C-D.** Details of the scales in tangential sections: grid-like base plates (**b**) and apical parts (**a**). **E.** Nucleus (note 2 peripheral nucleoli) and a dictyosome. **F.** Detail of the dictyosome: note microtubules (arrowheads) adjacent to the dictyosome's side opposite to the nucleus. **G.** Mitochondria. **H.** Cyst (note inner cyst wall underlying tectum; the latter covers only part of the surface; arrowheads indicate margins of an outer cyst wall formed by scales). Scale bar = 1 µm in all figures except **F**, where it is 0.2 µm.

***Cochliopodium minus* strain CPE**

Length of the locomotive form in this strain was 12 - 30 µm (average 20 µm), breadth 18 - 46 µm (average 28 µm), length / breadth ratio was 0.42 - 1.08 (average 0.73; n = 76). Amoebae, in above view, were generally broadly triangular, oval or fan-shaped during locomotion, and changed their outline very quickly (Figure 4A). The central mass of granuloplasm was located posteriorly and surrounded by a peripheral hyaline sheet (Figure 4C-D). Anterior and lateral parts of the hyaline sheet were usually much wider than the posterior one, their width was equal to approximately one-third of the cell length. The margin of the hyaline sheet was very uneven, producing numerous waves, irregularities and short subpseudopodia (Figure 4C-D), sometimes deeply cleaved into several lobes. Occasionally, the anterior area of the hyaloplasm retracted, and lateral parts became wider. In this case amoebae temporarily expanded in two opposite directions before resuming locomotion in one direction. Rear parts of the lateral hyaline margins, as well as the posterior end of the cell often adhered to the substratum producing a number of trailing filaments (Figure 4C-D), occasionally becoming as long as the remaining cell body. Non-directionally moving and stationary amoebae (Figure 4E) showed either irregular shapes with the cytoplasm split into several lobes, or were rounded, with the central granuloplasmic mass completely surrounded by the hyaline sheet of approximately equal breadth from each side. A floating form was only occasionally seen in cultures and occurred more frequently when amoebae were placed on a glass slide. When floating, amoebae usually contracted and their central granuloplasm became a spherical compact mass. The peripheral hyaloplasm folded and often produced several hyaline pseudopodia (Figure 4F). The floating form was usually maintained for a maximum of 20’ until amoebae settled back to the substratum and started locomotion.

The majority of cells possessed a single vesicular nucleus (Figure 4B), but cells with 2 or 3 nuclei were occasionally seen, that were usually larger than the uninucleate ones. The nucleus was spherical, with a conspicuous envelope and a large central nucleolus that sometimes contained one or several small cavities. The nuclear envelope was sometimes outlined with a coarse layer consisting of fine granules. The diameter of the nucleus was 4.3 - 9.2 µm (average 6.3 µm), that of the nucleolus 1.9 - 5.1 µm (average 3.6 µm) (n = 32). Amoebae possessed several asynchronously working contractile vacuoles. The granuloplasm

contained 5 to 15 refractile crystals of a bipyramidal shape 1 - 2 µm in length (Figure 4C, E). Several food vacuoles and a number of spherical or elongated granules below 1 µm in size were also present. The tectum was clearly visible in phase contrast or DIC optics covering the dorsal surface of the granuloplasm (Figure 4E) and the hyaloplasmic surface in non-directionally moving and stationary amoebae, as a number of granules over the surface. In cultures amoebae encysted regularly producing spherical or ovoid double-walled cysts ca 15 µm in diameter.

Scales reconstructed from TEM sections (Figure 4G-J) appeared to be identical in structure and dimensions to those of *Cochliopodium plurinucleolum* (Figure 2). A very slight difference in their appearance was a less dense periphery of the top part, with concentric rings being better visible, but this may vary between individual fixations. Dimensions of scales are given in Table 1. The nucleus was rounded in sections containing a central, electron-dense nucleolus (Figure 5A). Rounded or oval mitochondria had electron-dense matrix and tubular cristae (Figure 5A-B). Each cell possessed a single dictyosome located close to the nuclear envelope between nucleus and the dorsal surface of the cell. A Golgi attachment (Figure 5C) visible as a dense granular structure was located close to the dictyosome, opposite to the nucleus. Cysts of this strain had the same structure as those of *C. plurinucleolum* (Figure 5D-E). The dense inner wall was fibrous and contained a number of cavities filled with granular material in a young cyst that also occupied the space between the folded plasma membrane of the encysting cell and the cyst wall (Figure 5E).

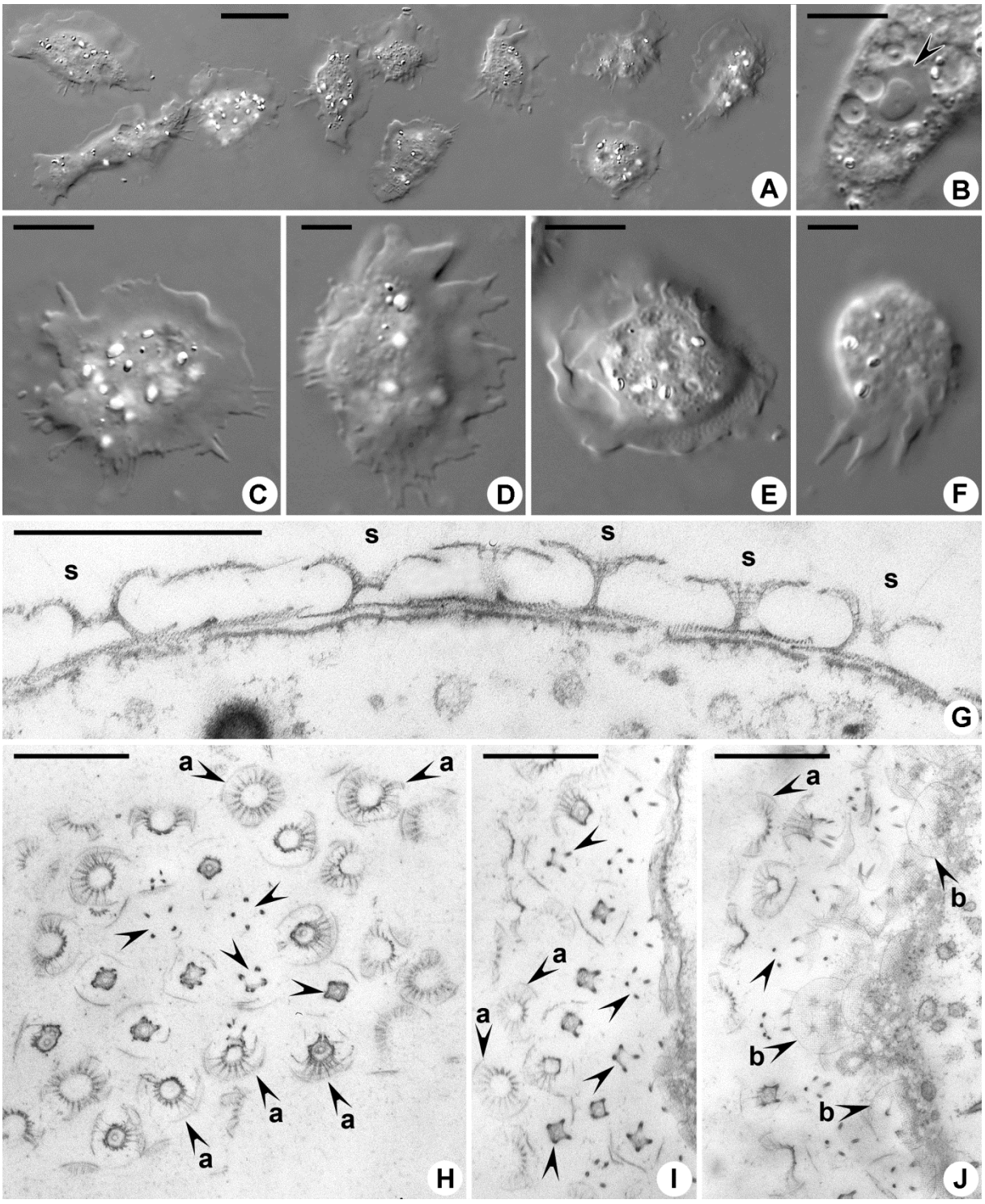

Figure 4. *Cochliopodium* sp. CPE. Light (**A-F**) and electron (**G-J**) micrographs. **A, C-D**. Locomotive forms on glass surface. **B.** Nucleus (indicated by arrowhead). **E.** Amoeba in non-directed movement. **F.** Floating form. **G.** Scales (**s**) on the dorsal surface of a cell in a vertical section. **H-J.** Serial tangential sections showing scales cut at different levels. Note tangentially sectioned base plates (**b**), apical parts (**a**) and central columns (arrowheads). Scale bar = 20 µm in Figure **A**, 10 µm in **B-F**, 1 µm in other figures.

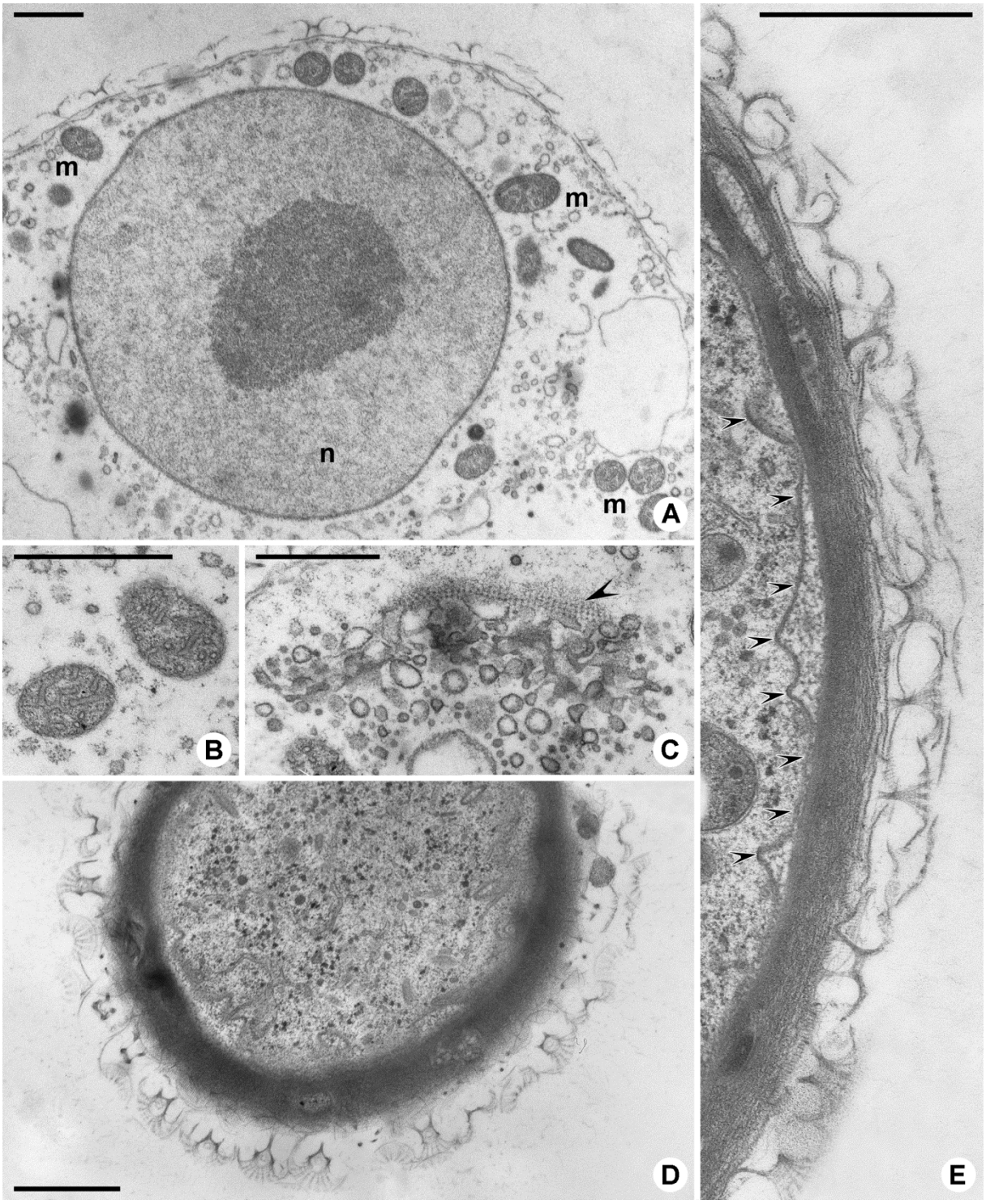

Figure 5. *Cochliopodium* sp. CPE, electron micrographs. **A.** Section of a trophic amoeba showing nucleus (**n**) and mitochondria (**m**). **B.** Mitochondria at a higher magnification. **C.** Dictyosome and a Golgi attachment (arrowhead). **D.** Cyst wall in oblique section. **E.** Transverse section of a (young) cyst wall; arrowheads indicate underlying plasma membrane. Scale bar = 0.5 µm in **A**, 1 µm in other figures.

Table 1. Comparison of the scale characteristics among *Cochliopodium* species with scales of "Category 1" (Anderson and Tekle 2013). Source references are in brackets after species names, numbers in brackets show average values where available

Species	Base plate diameter (µm)	Top part diameter (µm)	Scale height (µm)	Number of spokes in top part
C. plurinucleolum n. sp.	0.465-0.76 (0.627)	0.445-0.577 (0.5)	0.185-0.3 (0.25)	ca 15
Cochliopodium minus CPE	0.538-0.788 (0.671)	0.475-0.654 (0.56)	0.185-0.338 (0.25)	15-17
Cochliopodium sp. NYS strain (probably *actinophorum*; Nagatani et al. 1981, Yamaoka et al. 1984)	0.64	0.8	0.7	24
C. barki (Kudryavtsev et al. 2004)	0.7–1 (0.81)	0.6–0.85 (0.67)	0.35-0.45	20
C. minutoidum CCAP 1537/7 (Kudryavtsev 2006)	0.49-0.63 (0.56)	0.47-0.6 (0.54)	0.26-0.3	14-17
C. minus CCAP 1537/1A (Kudryavtsev 2006)	0.64-0.77	0.58-0.69	0.21-0.25	17-19
C. minus CCAP 1537/5 (Kudryavtsev 2006)	0.64-0.77	0.58-0.69	0.17-0.18	17-19
C. megatetrastylus (Anderson and Tekle 2013)	0.6-1	0.5-0.7	0.2-0.4 (0.3)	16

Sequence data analysis

SSU rRNA gene

Sequenced pieces of the SSU rRNA gene in *Cochliopodium plurinucleolum* strains 8 and 86 were identical to each other and had a GC content of 40 %. In other studied strains, cloning of the SSU rDNA amplicons demonstrated slight variation among different molecular clones obtained from the same PCR product. The range of this variation was 0.1 - 0.3 % in *Cochliopodium minus* CPE (n = 5), 0.4 - 1 % in *C. minutoidum* CCAP 1537/7 (n = 4), 1.8 - 1.9 % in *C. kieliense* and 0.3 % in *C. minus* CCAP 1537/5 (n = 2). Among 8 sequenced clones of *C. minus* CCAP 1537/1A 4 clones were identical, the other 4 varied by 0.1 - 0.3 %. *Cochliopodium minus* CCAP 1537/1A was obtained from the culture collection and re-investigated to facilitate a more precise molecular identification of *C. minus* CPE strain studied here, and to evaluate the identity of a sequence JF298257 previously assigned to *Cochliopodium minus* CCAP 1537/1A (Kudryavtsev et al. 2011). Several newly obtained light and electron micrographs of these amoebae are shown in Figure 6A-G. Our results show that the strain investigated in this study is identical to the one previously studied microscopically (Kudryavtsev 2006; several new micrographs of the scales are shown in Figure 6F-G). Yet, the SSU rRNA gene sequence of the newly obtained strain is highly divergent from a sequence JF298257 previously designated as *C. minus* CCAP 1537/1A (Kudryavtsev et al. 2011).

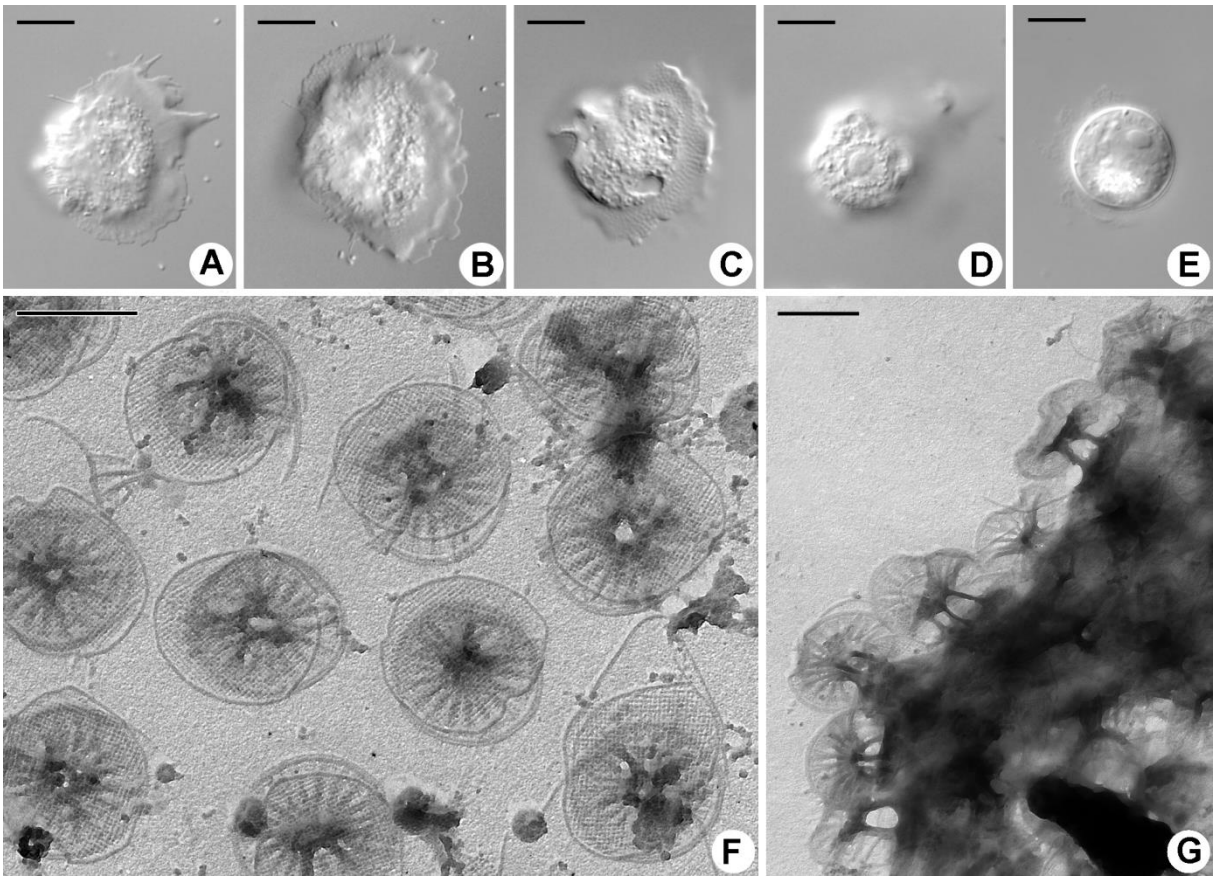

Figure 6. *Cochliopodium minus* CCAP 1537/1A, newly obtained light (**A-E**) and electron (**F-G**) micrographs of re-ordered and sequenced strain. **A-C.** Locomotive forms on glass surface; **C** showing scales of the dorsal surface of the cell. **D.** Nucleus in a living amoeba. **E.** Cyst. **F-G.** Scales in a whole-mount TEM preparation shadowed with chromium. Scale bar = 10 µm in **A-E**, 0.5 µm in **F-G**.

The use of improved primers and cycling conditions enabled us to update the available sequence database of *Cochliopodium* spp. by obtaining complete SSU rRNA sequences of *C. kieliense* and *C. minus* CCAP 1537/5. Previously, the SSU rRNA gene of both strains was partially sequenced from the amplicons obtained with the primer pair s6 - RibB, therefore missing approximately 600 nucleotides from the 5' end (Kudryavtsev et al. 2005; GenBank accession numbers AY785057 and AY785056 respectively), as the full-length amplicons could not be obtained. During this study we obtained and sequenced the full-length amplicons of SSU rRNA genes from the same DNA samples as used in the previous work, and additionally obtained partial Cox1 gene sequences of these species. The newly obtained SSU rRNA gene sequences are largely identical to the previous ones. Differences in several nucleotides are most probably due to a sequencing protocol used during the present study that applied a BigDye Terminator sequencing kit in a single reaction run on a capillary electrophoresis, while the previous one applied 4 separate sequencing reactions and gel electrophoresis (Kudryavtsev et al. 2005) that may have a higher error rate. We therefore substitute the

previously published sequences AY785056 and AY785057 with the new ones, to enable the inclusion of more nucleotide positions in phylogenetic analyses.

The phylogenetic trees based on maximum likelihood and Bayesian algorithms robustly placed sequences of *C. plurinucleolum* n. sp. (strains 8 and 86) within the monophyletic genus *Cochliopodium* in the same position. They branched as a sister to a monophyletic clade comprising C. *actinophorum*, *C. kieliense* and a *minus*-clade consisting of very shortly branching species *C. minus*, *C. minutoidum*, *C. megatetrastylus* and *C. pentatrifurcatum* (Figure 7) with strong support (PP / BS= 1.0 / 92).

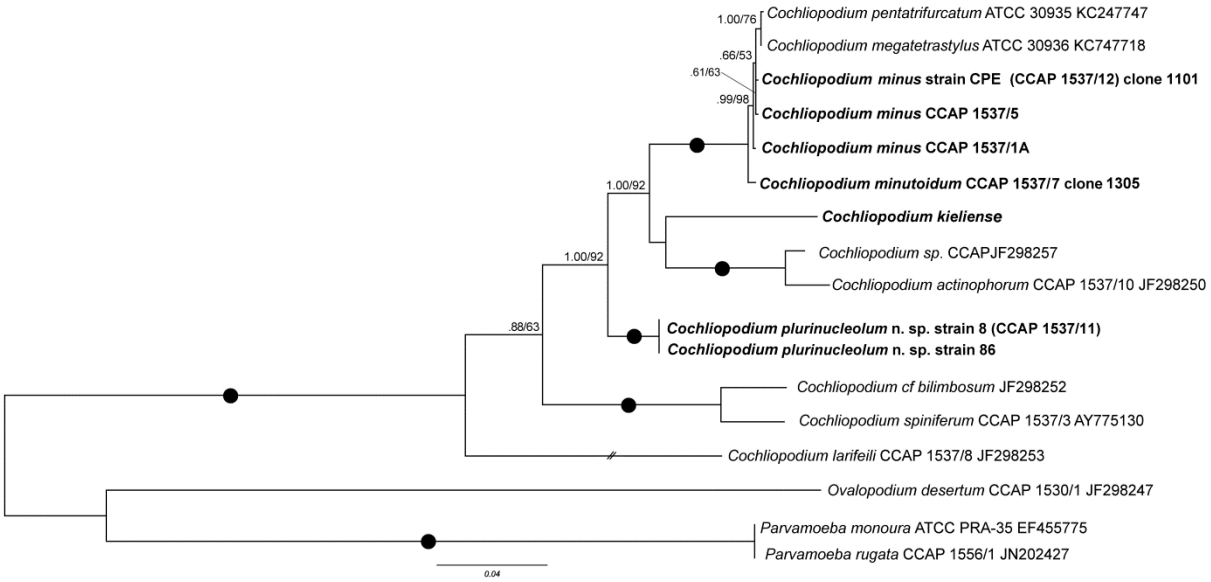

Figure 7. Maximum likelihood phylogenetic tree based on the SSU-rDNA gene (1,404 nucleotide positions) using RaxML program version 7.3.2 (Stamatakis 2006) with GTRGAMMAI model of nucleotide substitution. Numbers at nodes indicate Bayesian posterior probability / bootstrap values if above 0.5 / 50; solid circles = 1.00 / 100. The tree is rooted with *Parvamoeba* and *Ovalopodium* spp., scale bar = 0.04 substitutions / site.

In the phylogenetic trees *Cochliopodium minus* CPE branched closely together with *C. minus* CCAP 1537/1A and CCAP 1537/5, *C. pentatrifurcatum* ATCC 30935 and *C. megatetrastylus* ATCC 30936 in the *minus*-clade. *C. minutoidum* CCAP 1537/7 was always sister to the clade formed by different strains of *C. minus*, and the whole *minus*-clade was robustly sister to a clade comprising *C. kieliense* and *C. actinophorum*. Extremely short branches in the *minus*-clade were in agreement with very high sequence similarity between these strains (Supplementary Table 1). Yet, distinct nucleotide motifs could be identified in these sequences (Supplementary Table 2), so that each species and clade could be characterized by specific nucleotide signatures. Therefore, a robust topology obtained by phylogenetic analyses was in accordance with gene sequence features.

Cox1 mitochondrial gene

Amplified and sequenced fragments of the mitochondrial Cox1 gene of each strain studied were 666 base pairs long not including PCR primers, and did not contain any indels. Cloning of the amplicons has shown identical sequences among different molecular clones in *C. actinophorum*, *Cochliopodium minus* CPE and *C. minutoidum* CCAP 1537/7, whereas *C. minus* CCAP 1537/1A, *C. minus* CCAP 1537/5 and *C. kieliense* have shown differences in nucleotide sequences between molecular clones obtained from the same amplicon in the range of 1 - 3 variable nucleotide positions per sequence. Six out of 8 sequenced molecular clones of *C. minus* CCAP 1537/1A have shown a complete identity to the Cox1 gene sequence of *C. pentatrifurcatum* ATCC 30935 (KC489470; Tekle et al. 2013) at the nucleotide level. Partial Cox1 gene sequences of *C. actinophorum* CCAP 1537/10 and *C. minutoidum* CCAP 1537/7 obtained earlier (Nassonova et al. 2010; GQ354207 and GQ354208, respectively) were completely identical to sequences from the same strains obtained during the present study. The analysis of translated sequences revealed that differences between molecular clones within a single amplicon were synonymous in most cases, but caused variability in 1 to 2 amino acid positions in two molecular clones of *C. minus* CCAP 1537/1A and *C. minus* 1537/5 amplicons, respectively. *C. plurinucleolum* n. sp. differed from other *Cochliopodium* spp. included in this study by an average of 12.9 % at the nucleotide level (11.8 - 16.4 %), based on 495 nucleotide positions, and by 5.6 % (3.7 - 11.6 %) at the amino acid level. The identity matrix between all strains of the *minus*-clade is shown in Supplementary Table 3. The maximum likelihood phylogenetic analysis of Cox1 sequences of *Cochliopodium* spp. (Figure 8) basically resulted in the same tree topology and branch support values as in the tree based on the SSU rRNA gene revealing two clades of *Cochliopodium* spp., one comprising *C. actinophorum* and *C. kieliense*, and the second one corresponding to a *minus*-clade outlined above. *C. plurinucleolum* n. sp. forms the most basal branch of the *minus*-clade, being sister to a monophyletic branch comprised by the other strains. Phylogenetic analysis of amino acid sequences (not shown) resulted in the same topology. However, as most of the substitutions were synonymous in the *minus*-clade, the latter did not show any defined topology, while *C. plurinucleolum* n. sp. was sister to it.

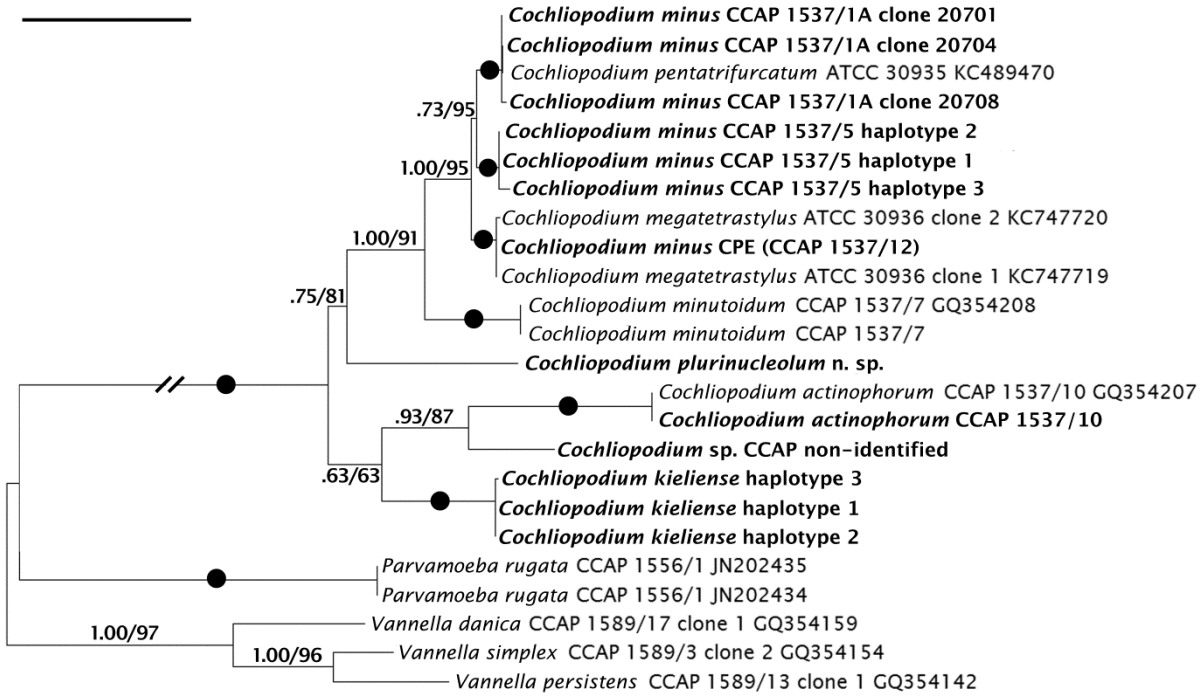

Figure 8. Maximum-likelihood phylogenetic tree of *Cochliopodium* spp. based on the partial nucleotide sequences of mitochondrial Cox1 gene (495 nucleotide positions). The tree shown was derived using a RaxML program version 7.3.2 (Stamatakis 2006) with GTRGAMMAI model of nucleotide substitution. Numbers at nodes indicate Bayesian posterior probability / bootstrap values if above 0.5 / 50; solid circles = 1.00 / 100. The tree is rooted with *Vannella* spp., scale bar = 0.1 substitutions / site.

Discussion

Morphological identification of the studied strains

Cochliopodium plurinucleolum n. sp.

Light and electron microscopic analyses unambiguously identify the studied strains 8 and 86 as a species of the genus *Cochliopodium* due to the presence of a tectum and a characteristic locomotive form. Several morphological characteristics specific for this species distinguish it from all other currently described *Cochliopodium* spp., including *C. barki* that is morphologically most similar to *C. plurinucleolum*, and is one of the few isolates reported so far to occur in soils (Kudryavtsev et al. 2004). Cells of *C. barki* are, however, bigger, sometimes produce subpseudopodia and occasionally contain more than one nucleus. The structure of the nucleus is the character that best distinguishes *C. plurinucleolum* from all other known *Cochliopodium* spp. This presence of multiple nucleoli appears to be a reliable character, because no variation of the observed nuclear structure among the studied cells was seen, and the results of the electron microscopic study corresponded to the light microscopic data. The only described species that has a similar nucleus with peripheral

nucleoli is *C. clarum* Schaeffer, 1926. However, this species is larger, was isolated from marine habitats and has generally a smoother outline of the cell with several short subpseudopodia (Schaeffer 1926). Other described species of *Cochliopodium* possess a vesicular nucleus containing a large central nucleolus (Dyková et al. 1998, Kudryavtsev 1999, Kudryavtsev 2000, Kudryavtsev 2004, Kudryavtsev et al. 2004, Kudryavtsev 2005, 2006, Kudryavtsev and Smirnov 2006, Anderson and Tekle 2013, Tekle et al. 2013). We therefore recognize *C. plurinucleolum* as a new species. The scale structure of this species is very similar to that of *C. barki*, *C. megatetrastylus*, *C. minus* and *C. minutoidum* (Dyková et al. 1998, Kudryavtsev 2004, 2006, Anderson and Tekle 2013). Size differences between scales in these species are summarized in Table 1. We have to admit that the structural pattern of the scales is identical, and the slight quantitative differences between them may be purely intraclonal, but no statistical data are available to check this at the moment. A study investigating statistical variation of the scale parameters in genetically identical isolates of *Cochliopodium* would be highly appreciated to reliably resolve boundaries between species.

Cochliopodium minus CPE

Based on the light microscopic characters of trophic amoebae and cysts, *Cochliopodium minus* CPE could be identified as most similar to *C. minus* and *C. minutoidum* as described by Kudryavtsev (2006). The most significant characters to identify *C. minus* CPE are oval and almost fan-shaped locomotive forms with a very uneven margin of the frontal hyaline area producing numerous subpseudopodia. This strain differs from *C. minutoidum* by forming cysts, but this character may vary depending on individual clones: for example, Page (1976b) mentioned “encystment present in some strains” for *C. minus*. Scales of *Cochliopodium* sp. CPE strain were most similar to those of *C. minutoidum* as described by Kudryavtsev (2006). Ambiguous identification of this strain based on the microscopic data demands a more precise identification based on the combination of morphology / ultrastructure and gene sequences. We therefore obtained and analyzed sequences of SSU rRNA and Cox1 genes from all available strains similar to *C. minus* and *C. minutoidum* in addition to *C. plurinucleolum*.

Molecular phylogeny and species distinction in *Cochliopodium*

Phylogenetic position and relationships of *C. plurinucleolum* n. sp.

Analysis of both SSU rRNA and Cox1 gene sequences were in accordance with morphological data, unambiguously placing *C. plurinucleolum* n. sp. as a distinct species within *Cochliopodium*. The new species branches deeply in the phylogenetic tree, but trees based on SSU rRNA and Cox1 genes demonstrate conflicting topologies: whereas in the SSU rRNA gene tree *C. plurinucleolum* n. sp. is a sister to the clade comprising *C. minus* and related species, as well as *C. actinophorum* and *C. kieliense*, Cox1 gene analysis places this species as a sister to the *minus*-clade, while *C. actinophorum* and *C. kieliense* branch outside this clade (Figures 7 - 8). Visual comparison of the aligned nucleotide sequences of Cox1 gene shows that no shared sequence motifs that may serve as molecular signatures to support the topology of either SSU rRNA or Cox1 gene trees can be found in *C. plurinucleolum* n. sp.; there are a number of positions with the motifs shared between *C. plurinucleolum* n. sp. and species of the *minus*-clade, as well as some positions that are identical in *C. plurinucleolum* n. sp. and *C. kieliense*, but differ in other species. Several explanations of this incongruence between different markers are currently possible, but none of them can provide sufficient clarifications based on the present data. As the position of *C. plurinucleolum* n. sp. in the tree based on the Cox1 gene was less resolved, as well as several other deep nodes of the tree, it is possible that the tree based on the Cox1 gene may be misleading due to incomplete taxon sampling compared to the tree based on the SSU rRNA gene, and a lower number of nucleotide positions available for phylogenetic analysis (495 nucleotide positions compared to 1,404 available for the SSU rRNA gene). Moreover, faster evolution of the Cox1 gene compared to SSU rRNA with unevenly conserved nucleotide positions across the gene (as Cox1 is a coding gene, third codon position is less conserved than the other two) may lead to the saturation of the 3rd codon positions resulting in homoplasies, favoring the evolutionary scenario reconstructed based on the SSU rRNA over Cox1 gene. We also cannot exclude the putative presence of multiple copies of the Cox1 gene in a genome or incongruence in the evolutionary history between nuclear and mitochondrial genomes in these species, in which case not all markers will yield gene trees congruent to a species tree.

Anyway, the position of *C. plurinucleolum* n. sp. in the phylogenetic tree reconstructed based on both markers is in accordance with scale structure and molecular synapomorphies

present in the SSU rRNA gene of this species. The scale structure of these amoebae is identical to that of the whole *minus*-clade (scales of Category 1 according to Anderson and Tekle 2013; Figure 2). Although no data on the scale structure of *C. actinophorum* CCAP 1537/10 have been published, our data show (Kudryavtsev, submitted) that scales of this species were described by Nagatani et al. (1981) and Yamaoka et al. (1984), as was first reasonably suggested by Page (1987). This means that the scale structure of *C. actinophorum* also belongs to Category 1 (Anderson and Tekle 2013). The suggestion that this type of scales is a synapomorphy of the clade unifying *C. plurinucleolum* n. sp., *C. actinophorum* and the *minus*-clade is rather attractive. But as this type of scales is not the only type of scales known among these species with *Cochliopodium kieliense* Kudryavtsev, 2006 and a recently described *C. pentatrifurcatum* Tekle et al. (2013) possessing completely different scale structures, while robustly branching close to *C. actinophorum* and among the *minus*-clade, respectively. Moreover, whereas *C. kieliense* forms a relatively long branch that is distantly related to *C. actinophorum* in the trees based on both, SSU rRNA and Cox1 genes, gene sequences of *C. pentatrifurcatum* are completely identical to the majority of molecular clones that we sequenced for the type strain of *C. minus*. Currently, we do not have a clear explanation for these discrepancies on morphological and molecular levels, but an artifact seems unlikely, as the same was independently observed by Y. Tekle (pers. comm.). If this identity is not due to the errors occurred at the stage of data collection caused by erroneous assignment of either the sequences or the scale structure, we have to suggest that this is the first case of sequence identity between distinct morphospecies that considerably differ from each other based on the scale structure. The first explanation for this case may be that both genetic markers sequenced are not capable to distinguish two different species that may differ from each other in other markers. The second possible explanation is that *C. pentatrifurcatum* and *C. minus* are indeed genetically identical, and differences in scale structures may reflect environmental plasticity within a single species, e.g. depending on life stage or environmental differences. Presently there are no data that might favor any of these explanations, and additional studies are necessary to clarify this situation.

The demonstrated position of *C. plurinucleolum* n. sp. in the phylogenetic tree is also in accordance with the molecular synapomorphies revealed within its SSU rRNA gene sequence. A secondary structure pattern of helices 28 - 30 of the SSU rRNA previously demonstrated for Cochliopodiidae (Kudryavtsev et al. 2011) is also present in this species

(Figure 9A). Comparison of this structure among different species of *Cochliopodium* reveals characters that appear to be synapomorphies of different phylogenetic lineages within this genus. In particular, there are several distinct patterns of nucleotides between helices E29-1 and E29-2. *C. plurinucleolum* n. sp., all species belonging to a clade that comprises *C. actinophorum*, *C. kieliense* and species related to *C. minus* share an 8- to 18-nucleotide AT-rich insertion that forms an extensive loop (Figures 9A-H). In the same site, *C. spiniferum* and *C.* cf. *bilimbosum* that form *Cochliopodium* clade contain only 2 or 3 adenines (Figures 9I-J), whereas *C. larifeili* branching separately at the base of the *Cochliopodium* tree possesses an additional helix consisting of 17 nucleotides in this site (Figure 9K).

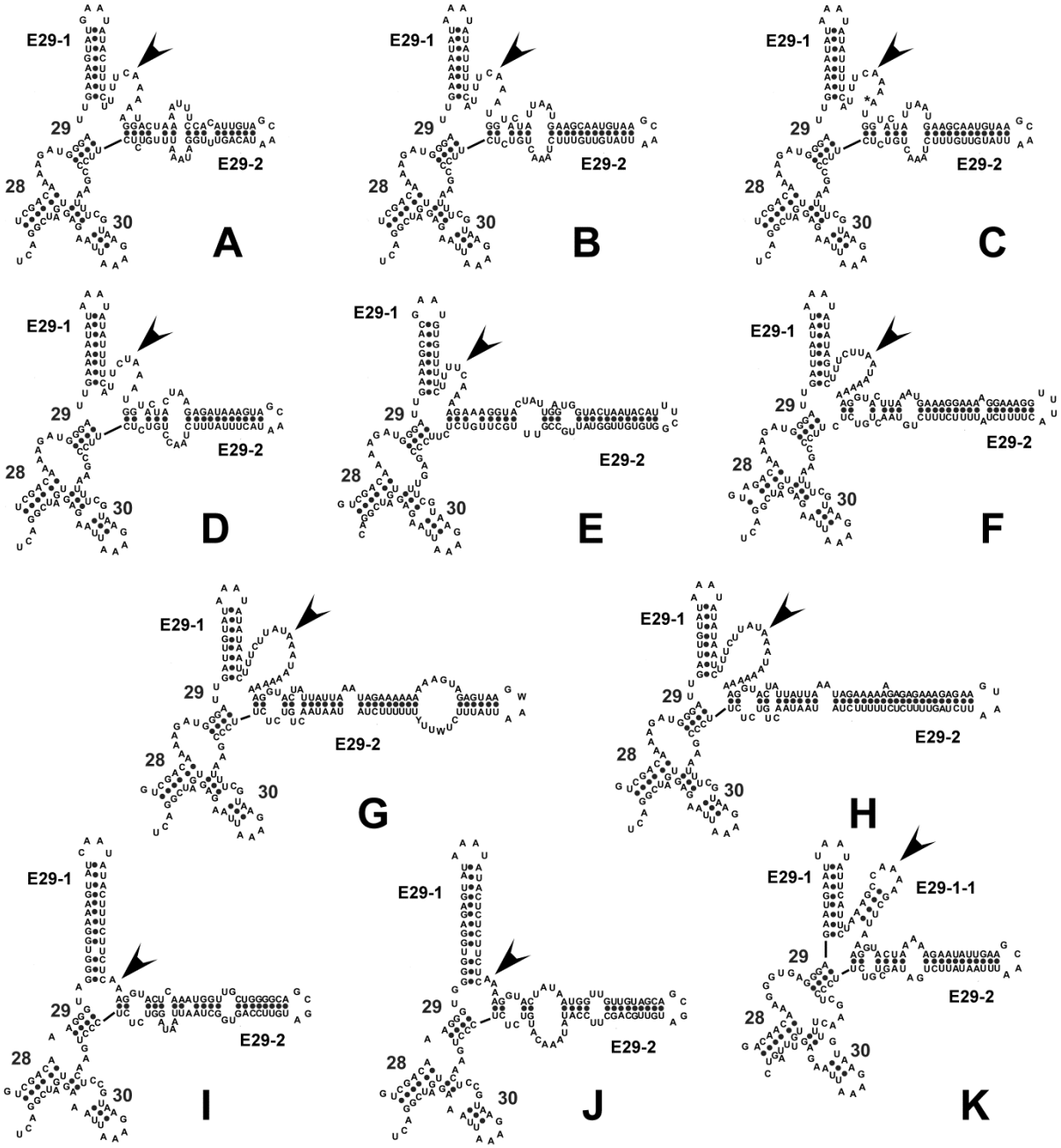

Figure 9. Secondary structure of the SSU rRNA helices 28 - 30 in *Cochliopodium* spp. **A.** *C. plurinucleolum* n. sp. **B.** *C. minus* CCAP 1537/1A and *C. pentatrifurcatum* ATCC 30935 (KC247747). **C.** *Cochliopodium* sp. CPE, *C. minus* CCAP 1537/5 and *C. megatetrastylus* ATCC 30936 (KC747718). **D.** *C. minutoidum* CCAP 1537/7. **E.** *C. kieliense*. **F.** *C. actinophorum* CCAP 1537/10 (JF298250). **G.** *Cochliopodium* sp. CCAP non-identified (JF298257). **H.** *Cochliopodium* sp. from rice (*Oryza sativa* cDNA clone OSIGCRA115O12, CT837767). **I.** *C. spiniferum* CCAP 1537/3 (AY775130). **J.** *C.* cf. *bilimbosum* (JF298252). **K.** *C. larifeili* CCAP 1537/8 (JF298253). Arrowheads mark a loop between helices E29-1 and E29-2 defining major clades of *Cochliopodium*. Asterisk in **C** marks a single extra nucleotide that distinguishes this structure from **B**.

Molecular identification of *C. minus* CPE and borders between species related to *C. minus*

Whereas identification and position of *C. plurinucleolum* n. sp. in the phylogenetic tree of *Cochliopodium* are relatively clear apart from incongruence of SSU rRNA and Cox1 phylogenies, identification of *Cochliopodium minus* CPE is more problematic, and

relationships between species comprising a *minus*-clade are obscure. Our results presented here reveal that the SSU rRNA sequence JF298257 was wrongly attributed to *C. minus* CCAP 1537/1A, and now we provide correct sequences of this strain. This error is due to the source of DNA material used to obtain the previous sequence, which was a genomic DNA sample available in the collection of amoebozoan DNA kept at the University of Geneva and designated as *C. minus* CCAP 1537/1A dating back to early 2000s. SSU rRNA, actin and Cox1 gene sequences were obtained from this DNA sample; sequences of the first two genes were published (Kudryavtsev et al. 2011) and attributed to *C. minus* CCAP 1537/1A as there was no reason to doubt the identity of the source DNA sample. However, accumulation of further sequences of amoebae similar to *C. minus* and *C. actinophorum* raised doubts about this identity. Therefore, a strain of *C. minus* 1537/1A was ordered from CCAP and sequenced again. The sequences obtained differ significantly from the one previously published by Kudryavtsev et al. (2011; JF298257). At the same time, the identity of the presently sequenced strain could be confirmed morphologically, whereas no morphological data that might help to identify the previously used DNA sample are available. In this case we have to conclude that the identity of the previously published sequence was erroneous due to a mislabeled DNA sample. Yet, this sequence robustly branches within *Cochliopodium* being most closely related to *C. actinophorum* (Figure 7). We therefore retain the published SSU rRNA gene sequence JF298257 and actin gene sequences JF298270-JF298272 (Kudryavtsev et al. 2011) in the database and designate them as *Cochliopodium* sp. to avoid confusion during subsequent studies. Correct sequences of a type strain of *C. minus* are now made available for analyses as the result of this study.

As mentioned above, all species of the *minus*-clade have virtually identical scales (Table 1), with the exception of *C. pentatrifurcatum*, whose scales are significantly different and mostly resemble those of *C. kieliense* (Tekle et al. 2013). Yet this species is virtually identical to *C. minus* based on sequence data. Another striking example is *C. megatetrastylus* that shares an identical scale structure and gene sequences with *C. minus*, while being significantly larger (up to 60 µm) and possessing a smoother outline without pronounced subpseudopodia (Anderson and Tekle 2013). Finally, *C. minutoidum* CCAP 1537/7 has virtually the same scales and only slightly differs from *C. minus* by the continuous peripheral hyaloplasmic sheet in most of the cells and the absence of cysts (Kudryavtsev 2006). *C. minutoidum* comprises the smallest members of this clade, despite the size range of this

species largely overlaps with that of the others. These differences may be due to variability between individual strains, yet *C. minutoidum* clearly differs from the remaining species on the molecular level (sequence difference of about 4 % in the SSU rRNA and about 7 % in Cox1, Supplementary Table 1, 3).

In the situation outlined, *Cochliopodium minus* CPE can hardly be assigned to any listed species on the basis of specific characters, as borders between species based on molecular data are only partly congruent with those that can be outlined from morphological and ultrastructural data. On the basis of gene sequences, we have to recognize that *C. megatetrastylus* seems to be the closest relative of *Cochliopodium minus* CPE that contradicts strongly the morphological dissimilarities of these species outlined above. *C. minus* strains CCAP 1537/1A and CCAP 1537/5 are the next closest relatives of *Cochliopodium minus* CPE; morphologically more similar to this strain than *C. megatetrastylus*. We therefore provisionally identify the studied amoeba as another strain of *C. minus*. At the same time our data clearly revealed that species borders in at least some of the phylogenetic lineages in *Cochliopodium* are far from being clear, and additional extensive studies on other closely related strains of the *minus*-clade, evaluating their morphological, ultrastructural and genetic diversity are necessary to clarify the taxonomy of this part of the *Cochliopodium* phylogenetic tree. To facilitate further studies, we provide a checklist of all described strains of *C. minus* and species closely related to it, with literature references and gene sequence data accession numbers where available.

"Golgi attachment" in *Cochliopodium* is a MTOC: electron microscopic evidence

Members of the genus *Cochliopodium* possess a characteristic cytoplasmic structure that looks like an electron-dense bar adjacent to a dictyosome usually referred to as "Golgi attachment" (Yamaoka et al. 1984, Kudryavtsev 2004, Kudryavtsev et al. 2004, Kudryavtsev et al. 2005, Kudryavtsev 2006). Previous results published by Kudryavtsev (2004) and Kudryavtsev et al. (2004) suggested that this structure is in fact a microtubule-organizing center (MTOC). However, we admit that the data on which this suggestion was based were rather ambiguous. Several published electron micrographs have shown elongated structures that were connected with one tip to the Golgi attachment and continuing into the cytoplasm (see Figure 13 in Kudryavtsev 2004 and Figure 8 in Kudryavtsev et al. 2004). These structures were cautiously interpreted as microtubules; therefore the Golgi attachment was suggested

to be a MTOC. Here we confirm this initial idea by using a fixation of better quality compared to the earlier works, clearly demonstrating that microtubules are radiating from the Golgi attachment into the cytoplasm (Figure 3F). Therefore, in addition to our recent data on *Stenamoeba* (Geisen et al. 2014), and earlier published results on Centramoebida (Bowers and Korn 1968), *Stygamoeba* (Smirnov 1996), *Gocevia* (Pussard et al. 1977), *Endostelium* (Bennett 1986), *Pellita* (Kudryavtsev et al., in press), and *Corallomyxa* and *Stereomyxa* (Benwitz and Grell 1971a, b, Grell and Benwitz 1978), *Cochliopodium* appears to be another lineage of amoebae where the presence of a cytoplasmic MTOC associated with dictyosomes is confirmed.

Diagnosis

Phylum Amoebozoa, Subphylum Lobosa, Class Discosea, Order Himatismenida, Family Cochliopodiidae, Genus *Cochliopodium*

***Cochliopodium plurinucleolum* n. sp.**

Length in locomotion 8.8 - 16.4 µm (mean 12.0 µm), breadth 10.2 - 14.8 µm (mean 12.0 µm), length: breadth ratio 0.7 - 1.3 (mean 1.0); shape variable, sometimes oval, triangular, fan-shaped, round or crescent; thin, hyaloplasmic veil surrounding the remaining cell smooth or slightly irregular; traversed by stripes with the hyaloplasm between slightly bulging out; hyaloplasm only in fast directed locomotion reduced in the posterior and sometimes replaced by one or more short trailing filaments; no sub-pseudopodia; one single spherical nucleus, 2.2 - 4.0 µm diameter (mean 3.4 µm) with one or more small, often decentralized nucleoli ranging in diameter from 0.6 to 1.2 µm (mean 1.0 µm). Stable double-walled cysts formed in older cultures. Scales made up of a circular, grid-like base plate with the mesh size of 0.015 µm, 4 stalks attached to the base plate converging towards a funnel-shaped apical part consisting of ca 15 radial spokes with very fine, poorly discernible concentric filaments between them. Diameter of a base plate 0.465 - 0.76 µm (average 0.627 µm), of the apical part, 0.445 - 0.577 µm (average 0.5 µm), height of a scale 0.185 - 0.3 µm (average 0.25 µm).

Etymology: The peculiar presence of several nucleoli inside the nucleus was eponymous for designating the species name "*plurinucleolum*"; type material: type culture is deposited with

CCAP (UK), accession number CCAP 1537/11; observed habitat: Grassland soil on Sardinia, Italy (40°46'N, 9°10'E).

Differential diagnosis: Due to its unique nuclear structure, *C. plurinucleolum* is only comparable to *C. clarum* Schaeffer, 1926. However, it differs from this species in being smaller, having a more irregular outline, and inhabiting soils in contrast to marine habitats.

Checklist for *Cochliopodium minus*-similar strains and species

C. minus Page, 1976 CCAP 1537/1A (type strain). References: Dyková et al. 1998, Kudryavtsev 2006, Page 1968 (as *Hyalodiscus actinophorus* var. *minor*), 1976, 1988

C. minus CCAP 1537/5 (strain perished in CCAP). References: Kudryavtsev 2006, Kudryavtsev et al. 2005

C. minus CPE (CCAP 1537/12). References: this study

C. barki Kudryavtsev et al., 2004 CCAP 1537/4 (type strain, perished in CCAP). References: Bark 1973, Kudryavtsev et al. 2004

C. megatetrastylus Anderson et Tekle, 2013 ATCC 30936 (type strain). References: Anderson and Tekle 2013; GenBank accession numbers: KC747718 (SSU rRNA), KC747719- KC747720 (Cox1)

C. minutoidum Kudryavtsev, 2006 CCAP 1537/7 (type strain). References: Kudryavtsev 2006, this study

C. pentatrifurcatum Tekle et al., 2013 ATCC 30935 (type strain). References: Tekle et al., 2013; GenBank accession numbers: KC247747 (SSU rRNA), KC489470 (Cox1).

Supplementary

Supplementary Table 1. Percentage difference between SSU rRNA gene sequences of different strains within the *Cochliopodium minus* / *C. minutoidum* / *C. megatetrastylus* / *C. pentatrifurcatum* clade

	C. minus CCAP 1537/1A	*C. minus* CCAP 1537/5	*C. pentatrifurcatum* ATCC 30935	*Cochliopodium* sp. CPE	*C. megatetrastylus* ATCC 30936
C. minus CCAP 1537/5	0.7				
C. pentatrifurcatum ATCC 30935	0.1	0.8			
Cochliopodium sp. CPE	0.9	0.9	0.9		
C. megatetrastylus ATCC 30936	0.9	0.4	0.8	0.1	
C. minutoidum CCAP 1537/7	3.9	4	4	3.9	4

Supplementary Table 3. Average percentage difference between Cox1 gene sequences of different strains within *Cochliopodium minus* / *C. minutoidum* / *C. megatetrastylus* / *C. pentatrifurcatum* clade based on nucleotide and amino acid (bold number in brackets) data

	C. minus CCAP 1537/1A	*C. minus* CCAP 1537/5	*C. pentatrifurcatum*	*Cochliopodium* sp. CPE	*C. megatetrastylus*
C. minus CCAP 1537/5	2.3 (0.5)				
C. pentatrifurcatum	Identical sequences	2.3 (**0.4**)			
Cochliopodium sp. CPE	2.8 (**0**)	2.7 (**0.4**)	2.7 (**0**)		
C. megatetrastylus	3.1 (**1**)	3 (**1.4**)	3 (**1**)	0.4 (**0.5**)	
C. minutoidum	6.8 (**0.7**)	7.3 (**1.1**)	6.7 (**0.7**)	6.7 (**0.7**)	7 (**1.6**)

Supplementary Table 2. SSU rDNA sequence motifs distinguishing the *Cochliopodium minus* / *C. minutoidum* / *C. megatetrastylus* / *C. pentatrifurcatum* clade; - : GAP position

Helix #	Start position (in *C. pentatrifucatum)*	Length	Seq. in *C. minutoidum* CCAP 1537/7	Seq. in *C. minus* CCAP 1537/1A	Seq. in *C. pentatrifurcatum*	Seq. in *C. megatetrastylus*	Seq. in *C.* CPE	Seq. in *C. minus* CCAP 1537/5
9-11	150	1	T	T	T	-	-	-
9-11	215	1	A	G	G	G	G	G
9-11	237	1	T	C	C	C	C	C
9-11	241	5-8	TAACAAAG	TTAAAG	TTAAAG	TTAAG	TTAAG	TTAAG
9-11	251	1	A	A	A	G	G	G
9-11	256	2-3	CTT	CTT	CTT	CT	CT	CTT
16	467	1	G	A	A	A	A	A
16	469	1	A	T	T	T	T	T
17	510	4	GGGA	AGGA	AGGA	AGGG	AGGG	AGGG
17/18	543	1	-	-	-	C	-	-
E23-1-7	775	1	A	A	A	T	T	A
E23-13	895	1	T	G	G	A	A	G
E23-13	906	1	G	C	C	T	T	T
24	1033	1	T	A	T	T	T	T
29	1146	6-7	TCTAAA	TTCAAA	TTCAAA	TTCAAAA	TTCAAAA	TTCAAAA
E43	1489	1	C	T	T	C	C	C
E43	1495	2	AG	AA	AA	GA	GA	GA
E43	1507	1	T	T	T	C	C	C
E43	1513	1	A	G	G	A	A	A
E43	1559	1	T	C	C	T	T	C
E43	1578	2	GA	AA	AA	GG	GG	GG
E43	1588	1	T	C	C	T	T	T
E43	1612	1	T	C	C	T	T	T
43	1624	1	T	T	T	T	T	A
49	1979	1	T	A	A	A	A	G
49	2000	1	T	C	C	T	T	C
49	2007	1	A	A	A	A	G	A

Part 1 – Chapter 3

Expansion of the 'reticulosphere': Diversity of novel branching and network-forming amoebae helps to define Variosea (Amoebozoa)

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Abstract

Amoebae able to form cytoplasmic networks or displaying a multiple branching morphology are found across the eukaryote tree of life but remain very poorly studied. We sequenced the small-subunit ribosomal RNA gene of 14 new amoeboid isolates, 13 of which are branching or network-forming amoebae (BNFA). Phylogenetic analyses showed that these isolates are all grouping within Variosea, a poorly-known and weakly-defined group within Amoebozoa. They are resolved into six lineages corresponding to distinct new morphotypes, and we describe them as new genera *Angulamoeba* (type species *Angulamoeba fungorum* n. gen., n. sp), *Arboramoeba* (type species *Arboramoeba reticulata* n. gen., n. sp), *Darbyshirella* (type species *Darbyshirella terrestris* n. gen., n. sp), *Dictyamoeba* (type species *Dictyamoeba vorax* n. gen., n. sp), *Heliamoeba* (type species *Heliamoeba mirabilis* n. gen., n. sp), and *Ischnamoeba* (type species *Ischnamoeba montis* n. gen., n. sp). We also isolated and sequenced four additional variosean strains, one belonging to the genus *Flamella*, one related to *Telaepolella tubasferens*, and two members of the cavosteliid protosteloid lineage. Using the sequences from our new isolates and the members of eleven previously characterized variosean lineages, we identified a further 104 putative variosean environmental clone sequences in Genbank, comprising up to 14 lineages that may prove to represent additional novel morphotypes when characterised. We show that BNFA are phylogenetically widespread in Variosea; they are morphologically very variable, both within and between lineages, and in some cases this variability is influenced by food source.

Introduction

Naked heterotrophic reticulose and branching amoebae are characterized by more or less thin cytoplasmic extensions from, or as part of, their cell bodies. The appearance of the cytoplasmic extensions varies considerably. Some lineages are able to fuse (anastomose) cytoplasm from different parts of their own cells or different cells, whereas others do not do this. While this characteristic is known for some taxa, in general the distinction is difficult to make because most lineages are highly understudied. Some highly branching amoebae are not known to form networks (e.g. *Mesofila*; Bass et al. 2009), whereas others appear to form networks under some conditions but perhaps not others. Where networks occur they can be very fine, with or without distinct cell bodies (e.g. *Filoreta* and *Reticulamoeba*; Bass et al. 2009, 2012), or more compact with proportionally smaller lacunae (e.g. *Leptomyxa*, *Protomyxa*, and some vampyrellids; Berney et al. 2013; Goodley, 1915; Hess et al. 2012; Rhumbler, 1904; Smirnov et al. 2008), sometimes resembling 'sheets' of cytoplasm within which lacunae may occur, e.g. *Thalassomyxa* (Berney et al. 2013). In these cases lacunae are often formed by cleavage of the cytoplasm into separate streams, so there is no clear distinction between cell body and cytoplasmic network. A large range of intermediate morphotypes can be found between these main forms.

Despite their morphological distinctiveness, these branching and network-forming amoebae (BNFA) are remarkably poorly known. Ultrastructural and molecular phylogenetic studies have revealed that BNFA can be found in many places across the eukaryotic tree of life within major groups such as Amoebozoa, Rhizaria, and Stramenopiles. However there are also many such amoebae described in the literature for which sequence data are not available yet (e.g. Adl et al. 2012; Lee et al. 2000; Bass et al. 2009; 2012). Their range of food items is strikingly wide, including bacteria, other protists, diatoms, algae, fungi, and even small metazoans. In other cases feeding behaviour has been elusive, further emphasizing the interesting nutritional modes shown by BNFA. It is thought that cytoplasmic networks enable BNFA to be more efficient than many heterotrophic protists in finding and ingesting surface-attached prey, and exploiting food sources in interstitial spaces in particulate sediments (Rogerson et al. 1996, Butler and Rogerson 1997). Networks are often very thin and flat and maximize the cell-surface to volume ratio (i.e. foraging area), making them theoretically more energetically efficient in certain microhabitats in comparison with larger, rounder cells. Per cell volume they have been

shown to consume bacteria more quickly than non-reticulose cells, and there is evidence that some lineages can digest bacteria or other prey such as diatoms within the pseudopodia themselves (Grell 1994, 1995, Rogerson et al. 1996)

The current state of knowledge of BNFA suggests that, although they can be found across the whole eukaryotic tree of life, there are only a small number of lineages with these morphological characteristics, and that they are relatively rare in the environment. However, this perception is influenced by some important factors: the cells are easily disrupted and broken by standard sample collection methods, they are often very slow growing in culture or do not thrive in standard laboratory culturing conditions (which usually favour relatively fast-growing bacterivores and ecological generalists). They are not readily seen or recognized due to lack of dispersed expertise and because they are rarely the focus of experimental work. Rogerson et al. (1996) found that although they were not as numerous as other heterotrophic protists, direct counting methods suggested they were an order of magnitude more abundant in some coastal marine sediments than culturing-based estimates allowed. Feest and Campbell (1986) found BNFA in most soils they investigated, and correlated abundance of BNFA and dictyostelids with low levels of take-all disease. Branching amoeboid morphologies may also confer nutritional advantages for parasites and predators of relatively large organisms: *Grellamoeba* has recently been identified from fish kidney tissue (Dyková et al. 2010a), the stramenopile labyrinthulids include parasites of seaweeds and green plants, *Protogenes* and *Protomyxa* are particularly associated with the seaweeds *Gelidium* and *Bryopsis* respectively (perhaps ectoparasitically), and some vampyrellids enter host algal cells in the process of consuming their contents (Hess et al. 2012). It is perhaps significant that some BNFA taxa are evolutionarily very close to important parasite groups, for example vampyrellids to phytomyxids (Berney et al. 2013) and *Filoreta* and *Gromia* to Ascetosporea (Bass et al. 2009a).

The large physical extent / reach of the cells, the ability to take up and metabolize prey at relatively high rates, and the wide prey range suggest that BNFA may play diverse, distinct, and significant ecological roles. We provide new evidence for this by revealing previously unknown but apparently common variosean (Amoebozoa) BNFA lineages from soils that represent novel morphotypes and are also phylogenetically very diverse.

Materials and Methods

Sample collection, culture isolation, microscopy, and DNA extraction

Localities and dates of collection of samples for isolation are given in Table 1. In the culture lab, samples were placed in Volvic mineral water (Danone, Paris, France) or Prescott-James medium (Page 1991), enriched with 0.15 % wheat grass (Weizengras, Sanatur GmbH, Singen, Germany) and grown at room temperature and / or in a 16 °C incubator. Dishes were checked regularly for the presence of BNFA. Amoebae of interest were subcultured and when possible purified by serial dilution until devoid of any other eukaryotic organism. For some observed morphotypes, a subset of dishes was supplemented with distinct selected food sources (bacteria alone, baker's yeasts, or a freshwater diatom *Achnanthes* sp.) to see if it had any influence on the growth rate and morphology of the amoeba's active trophic stage. For differential interference contrast (DIC) light micrographs, we used either a Nikon (Tokyo, Japan) Eclipse 80i microscope with x 40 (NA 0.6) and x 60 (NA 1.0) DIC water immersion lenses, or a Nikon Eclipse 90i with x 63 (NA1.0) DIC oil immersion lenses. Phase contrast micrographs were taken from a Leica (Wetzlar, Germany) DM IRB microscope or a Nikon Eclipse TS100 microscope. Images were recorded on a Sony (Tokyo, Japan) HD HDR-XR155 camcorder, a HDV 1080i Handycam®, or Nikon DIGITAL SIGHT DS-fi1 camera. Frames were captured using the software PMB version 5.2 (Sony) or Final Cut Express HD 3.5.1 (Apple Inc., Cupertino, CA, USA). DNA was extracted either early on from mixed cultures where a single BNFA morphotype was observed and had reached a reasonable density or, whenever possible, later on from purified clonal cultures. In some cases, this was done by picking individual cells (20 to 50) with a micropipette. DNA extraction was then performed either by using the UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the Maximum Yield Protocol, or by placing the cells in guanidine thiocyanate buffer and using a protocol described in Sambrook et al. (1989). In other cases, total DNA extractions were performed on the whole culture. Most of the culture medium was decanted off, and using a sterile scraper, cells were collected from the bottom of the culture dish. Total DNA was then extracted from the pellet of organic material using the UltraClean™ Soil DNA Isolation Kit as above.

Ethics statement: No specific permission or permits were required for the described field studies. The sites were not privately owned or protected in any way and were fully open to public access. No endangered or protected species were involved in this study.

Amplification and sequencing of the SSU rDNA

The complete or nearly complete SSU rDNA sequence was amplified from all isolates except F2. For the latter we only amplified a 644 bp fragment spanning the variable regions V4 and V5, which turned out to be more than 98 % identical to the sequence of isolate 23-6A. SSU rDNA sequences were obtained in a single or up to three overlapping fragments, using different possible combinations of universal (eukaryotic) and lineage-specific primers. PCR amplifications were done in a total volume of 30 µl with an amplification profile typically consisting of 35 cycles with 30'' at 95 °C, 30'' at 56 °C, and 90'' at 72 °C, followed by 5' at 72 °C for the final extension. PCR products of clonal strains or individually picked cells were quality controlled for the expected length of amplification product on a 1.5 % TAE agarose gel and subsequently purified by adding 0.15 µl Endonuclease I (20 U * μl^{-1}, Fermentas, St. Leon-Rot, Germany), 0.9 µl Shrimp Alkaline Phosphatase (1 U * μl^{-1}, Fermentas, St. Leon-Rot, Germany) and 1.95 µl H_2O. The resulting mixture was incubated for 30' at 37 °C, followed by 20' at 85 °C. In non-clonal cultures, bands of the appropriate length were excised, and cleaned following the protocol of the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR amplicons were sequenced directly, or when necessary cloned into StrataClone™ SoloPack® Competent Cells using the StrataClone™ PCR Cloning Kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA). White colonies were screened using the primers M13for (5' - CGT TGT AAA ACG ACG GCC AGT - 3') and M13rev (5' - CAC AGG AAA CAG CTA TGA CCA - 3'). Positive PCR products were cleaned using a polyethylene glycol (PEG) protocol: for 20 µl PCR reactions, 20 µl of a 20 % PEG * 2.5 M^{-1} NaCl mixture was added to each tube. The tubes were mixed by vortexing and incubated for 30' at 37 °C, then centrifuged at 3,000 rpm for 30' to pellet the PCR products. Supernatant was discarded by pulse-spinning the inverted tubes at 600 rpm. The pellet was then washed with ice-cold 75 % ethanol, spun for ten minutes at 3,000 rpm, again inverted and pulse-spun to remove the supernatant. The ethanol wash was repeated; the PCR pellet was re-suspended in de-ionised water, and stored at –20 °C. Sequencing was performed with the Big Dye Terminator v1.1 Cycle Sequencing Kit, and analysed with an ABI-3730xl DNA sequencer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA).

BLASTn searches and construction of the sequence datasets

The new SSU rDNA sequences were edited and aligned manually using the BioEdit software (Hall 1999), following the secondary structure model proposed by Wuyts et al. (2000). Visual

screening of the sequences in search of sequence signatures, BLASTn searches (Altschul et al. 1990) against the NCBI GenBank database using default parameters, and preliminary phylogenetic analyses performed on a large dataset including a wide range of eukaryotes congruently suggested that our isolates all belong to class Variosea within phylum Amoebozoa. A first dataset was constructed to confirm this phylogenetic placement of our isolates; it contains 1,350 unambiguously aligned positions and 115 taxa, and includes all our isolates, representatives of all morphologically identified lineages within class Variosea and of all other morphologically defined higher-order taxa of lobose amoebae, and ten outgroup sequences (belonging to opisthokonts and apusomonads). The GenBank accession numbers of all sequences used in this dataset are given in Supplementary Table 1. The highly divergent sequences of Macromycetozoa (cellular and acellular slime moulds) and Archamoebae (entamoebids and pelobionts) were excluded from the analyses (see Discussion).

Exhaustive BLASTn searches (default parameters) were then performed using both our new and existing Variosea SSU rDNA sequences to seed searches to identify all Variosea clone sequences from environmental libraries present in the NCBI GenBank database. Further BLASTn searches were performed using manually truncated sequences (both at the 5’ and 3’ ends) as queries. This allowed retrieval of shorter clone sequences that would escape identification when using complete sequences as queries because of lower overall similarity scores compared to more distantly related but full-length sequences. All identified Variosea sequences were used as additional queries in further rounds of BLASTn searches until no more new unambiguous Variosea sequences could be found. Clones shorter than 500 bp were not considered. The presence of sequence chimeras was assessed by visual screening of the alignment in search for contradictory sequence signatures, and potential chimeric clones were confirmed as such by distance analyses based on different subsets of unambiguously aligned regions (as described in Berney et al. 2004). In total, we identified 20 environmental clones as chimeric out of a total of 114. A second dataset was then constructed to refine phylogenetic relationships and highlight lineage diversity within Variosea. It contains 1,375 unambiguously aligned positions and 100 taxa, and includes all our isolates, most morphologically identified members of class Variosea, a selection of identified Variosea environmental clones, and four outgroup sequences from other Amoebozoa lineages. Only environmental clones longer than 1,000 bp and spanning both the V4 and V7 variable regions of the SSU rDNA were included. Whenever multiple highly similar clones from a same environmental library had been identified, only one was kept to

avoid over-populating the figure. Missing data in partial environmental sequences were encoded as such (Ns). The non-Variosea part of any chimeric sequence included in this dataset was also encoded as missing data.

Maximum likelihood and Bayesian phylogenetic analyses

All phylogenetic analyses for both datasets were performed on the CIPRES server (Miller et al. 2010). Maximum likelihood (ML) analyses (Felsenstein 1981) were performed with the program RaxML version 7.6.6 (Stamatakis 2006), using the GTRGAMMA model with 25 rate categories. All necessary parameters were estimated from the datasets. The best ML topology was selected from 200 inferences with distinct maximum parsimony starting trees. The reliability of internal branches was assessed with the bootstrap method (Felsenstein 1985) using 2,000 replicates. In addition, Bayesian analyses were performed with MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) using a GTR + gamma model with 8 rate categories. Two runs of four simultaneous chains were run for 2,500,000 generations (heat parameters set to default), and trees were sampled every 100 generations. For each run 25,000 trees were sampled, 5,000 of which were discarded as the burn-in. Posterior probabilities of the branching pattern were estimated from the 40,000 remaining trees and mapped onto the ML tree when present. For both datasets the Bayesian posterior probability 50 % majority-rule consensus tree was fully compatible with the corresponding ML tree.

Results

Morphology and phylogenetic placement of our new isolates

We isolated 18 strains of amoebae (most with a BNFA morphotype), information about which is summarised in Table 1, and obtained SSU rDNA sequences from all of them.

Table1. Origin of the 18 new variosean strains isolated in this study; NA: Information not available

Isolate	Taxonomic identity	Collection date	Geographic origin	Sample type	Phylogenetic position
Tib190	*Telaepolella* sp.	2011	Mila mountain, Tibet	High altitude grassland soil	lineage V07
FN414	*Flamella* sp.	NA	NA	NA	lineage V08
M71	*Schizoplasmodiopsis* sp.	2011	Veluwe, the Netherlands	Grassland soil	lineage V11
F3	undetermined cavosteliid	2012	Flörsheim, Germany	Grassland soil	lineage V11
Tib85	*Ischnamoeba montis*, gen. et sp. nov.	2011	Sejila Mountain, Tibet	High altitude grassland soil	lineage V12
F4	*Ischnamoeba* sp.	2012	Flörsheim, Germany	Grassland soil	lineage V12
FN352	*Ischnamoeba* sp.	NA	NA	NA	lineage V12
Tib177	*Darbyshirella terrestris*, gen. et sp. nov.	2011	Sejila Mountain, Tibet	High altitude grassland soil	lineage V13
Esthw	*Darbyshirella* sp.	NA	Oxford, England, UK	NA	lineage V13
M68	*Darbyshirella* sp.	2011	Veluwe, the Netherlands	Grassland soil	lineage V13
JDgam	*Darbyshirella* sp.	NA	Oxford, England, UK	soil from a garden?	lineage V13
M77	*Darbyshirella* sp.	2011	Veluwe, the Netherlands	Grassland soil	lineage V13
WalEn	*Dictyamoeba vorax*, gen. et sp. nov.	NA	Gregynogg, Wales, UK	mixture of soil, moss and lichens from garden	lineage V14
M134	*Heliamoeba mirabilis*, gen. et sp. nov.	2011	Veluwe, the Netherlands	Grassland soil	lineage V15
Tib182	*Arboramoeba reticulata*, gen. et sp. nov.	2011	Sejila Mountain, Tibet	High altitude grassland soil	lineage V16
23-6A	*Angulamoeba fungorum*, gen. et sp. nov.	NA	Oxford, England, UK	NA	lineage V17
F2	*Angulamoeba* sp.	2012	Flörsheim, Germany	Grassland soil	lineage V17
FN806	undetermined angulamoebid	NA	NA	NA	lineage V17

An appropriate sequence dataset was constructed to refine the phylogenetic placement of our isolates with respect to morphologically characterised amoebozoan taxa. The results of our analyses are shown in Figure 1 and confirm that all isolates belong to Variosea. The isolates fall into nine main lineages. Some were closely related to previously characterised lineages, congruently with their morphology: FN414 is a *Flamella* sp., Tib190 is related to and resembles the recently described species *Telaepolella tubasferens* (Lahr et al. 2012), F3 is very close to *Schizoplasmodiopsis vulgaris* (EF513180) within the cavosteliid lineage, and M71 branches at the base of that lineage and is likely to represent a novel or previously unsequenced species within or near the genus *Schizoplasmodiopsis*. The 14 other isolates were not closely related to known taxa and form six new variosean lineages with novel, distinct morphotypes. We describe below six new genera for these lineages, with one isolate designated as a type species in each one. One of the novel lineages, *Angulamoeba* n. gen., of which we isolated three strains, is highly divergent from all described varioseans in terms of SSU rDNA sequence (Figure 1). Another substantial, but short-branched clade was identified by our isolates, comprising two lineages - one with five reticulose isolates (*Darbyshirella* n. gen.) and one with three branching but not reticulose isolates (*Ischnamoeba* n. gen.). The remaining three isolates branch individually as three additional lineages; one of them is filose / ramose (M134, *Heliamoeba* n. gen.) while the other two are reticulose (WalEn, *Dictyamoeba* n. gen. and Tib182, *Arboramoeba* n. gen.).

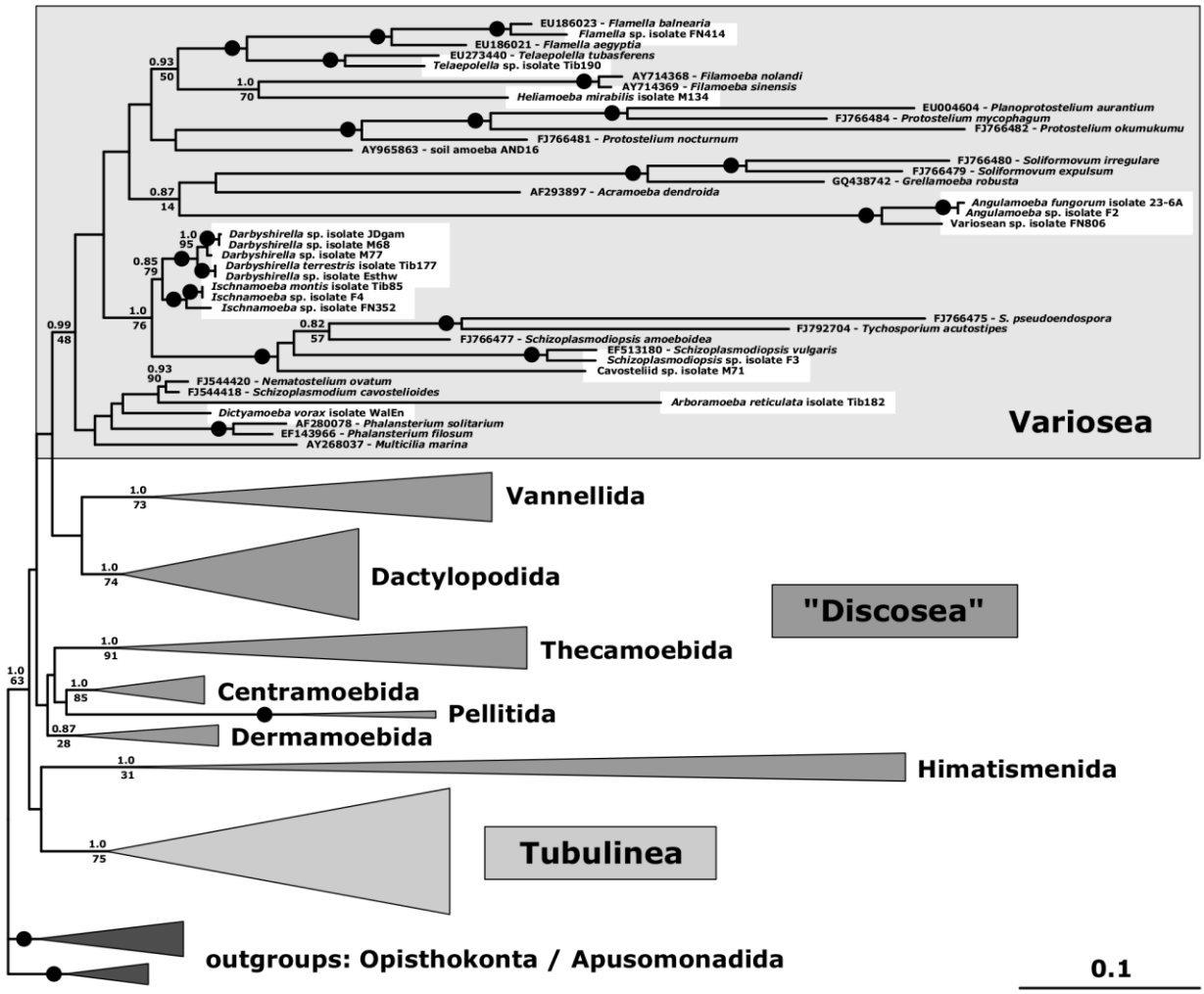


Figure 1. Maximum likelihood tree showing the monophyly of Variosea as sister to the orders of the class Discosea inside the supergroup Amoebozoa; new taxa described in this study highlighted in white.

With the exception of FN414 (*Flamella* sp.) and M134 (*Heliamoeba* n. gen.; Figure 5), all of our isolates were markedly branching (ramose) or network-forming (reticulose). None were seen to produce fruiting bodies of any kind, despite some being maintained in variable culture conditions for up to several years. However at least two (F3 and M71) clearly belong to a protosteloid lineage (the cavosteliids) where described taxa can produce fruiting bodies. Morphological variation between individual cells within each isolate is typically very high, and can exceed that between genetically different isolates within a lineage or even that between distantly related lineages, especially in younger, smaller cells. Nevertheless, by observing many cells within an isolate it becomes clear that each lineage corresponds to a distinct morphotype and is distinguishable from all others based on the shape, size, and / or organisation of the cells and networks.

The reticulose genera *Darbyshirella* (five isolates), *Dictyamoeba* (isolate WalEn), and *Arboramoeba* (isolate Tib182) all form indefinitely large networks. In *Darbyshirella* (Figure 4) the branches of the network are relatively long and thin and regularly spaced, and the network is formed through what appear to be random anastomoses between branches. The density of the network is relatively regular but with lacunae of various sizes, and often no clear direction of movement can be determined. In cases where a clear direction of movement can be observed, the network does appear denser at the anterior front. In *Dictyamoeba* young reticulose trophozoites have quite thin branches, with one or few non-branching, non filose posterior endings and a visibly denser branching / reticulose area at the front; they resemble small networks of the genus *Darbyshirella*. As the network grows in presence of suitable food (yeasts seem to be preferred) the branches become markedly thicker and more evenly spaced and the network can then grow in all directions, with many “terminal” areas where denser, fine branching extensions gradually replace the network. *Arboramoeba* (Figure 6) exhibits the densest and most impressive networks and in older cultures always looks “tree-like”, with one or few non-branching, non filose posterior endings and an extremely branching and reticulose anterior front forming a wide, non-permeable frontier, significantly denser than the posterior part of the network. These three morphotypes are not directly related according to our phylogenetic analyses.

The branching genera *Ischnamoeba* (three isolates) and *Angulamoeba* (three isolates) were never observed to become reticulose. In both morphotypes the branching pattern usually remains quite simple, more so than in the similar genus *Acramoeba* (Smirnov et al. 2008). In *Angulamoeba* (Figure 7), the cell body separates into three main branches on average (rarely more than four), which can sometimes (but rarely) be further subdivided. The branches are thicker at the base and quite short, ending with filose extensions. In *Ischnamoeba* (Figure 3), branches are thinner overall on average, more commonly further subdivided, and more randomly distributed, not all originating from a common central point as in *Angulamoeba*. They vary more in size and length but also end in filose extensions. Finally, isolate Tib190 displays many of the morphological characteristics of *Telaepolella tubasferens* (ATCC 50593), with young amoebae resembling *Flamella* spp. while older ones become much larger, branching forms, sometimes reticulose with lacunae.

Like many Variosea, our new isolates strongly vary in size, often even within clonal cultures. The number of nuclei per cell varies widely. Directed movement as present in most representatives within the supergroup Amoebozoa and even in the related variosean genera *Filamoeba* and *Flamella* was not easily visible in most of our BNFA isolates because of its extreme slowness; only in a time series of micrographs taken over several hours does it become clear that these amoebae can display directed movement. A shared feature of all isolates was the presence of fine filose pseudopodia, typical for amoebae in the class Variosea but not generally across Amoebozoa (Lahr et al. 2012). These pseudopodia can be observed all around the cell but are typically denser at the end of branches or at the anterior front of the reticulose genera. Movement could only be observed when the filose pseudopodia attached or detached from the substratum, while locomotion of entire trophozoites was very slow and could usually not be observed microscopically. In contrast, intracellular activity was high as granuloplasm movement and vacuolar activity was permanently visible. All isolates formed cysts in older cultures, which often varied strongly in size and shape.

Variosea: phylogeny and lineage diversity

Five species of the newly described *Darbyshirella* have been isolated and described as the first described taxon in a clade including a single environmental sequence (JN825696). All were morphologically indistinguishable. Three *Ischnamoeba* cultures formed the sister genus separated only by little genetic difference, but were morphologically smaller and therefore distinguishable from *Darbyshirella*. *Dictyamoeba* branches separately from all of our other isolates in a heterogenous short-branch group including *Multicilia* and the schizoplasmodiids (Lahr et al. 2011). Similarly, *Arboramoeba* forms a long-branch in this clade. *Heliamoeba* forms a long-branch sister genus to *Filamoeba* (Figures 1 + 2).

Using existing variosean sequences plus our new sequences as BLASTn search seeds against NCBI GenBank we recovered further variosean sequences. Phylogenetic analysis of all these sequences resulted in 31 phylogenetic clusters, based on shared morphotype where known and / or phylogenetic clustering with bootstrap support > 50 % (Figure 2). Only 16 of these 31 lineages have a known morphological identity. Of the remaining 15 we provide the first morphological data for six: lineage 12 – *Ischnamoeba*, lineage 13 – *Darbyshirella*, lineage 14 – *Dictyamoeba*, lineage 15 – *Heliamoeba*, lineage 16 – *Arboramoeba*, lineage 17 – *Angulamoeba*. Figure 1, Figure 2 and Supplementary Figure 2 show phylogenetic analyses for these including

more identified sequences placing in Variosea which are shown in Supplementary Table 1.

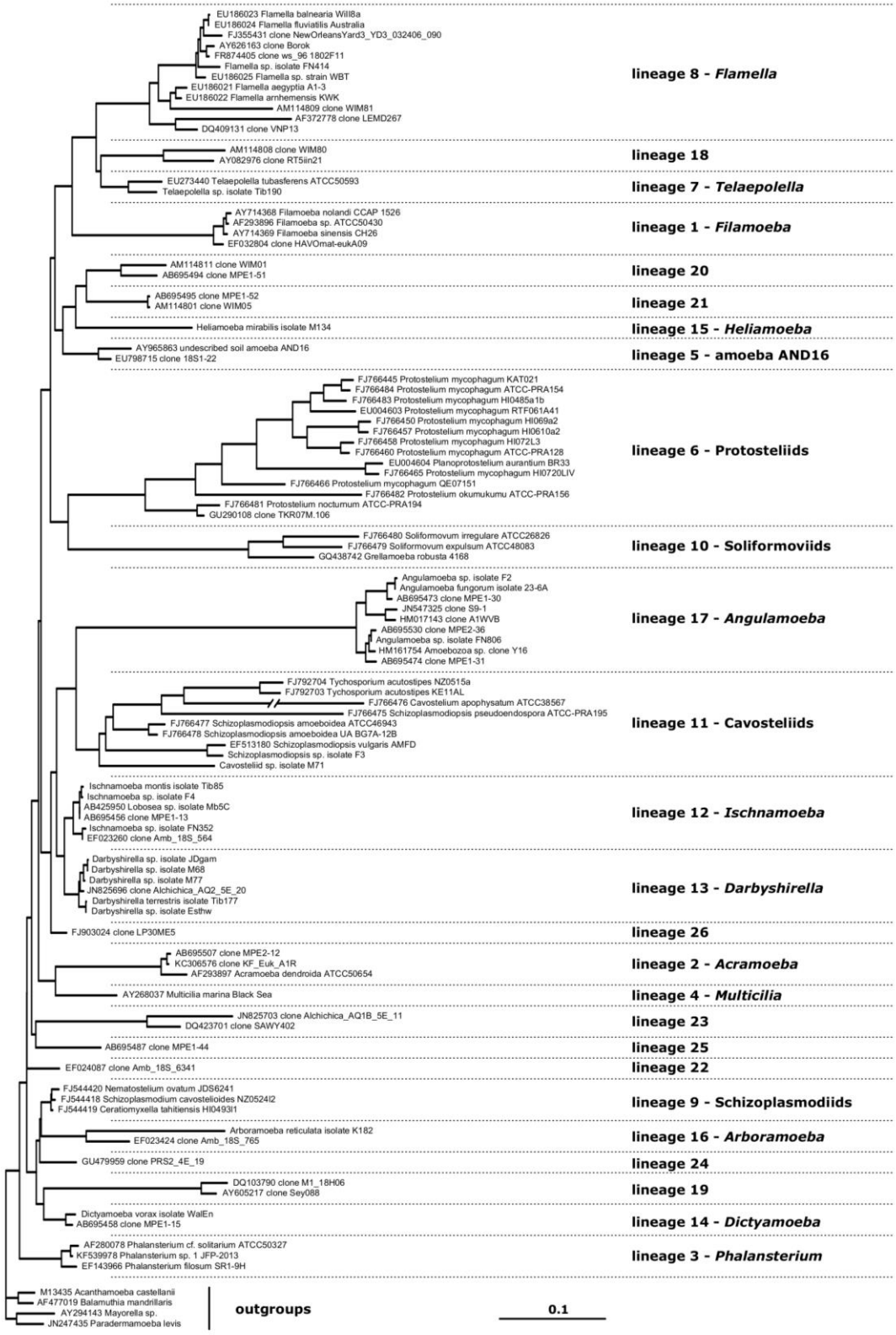


Figure 2. Maximum likelihood tree showing major variosean lineages where clones including both marker sequences in V4 and V7 were represented.

The length of the SSU rDNA sequences of our new isolates ranged from around 1,850 bp to almost 2,100 bp. This is above the average length of the SSU rDNA for most eukaryotes (typically in the range 1,750 - 1,850 bp), and in all isolates we observed an expansion of helix 43 in the variable region V7. The presence of a highly specific secondary structure signature associated with this expansion of helix 43 (see below) suggested that all isolates belong to a diverse, morphologically heterogeneous clade known as Variosea, which is part of the supergroup Amoebozoa. All isolates of the genera *Ischnamoeba* and *Darbyshirella* also exhibited expansions in the variable region V4 of the SSU rDNA. This region (that has been defined as a universal "pre-barcode" for protists; (Pawlowski et al. 2012) has an average length of 380 - 400 bp in eukaryotes (and in all of our other isolates) but is about 450 bp long in the *Ischnamoeba* isolates, and 530 - 570 bp long in the *Darbyshirella* isolates.

Diagnosis

Genus descriptions

V12 *Ischnamoeba* n. gen. (Figure 3)

Uninucleated soil naked amoeba extending over a distance from 37 to 142 µm with narrow cell body width of often only 2 µm (up to 12 µm in rarely found non-elongated amoebae). Cells usually thin, extended and flat, showing no well-defined cell body. The nucleus containing part often only slightly broadening. Whole cells often bent, but little branching. Branching more pronounced in condensed cells or in condensed parts of individual cells, usually not at intermediate, connecting parts of the cell body. Filose pseudopodia produced almost exclusively at condensed and distal parts of cells and more pronounced in condensed organisms. Pseudopodia often branching into subpseudopodia. Never reticulate.

Movement of entire cells not directly observable. No distinct floating form. Cysts round, sometimes oval, from 6 to 16 µm in diameter. Exclusively bacterivorous.

Ischnamoeba montis Tib85 as type species; with "85" as a type species. Etymology: from Greek "Ischnos" (thin).

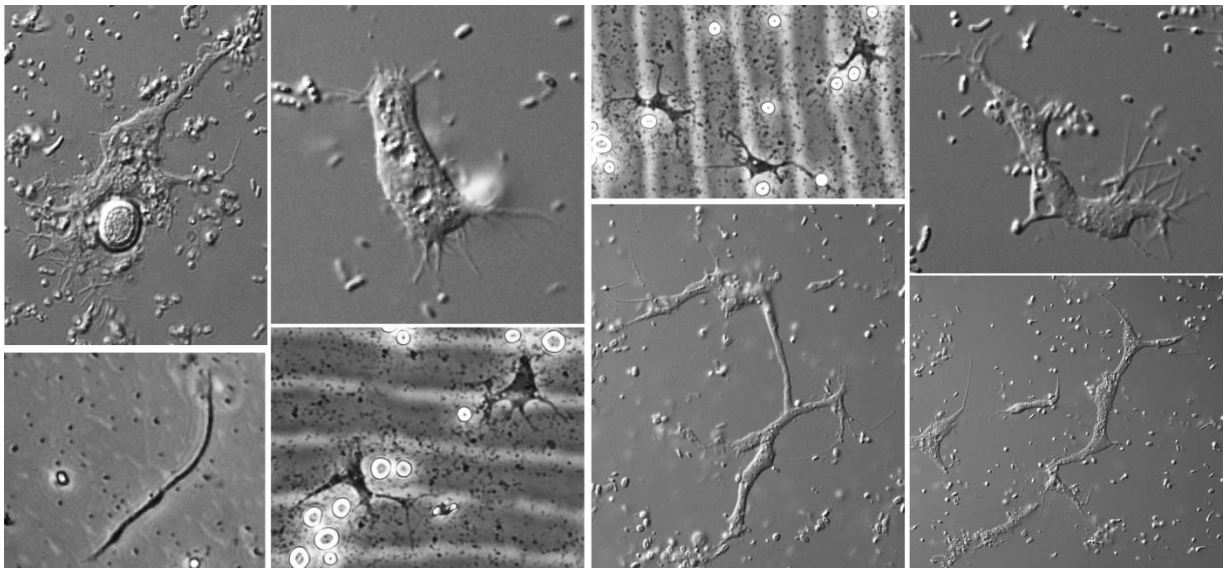


Figure 3. Pictures illustrating different morphological adaptions of *Ischnamoeba montis.*

V13 - *Darbyshirella* n. gen. (Figure 4)

Multinucleated soil naked amoeba extending over a distance of more than 600 µm. Cell body usually extended over the entire cell with the exception of pseudopods. Usually narrow cell body especially in extending parts, often below 2 µm, while more condensed parts reached width of 34 µm. Pronounced branching pattern across the entire cells with branching pronounced in condensed parts, also containing the majority of (sub)pseudopodia. Pseudopodia and branches usually formed when cells condensed in the anterior extending regions, resulting in up to three new branches. Posterior usually pointed with no or few pseudopodia and no branching. Grade of reticulosity strongly differing between amoebae, but branching parts of the cell body, even distal parts fusing on contact. Elongated amoebae consequently less reticulate than more condensed amoebae. Vacuoles in high numbers and activity present in entire cell.

Movement of entire cells not observable under the microscope. No distinct floating form was observed. Cysts with two clearly separate cell walls varying both in size and shape. Sizes from 9 to 42 µm in diameter and shape spherical, oval or bean shape. Bacterivorous.

Darbyshirella terrestris Tib177 as type species in honour of John F. Darbyshire.

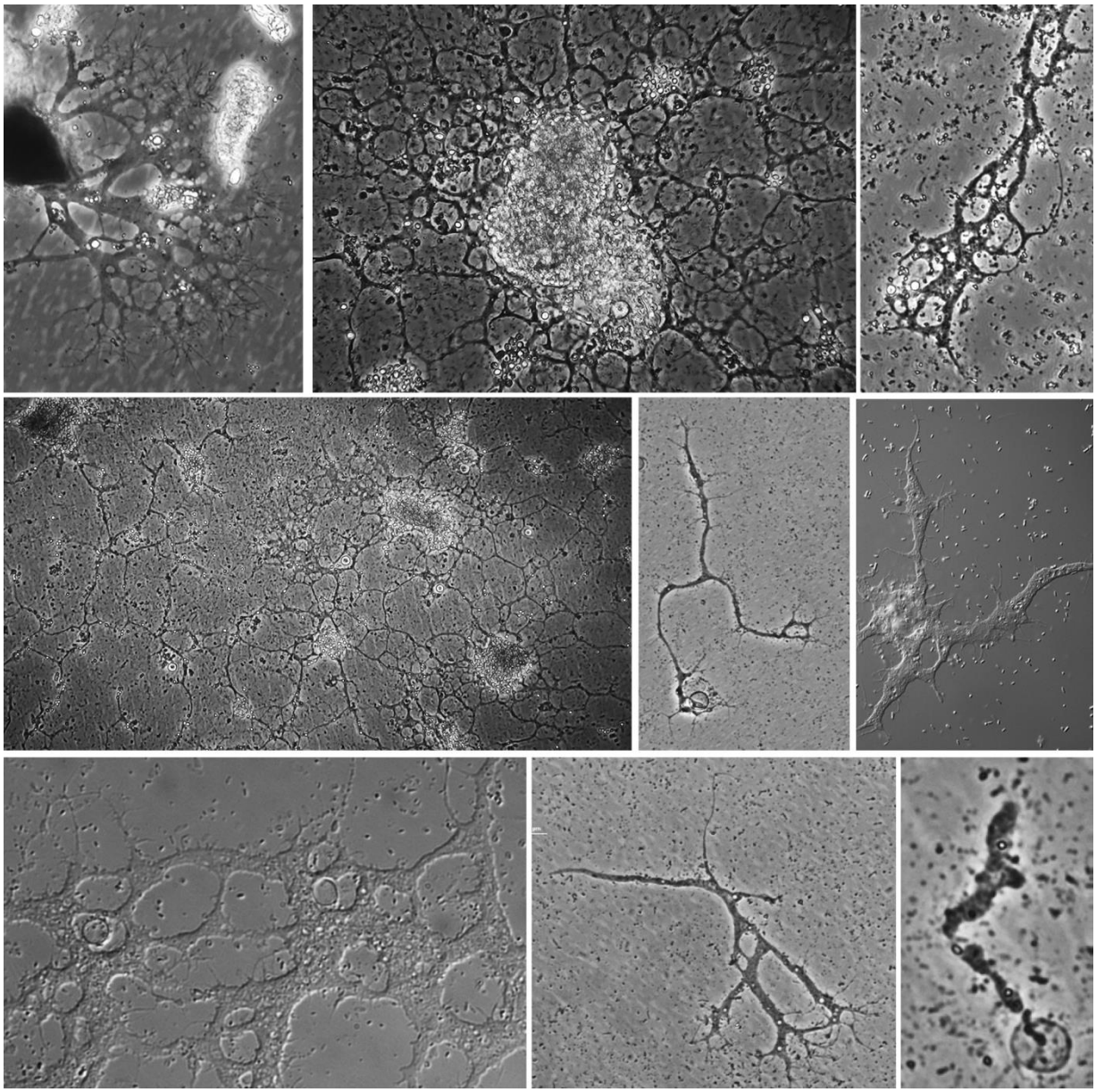


Figure 4. *Darbyshirella terrestris* showing high morphological plasticity.

V14 – *Dictyamoeba*, n. gen.

Very large (several 100 µm across), flattened, highly branching and reticulate naked amoebae with slender, pointed, sometimes branched subpseudopodia, lacking cilia or flagella. Simple life cycle including trophozoite and cyst stages. Trophozoites moving too slowly to be seen in light microscopy; main cell body multiply branched and anastomosing even in its simplest form (up to 100 µm), which can grow into gigantic networks (several hundred µm) with intersecting segments of varying width and numerous terminal branching areas; abundant fine subpseudopodia concentrated mostly at the extremity of lateral and terminal branches, especially in complex networks, but can be formed anywhere around the cell body in simpler forms; numerous contractile vacuoles observed everywhere in the network, but more

commonly at intersections of the main branches. Cysts of varying sizes and shapes, the simplest ones rounded and about 35 µm in diameter, the larger ones irregular in shape and > 300 µm across; at encystment a large network contracts simultaneously in different areas, leading to the formation of very many cysts of all possible sizes and shapes, the position of which mirrors the original layout of the network. Type species: *Dictyamoeba vorax* n. sp.

Etymology: from the Latin "vorax" (insatiable) in reference to the very rapid rate of consumption of yeasts when available, and "rete" (network) in reference to the very large and complex reticulate form adopted by the amoeba in presence of abundant food.

V15 – *Heliamoeba* n. gen. (Figure 5)

Binucleated soil naked amoeba reaching up to 168 µm from the most distal parts. Single, clearly distinct cell body always present with maximum length of 58 µm (width 10 µm), with pronounced pseudopodia at usually at anterior and posterior ends, contributing mostly to total cell dimensions. Very rarely and little branching at edges of the cell body. Pseudopodia, often branching into subpseudopodia present in anterior and posterior regions of cells if fully extended, everywhere around the main cell body in condensed cells. When disturbed under a coverslip, cells often condense, form filose pseudopodia all around the main cell body and eventually produce short, round, lobose-like extensions all around the main cell body formed. Never reticulate.

Slow movement of entire cells observable under the microscope. For movement, cell bodies narrowed at the posterior and widened at the anterior, where new pseudopodia are formed. A distinct floating form with the cell body condensed in a sphere and numerous filose pseudopodia radially extending is produced if detached from substratum. Amoebae rapidly form an extended shape when re-attaching. Cysts similar; round, with two clearly separate cell walls ranging from 10 to 15 µm in diameter. Feeding on bacteria, fungi and small flagellates.

Heliamoeba mirabilis M134 as a single type species; Etymology: from Greek “helios” (sun) due to the often sun-like morphology of trophozoits

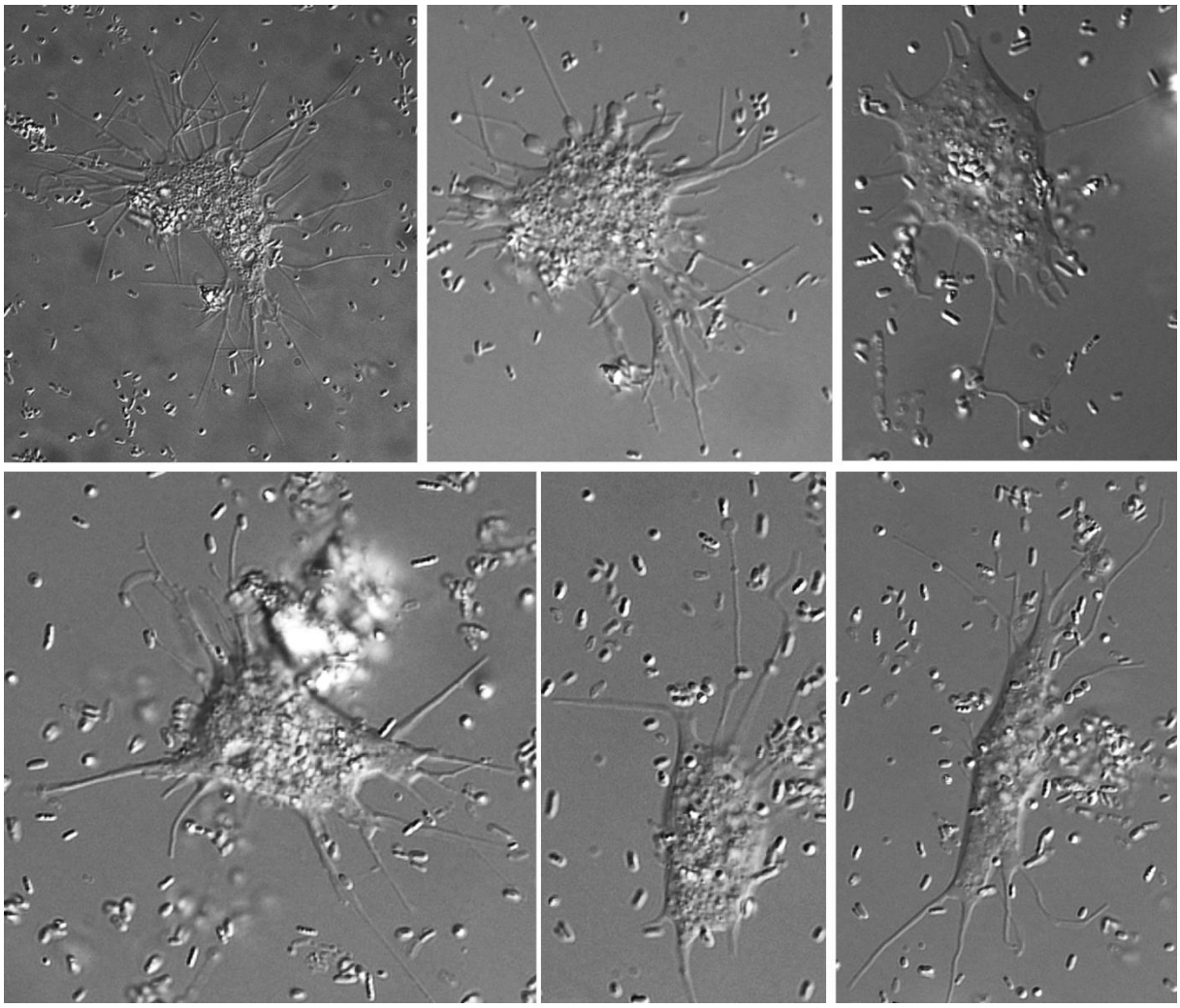


Figure 5. *Heliamoeba mirabilis* in different growth stages.

V16 – *Arboramoeba* n. gen. (Figure 6)

Multinucleated soil naked amoeba extending over a distance of up to 600 µm. Cells containing no distinct cell body, but a large reticulate network filled entirely filled with cell contents, such as nuclei. Pronounced branching pattern and high reticulosity most pronounced in anterior where a large “boarder” towards prey organisms is formed leaving behind a nearly sterile zone in posterior. Posterior of cells little reticulate and branching, ending in few single points. Filose, branching pseudopodia most pronounced in anterior, but also found all along the cell body, regularly fusing with other parts of the cell. Extreme load and activity of vacuoles in cells.

Movement of entire cells not observable under the microscope; No distinct floating form was observed. Cells completely retain shape when carefully detached from substratum; Cysts with two clearly separate cell walls varying both in size and shape. Sizes from 10 to 21 µm in

diameter and shape never perfectly round, often ovoid. Feeding on bacteria, fungi and small flagellates.

Arboramoeba reticulate Tib182 as a single type species. Etymology: from Latin “arbor” (tree) as expanding amoeba often take on the shape of a tree.

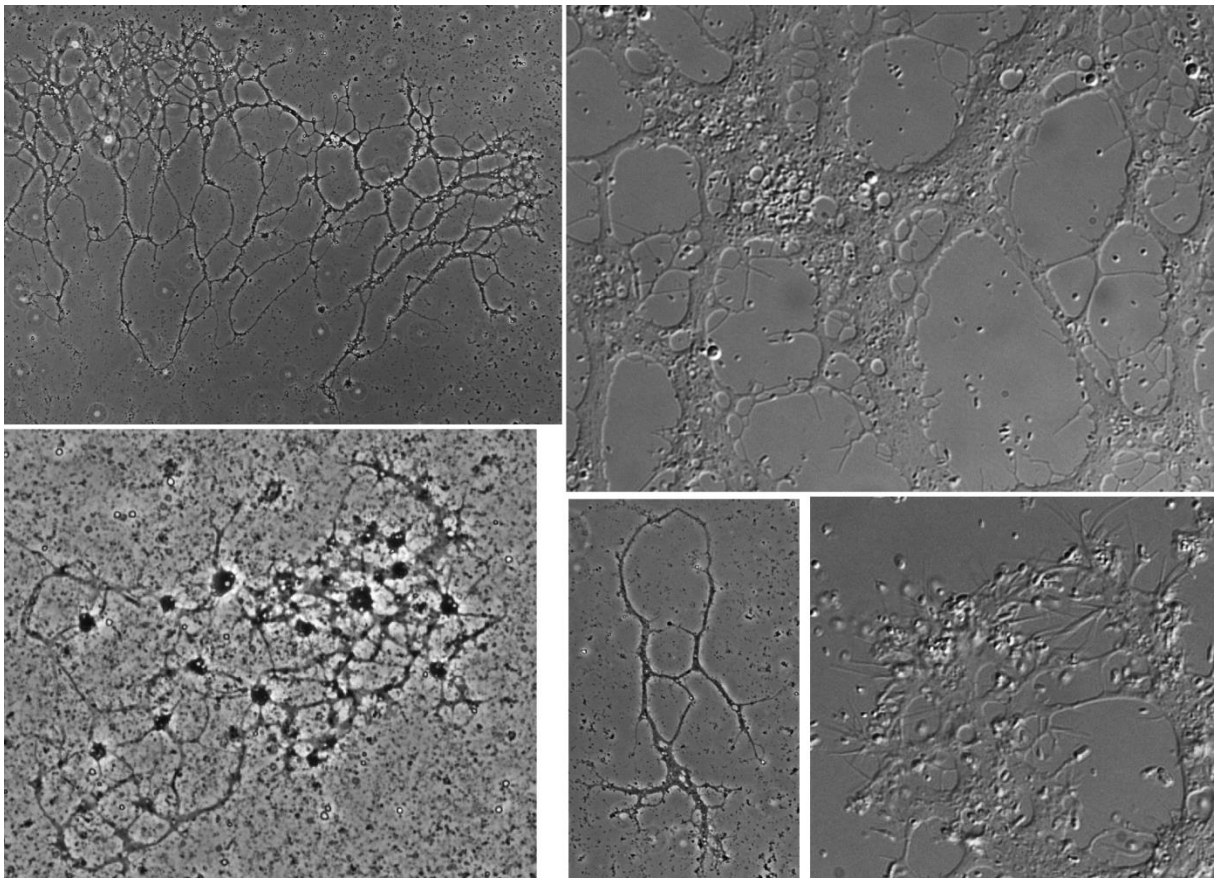


Figure 6. Expanding *Arboramoeba reticulate* trophozoits.

V17 -- *Angulamoeba* n. gen. (Figure 7)

Medium-sized (50 to 300 µm across), flattened, branching naked amoebae with slender, pointed, sometimes branched subpseudopodia, lacking cilia or flagella. Simple life cycle including trophozoite and cyst stages.

Trophozoites moving too slowly to be seen in light microscopy; main cell body elongated, consisting of up to four main branches often with several small lateral branches, never forming a network; numerous fine subpseudopodia concentrated mostly at the extremity of the lateral and terminal branches, but can be formed anywhere around the cell body; several contractile vacuoles can be observed. Cysts small (about 20 µm in diameter) and rounded, of very regular size and shape.

Type species: *Angulamoeba fungorum*. Etymology: latin “angulus” (angle) due to the often reticulate morphology.

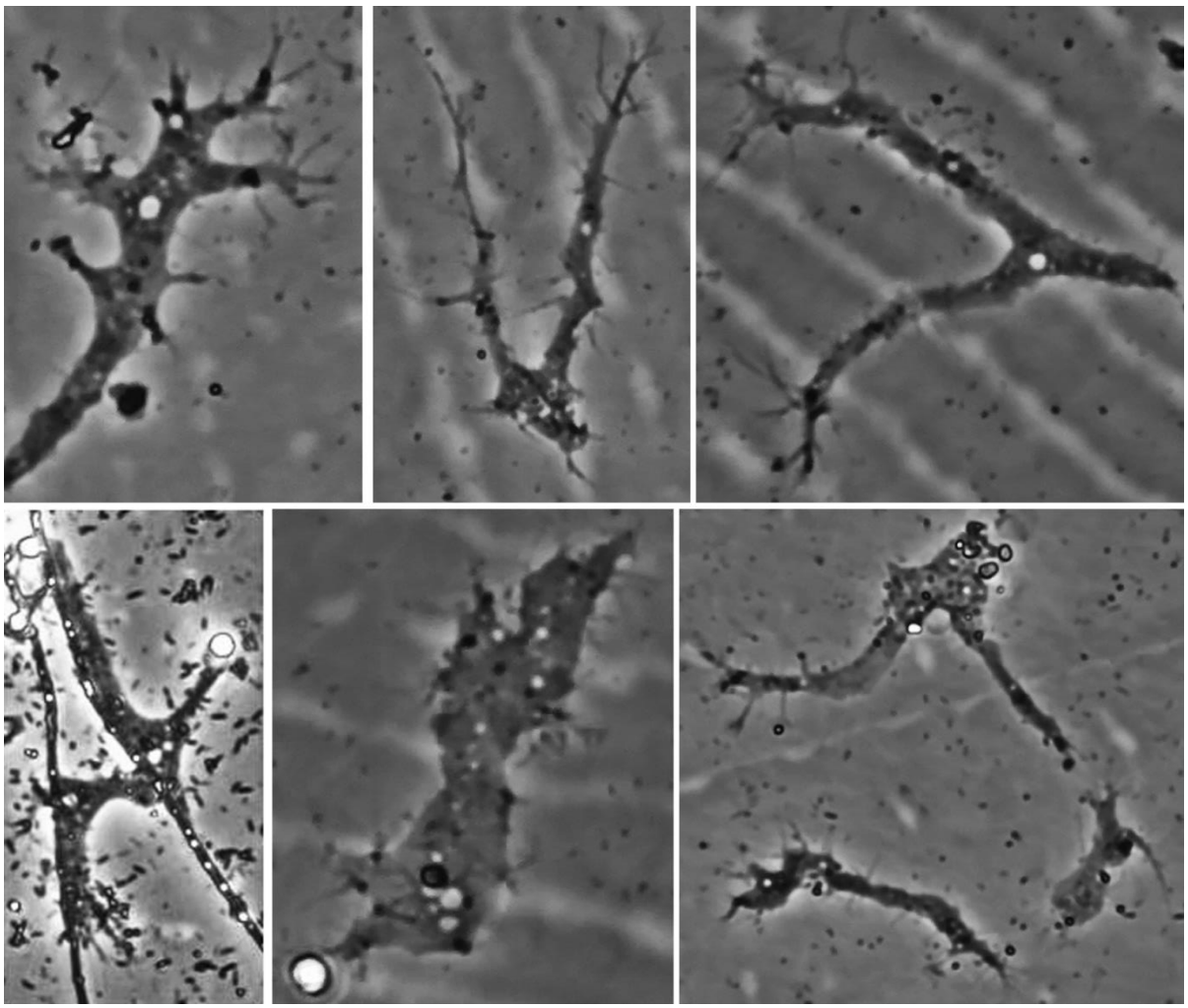


Figure7. *Angulamoeba fungorum* in different growth stages, often showing the angle-like morphology.

Discussion

Expanding our understanding of Variosea

Class Variosea is a morphologically puzzling assemblage of mostly amoeboid but sometimes flagellated organisms within Amoebozoa. It was never recognised as a potential taxon before the advent of molecular phylogenies because of the highly disparate morphologies observed among its members. Variosea provide an excellent example of the many challenges that modern protistologists have to face when defining higher-level clades based primarily on molecular data, in absence of an obvious morphological identity. In the case of Variosea, that task was made all the more difficult for two reasons. Firstly, amoeboid organisms can be particularly difficult to distinguish and more easily misidentified as other taxa. Until very

recently, their taxonomy has suffered greatly from a striking underestimation of their true diversity; as a result new morphotypes would often more likely be mistaken for members of a few long-known genera instead of being correctly described as new ones. Secondly, the molecular marker used to define the clade (here the SSU rDNA) is unfortunately highly prone to phylogenetic reconstruction artefacts in Amoebozoa, because of the unusually wide range of evolutionary rates observed between the various sequenced lineages. As a result, the history of the recognition of class Variosea has been plagued with several issues that obscured the evolutionary and ecological significance of the group for more than a decade. Classically, the various genera and / or morphotypes that we now know belong to Variosea fell in one of the three following categories: (1) described genera that were previously classified with non-Variosea amoebae based on convergent characters such as general pseudopod morphology, for instance the genera *Filamoeba* and *Flamella* (see below); (2) described genera that had been left *incertae sedis* in classification systems of eukaryotes, or placed in monotypic higher-level taxa, such as *Phalansterium* and *Multicilia*; and (3) organisms that represent amoeboid morphotypes that had never been properly described before, and when observed were generally ignored as unidentifiable filose or reticulose amoebae - this is true for most of the new isolates described in the present study.

The first molecular evidence for the existence of Variosea was published when Amaral-Zettler et al. (2000) showed a relationship between the genus *Filamoeba* and an ATCC strain (50654) that was then believed to belong to the leptomyxid genus *Gephyramoeba*. This result was surprising (see, e.g. Smirnov et al. 2008) because *Filamoeba* was believed to be closely related to the tubulinid amoebozoan genus *Echinamoeba* based on seemingly shared pointed subpseudopodia, since then revealed as the result of convergent evolution (category 1 above). In addition, later re-investigation of the ATCC strain 50654 by Smirnov et al. (2008) conclusively showed that it is actually not the leptomyxid described by Goodey (1915) but corresponds to a new genus that they named *Acramoeba*. Class Variosea was introduced by Cavalier-Smith et al. (2004) when a third member, the flagellate *Phalansterium* was sequenced. Later Nikolaev et al. (2006) showed that the mysterious multiflagellated taxon *Multicilia* also belongs to this assemblage; both genera are typical examples of taxa belonging to category 2 above. Since then, Kudryavtsev et al. (2009b) added the long known genus *Flamella* to the list of taxa belonging to Variosea, showing that it is not related to flabellinids as traditionally believed. Another ATCC strain (50593) has been shown to belong to Variosea (Tekle et al. 2008); initially

it was tentatively identified as a potential member of the cercozoan genus *Arachnula* but was since described as a new genus *Telaepolella* (Lahr et al. 2012). Finally, several lineages of amoebae with a protosteloid life cycle have been shown to be possibly related to or part of Variosea (Brown et al. 2007, Shadwick et al. 2009).

In the present study, the combined effect of increased taxon sampling and use of secondary structure information in the variable region V7 of the SSU rDNA allows us to indisputably define which of the Amoebozoa morphotypes sequenced to date are part of class Variosea, and how many clones from environmental SSU rDNA libraries represent additional lineages of yet unknown morphology that also belong to Variosea. We identify 31 lineages that clearly belong to Variosea based on both strong phylogenetic support and V7 secondary structure patterns (see Supplementary Table 1; Figure 2). Of these, 16 have a known morphotype, six of which are novel and described here for the first time as new genera. In particular, we confirm that four of the sequenced lineages of protosteloid amoebae belong to Variosea (protosteliids, schizoplasmodiids, soliformoviids, and cavosteliids), but show that the protosporangiids do not, and that their position in the tree presented by Shadwick et al. (2009) must have resulted from a long-branch attraction artefact. The other eight Variosea lineages presented in Figure 1, Supplementary Figure 1 and Table 1 are known only from environmental clone libraries so far.

Members of the Macromycetozoa (dictyosteliids and myxogastrids) and Archamoebae (pelobionts and entamoebids) have very divergent SSU rDNA sequences. In recent phylogenetic studies containing a good taxon sampling for Amoebozoa, they often appear to branch within class Variosea, either together or separately, usually next to some of the fast evolving protosteloid lineages within that class (Nikolaev et al. 2006, Tekle et al. 2008, Shadwick et al. 2009). This apparent paraphyly of Variosea with respect to Macromycetozoa and Archamoebae could be genuine, especially for Macromycetozoa, in agreement with the idea that cellular and acellular slime moulds evolved from a protosteloid ancestor. However it could also be the result of long-branch attraction artefacts. Importantly, no member of the Macromycetozoa and Archamoebae seem to possess the V7 secondary structure signature of the Variosea, but again this could be a result of the highly divergent nature of their SSU rDNA sequences. Multigene datasets including protein-coding genes will be necessary to resolve this issue. Therefore, because SSU rDNA alone cannot be expected to resolve the position of Macromycetozoa and Archamoebae within Amoebozoa, we decided to exclude these taxa from the present analyses

to minimise artefacts when inferring internal relationships within Variosea. Our results suggest that even in absence of these highly divergent taxa, the range of evolutionary rates observed in SSU rDNA sequences between the various Variosea lineages probably precludes confident resolution of their relationships based on that gene alone. Only a few well-supported relationships can be inferred from our analyses, such as that between the *Telaepolella* lineage, the *Flamella* lineage, and environmental lineage V18. Again, other molecular markers will evidently be needed to resolve further the relationships between the various Variosea lineages identified in this study.

Variosean BNFA: ecology, abundance, food preference, etc.

As well as improving the resolution and limits of the variosean phylogeny, our results also provide the first morphological data for lineages previously only represented by environmental sequences. In addition, our new data also demonstrate that other sequences in Genbank also belong to Variosea, even though they are annotated differently, e.g. some sequences classified as “Eiimeridae”, *Acanthamoeba* (EF023424) and Choanozoa (AY969212) (O'Brien et al. 2005, Lesaulnier et al. 2008)

It is striking that most of our new isolates require to be described as new genera; we found no corresponding taxa previously described in the literature. Recently, sequence data has been published for several non-sporocarp-forming branched and reticulate amoebae taxa that were previously only represented by (sometimes basic) morphological descriptions, e.g. the rhizarians *Thalassomyxa*, *Penardia*, *Vampyrella*, *Leptophrys* (Hess et al. 2012, Berney et al. 2013), *Reticulamoeba* (Berney et al. 2013), *Arachnula* (Bass et al. 2009a), the stramenopile *Leukarachnion* (Grant et al. 2009), and the amoebozoans described above. Others, superficially similar to those described in this paper remain unsequenced, e.g. *Asterocaelum*, *Branchipocola*, *Chichkovia*, *Cinetidomyxa*, *Enteromyxa*, *Gymnophrydium*, *Protomyxa*, *Synamoeba* (from Lee et al. 2000). However, none of these corresponded to our new isolates.

This high rate of lineage discovery is particularly interesting in light of the fact that BNFA were encountered in almost all soil samples analysed, suggesting that they are numerically abundant in many soils, as well as being phylogenetically highly diverse. A modified version of the liquid aliquot culturing method (Chapter 4) did not detect known fruiting body-forming protosteliids or BNFA from other groups, for example the rhizarian vampyrellids. Some Mycetozoa were

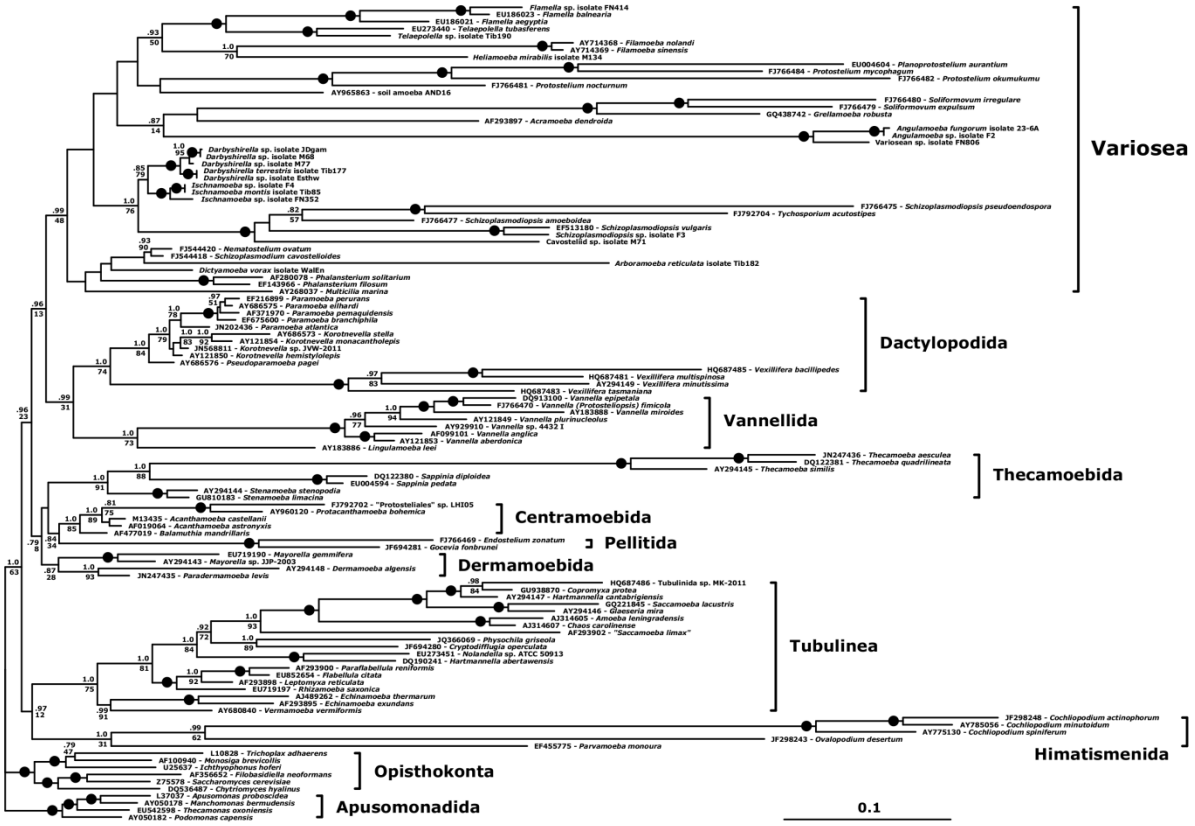
isolated, but at low frequency – fewer than 1,000 per gram of soil, which is at the lower limit of detection using LAM. In contrast, BNFA were recovered at a rate of 1,000 – 5,000 per gram of soil, and represented 5 - 20 % of all amoebae isolated per sample. Every culturing / cell isolation method has biases, so we cannot use our results to postulate that variosean BNFA are necessarily more abundant in soils than other BNFA taxa, although this may well be the case. The liquid aliquot method is likely biased towards bacterivores that can survive the physical disruption of this approach, or survive as resistant encysted stages until the culture conditions favour them. Nonetheless our results show that BNFA are likely to be key ecological players in soil habitats, and raises the hypothesis that non-sporocarp-forming variosean amoebae are the dominant BNFA taxa in soils. There are several reasons why their diversity and prevalence has not previously been recognised using culturing methods: 1) they take longer to appear and grow in culture than protists normally detected by these methods, 2) at least in their early stages they are morphologically similar, which without molecular analyses would give no indication of their genetic diversity, and means they could even be misidentified as smaller amoebae such as *Filamoeba*, and 3) their unusual morphotypes are often not recognised, or confused with other organisms, e.g. fungi. Even molecular sequencing studies have drastically underestimated them; their SSU rDNA sequences are often divergent in sequence and length, leading to biases against them in general eukaryote surveys, while misidentifications due to wrongly labelled sequences as detailed above lead to further confusion.

The physical nature of BNFA also points to their exerting a significant and distinctive ecological impact in soils. The networks / branches comprise a great range of cell body and pseudopodial dimensions, of extreme morphological plasticity, allowing the amoeba to probe interstitial spaces of many dimensions, range of relatively large areas, cross and both exploit and survive chemical and ecological microgradients, and potentially acquire and re-locate large amounts of resource around the cells. Therefore it is viable for them to exploit unusually small (or difficult to access) and large food resources, from bacteria to small metazoans. The fact that the BNFA morphology has evolved several times across the eukaryotic tree of life, often followed by significant radiations (in many amoebozoan and rhizarian lineages, stramenopiles, fungi, etc.) demonstrates the success of this foraging strategy. In culture their ability to densely cover large areas can rapidly virtually eliminate bacterial and eukaryote prey populations. Their eukaryote prey range is large, including fungi, diatoms, green algae, and possibly other heterotrophic protists and small metazoans. Some lineages, in particular *Darbyshirella* and *Dictyamoeba*,

develop particularly dense and extensive networks in the presence of eukaryotic prey, but will also grow in the presence of bacteria alone. *Heliamoeba* and *Arboramoeba* may require other eukaryotes as food, but this requires further testing. Their flat, extensive cells are often closely associated with soil particles, which as well as allowing highly efficient food acquisition may also protect them from being eaten themselves; in any case they are multinucleate and individuals can be fragmented by physical disruption and presumably also attack by other organisms, and then re-grow.

In conclusion, the new genera and species we describe in this paper possibly represent a much larger diversity of branching or network-forming variosean Amoebozoa that are abundant in many soil / sediment types, where they occupy a very distinctive niche. We infer from their morphology and behaviour in culture that they are significant grazers of bacteria and predators of a wide range of other eukaryotes, and therefore likely to have a large impact on soil ecology, which nonetheless has been largely overlooked in ecological studies. Other BNFA groups have been better studied in soils, for example amoebae feeding on pathogenic fungi and plant parasitic nematodes (Sayre 1973, Old and Darbyshire 1980, Old and Oros 1980), but those are either in different groups (*Arachnula* and *Theratromyxa* are vampyrellids) or do not correspond to our isolates. Furthermore it will be necessary to ensure that these often highly genetically divergent lineages are accounted for in molecular soil ecology studies, by revising existing primers, designing new ones, and / or by RNA-based and metagenomic approaches that are perhaps more likely to detect such lineages.

Supplementary



Supplementary Figure 1. Maximum likelihood tree showing all sequences included in the phylogenetic analysis of Figure 1.

Supplementary Table 1. Informtation on new and published Variosean sequences indicating newly defined Variosean lineage numbers and name, accession numbers with respective species name, origin of isolation, size of published SSU rDNA fragment (entire length / length of variosean specific sequence in chimeras), variosean specific signature sequences in V4 and V7 region and the reference; Lin = Variosean lineage; (T) = Type strain

Lin	Name	Accession	Species	Habitat	Size	V4	V7	Reference of 18S sequence
V01	*Filamoeba*	AF293896	*Filamoeba "nolandi"* ATCC 50430	MA - USA, offshore sediment core, North Carolina	1846	Y	Y	Amaral Zettler et al. 2000
		AY714369	*Filamoeba sinensis* CH26 (T)	FW - China, gills of Carassius gibelio	1839	Y	Y	Dyková et al. 2005
		AY714368	*Filamoeba nolandi* CCAP 1526/1 (T)	FW - USA, shore of Little Deer Lake, Minnesota	1839	Y	Y	Dyková et al. 2005
		AB425944	*Filamoeba* sp. H9a_6E	FW - Italy, rice field soil	1802	Y	Y	Murase & Frenzel 2008
		AB425947	*Filamoeba* sp. I4_5E	FW - Italy, rice field soil	1802	Y	Y	Murase & Frenzel 2008
		EF032804	clone HAVOmat-eukA09	FW - Hawaii, lava cave cyanobacterial mat	778	N	Y	Brown et al. unpub. (2006)
		GU320603	*Filamoeba* sp. COHH87	BR - USA, shore of Mt Hope Bay, Massachusetts	1784	Y	Y	Gast et al. unpub. (2011)
		GU320604	*Filamoeba* sp. COHH88	BR - USA, shore of Mt Hope Bay, Massachusetts	1139	Y	N	Gast et al. unpub. (2011)
		GU320578	*Filamoeba* sp. COHH101	BR - USA, shore of Mt Hope Bay, Massachusetts	1845	Y	Y	Gast et al. unpub. (2011)
		GQ371176	*Filamoeba* sp. JIH56	FW - Czech Republic, hot-water piping system	1840	Y	Y	Peckova et al. unpub. (2012)
V02	*Acramoeba*	AF293897	*Acramoeba dendroida* ATCC 50654 (T)	FW - USA, pond in Grand Haven, Michigan	1859	Y	Y	Amaral Zettler et al. 2000; Smirnov et al. 2008
		AB695507	clone MPE2-12	FW - Antarctica, lake moss pillars	1817	Y	Y	Nakai et al. 2012
		AB695457	clone MPE1-14	FW - Antarctica, lake moss pillars	1817	Y	Y	Nakai et al. 2012
		KC306576	clone KF_Euk_A1R	FW - Germany, groundwater of a karstic aquifer	1186	Y	N	Risse-Buhl et al. 2013
		KC306569	clone KF_Euk_F7	FW - Germany, groundwater of a karstic aquifer	1157	Y	N	Risse-Buhl et al. 2013
		KC306572	clone KF_Euk_A9R	FW - Germany, groundwater of a karstic aquifer	1162	Y	N	Risse-Buhl et al. 2013
		KC306575	clone KF_Euk_F7R	FW - Germany, groundwater of a karstic aquifer	1157	Y	N	Risse-Buhl et al. 2013
		KC306581	clone KF_Euk_A1	FW - Germany, groundwater of a karstic aquifer	1186	Y	N	Risse-Buhl et al. 2013
		KC306594	clone KF_Euk_A9	FW - Germany, groundwater of a karstic aquifer	1162	Y	N	Risse-Buhl et al. 2013
		KC306611	clone KF_Euk_D12R	FW - Germany, groundwater of a karstic aquifer	1186	Y	N	Risse-Buhl et al. 2013
		KC306613	clone KF_Euk_D12R	FW - Germany, groundwater of a karstic aquifer	1186	Y	N	Risse-Buhl et al. 2013
V03	*Phalansterium*	AF280078	*Phalansterium* cf. solitarium ATCC 50327	FW - USA, outdoor bath at hot springs area, West Virginia	1876	Y	Y	Cavalier-Smith et al. 2004
		EF143966	*Phalansterium filosum* Thailand (T)	FW - Thailand, forest soil near flooded stream	1858	Y	Y	Smirnov et al. 2011
		FK539978	*Phalansterium* sp. 1 JFP-2013	FW - contaminant in Monomorphina aenigmatica UTEX 1284	1873	Y	Y	Pombert et al. 2013
V04	*Multicilia*	AY268037	*Multicilia marina*	MA - Ukraine, surface of brown algae, Black Sea	2746	Y	Y	Nikolaev et al. 2006
V05	AND16	AY965863	undetermined amoeba AND16	FW - Spain, silty clay soil, Andújar	1831	Y	Y	Lara et al. 2007
		EU798715	clone 18S1-22	FW - China, lignocellulose decomposing forest soil	1024	Y	N	Huang et al. unpub. (2008)
V06	Protosteliids	EU004603	*Protostelium mycophagum* RTF06-1A-4-1	FW - habitat where strain was isolated not provided	1790	Y	Y	Brown et al. 2007

EU004604	*Planoprotostelium aurantium* BR33	FW - habitat where strain was isolated not provided	1865	Y	Y	Brown et al. 2007
FJ766481	*Protostelium nocturnum* ATCC PRA-194	FW - habitat where strain was isolated not provided	1800	Y	Y	Shadwick et al. 2009
FJ766482	*Protostelium okumukumu* ATCC PRA-156 (T)	FW - USA, Hawaii, on plants	1813	Y	Y	Shadwick et al. 2009
FJ766483	*Protostelium mycophagum* HI04-85a-1b	FW - habitat where strain was isolated not provided	1819	Y	Y	Shadwick et al. 2009
FJ766484	*Protostelium mycophagum* ATCC PRA-154 (T)	FW - USA, on reed dead inflorescence, New Jersey	1809	Y	Y	Shadwick et al. 2009
FJ766440	*Protostelium mycophagum* HI07-6L-3	FW - habitat where strain was isolated not provided	1808	Y	Y	Shadwick et al. unpub. (2010)
FJ766441	*Protostelium mycophagum* HI07-22L-4	FW - habitat where strain was isolated not provided	1808	Y	Y	Shadwick et al. unpub. (2010)
FJ766442	*Protostelium mycophagum* TB-A2-1	FW - habitat where strain was isolated not provided	1808	Y	Y	Shadwick et al. unpub. (2010)
FJ766443	*Protostelium mycophagum* PBR-A3-1	FW - habitat where strain was isolated not provided	1808	Y	Y	Shadwick et al. unpub. (2010)
FJ766444	*Protostelium mycophagum* BRNFDF05-11A-3	FW - habitat where strain was isolated not provided	1811	Y	Y	Shadwick et al. unpub. (2010)
FJ766445	*Protostelium mycophagum* KA-T02-1	FW - habitat where strain was isolated not provided	1812	Y	Y	Shadwick et al. unpub. (2010)
FJ766446	*Protostelium mycophagum* KEN-5A-2	FW - habitat where strain was isolated not provided	1811	Y	Y	Shadwick et al. unpub. (2010)
FJ766447	*Protostelium mycophagum* HI06-7A-1	FW - habitat where strain was isolated not provided	1814	Y	Y	Shadwick et al. unpub. (2010)
FJ766448	*Protostelium mycophagum* HI07-4a-III	FW - habitat where strain was isolated not provided	1830	Y	Y	Shadwick et al. unpub. (2010)
FJ766449	*Protostelium mycophagum* WWL06-3a-1	FW - habitat where strain was isolated not provided	1828	Y	Y	Shadwick et al. unpub. (2010)
FJ766450	*Protostelium mycophagum* HI06-9a-2	FW - habitat where strain was isolated not provided	1812	Y	Y	Shadwick et al. unpub. (2010)
FJ766451	*Protostelium mycophagum* KA-T15-1	FW - habitat where strain was isolated not provided	1800	Y	Y	Shadwick et al. unpub. (2010)
FJ766452	*Protostelium mycophagum* KA-T15-3	FW - habitat where strain was isolated not provided	1811	Y	Y	Shadwick et al. unpub. (2010)
FJ766453	*Protostelium mycophagum* OMBS04-1-1	FW - habitat where strain was isolated not provided	1800	Y	Y	Shadwick et al. unpub. (2010)
FJ766454	*Protostelium mycophagum* SI04-01A-1	FW - habitat where strain was isolated not provided	1791	Y	Y	Shadwick et al. unpub. (2010)
FJ766455	*Protostelium mycophagum* MFB-b	FW - habitat where strain was isolated not provided	1789	Y	Y	Shadwick et al. unpub. (2010)
FJ766456	*Protostelium mycophagum* BRNFDF05-11A-1	FW - habitat where strain was isolated not provided	1789	Y	Y	Shadwick et al. unpub. (2010)
FJ766457	*Protostelium mycophagum* HI06-10a-2	FW - habitat where strain was isolated not provided	1810	Y	Y	Shadwick et al. unpub. (2010)
FJ766458	*Protostelium mycophagum* HI07-2L-3	FW - habitat where strain was isolated not provided	1819	Y	Y	Shadwick et al. unpub. (2010)
FJ766459	*Protostelium mycophagum* HI07-2a-2	FW - habitat where strain was isolated not provided	1819	Y	Y	Shadwick et al. unpub. (2010)
FJ766460	*Protostelium mycophagum* ATCC PRA-128	FW - USA, plants on lake shore near Seattle, Washington	1791	Y	Y	Shadwick et al. unpub. (2010)
FJ766461	*Protostelium mycophagum* BRNFDF05-7C	FW - habitat where strain was isolated not provided	1794	Y	Y	Shadwick et al. unpub. (2010)
FJ766462	*Planoprotostelium aurantium* LEE06-21-3-1	FW - habitat where strain was isolated not provided	1815	Y	Y	Shadwick et al. unpub. (2010)
FJ766463	*Protostelium mycophagum* MFA-c	FW - habitat where strain was isolated not provided	1794	Y	Y	Shadwick et al. unpub. (2010)
FJ766464	*Protostelium mycophagum* KA-T15-2	FW - habitat where strain was isolated not provided	1803	Y	Y	Shadwick et al. unpub. (2010)
FJ766465	*Protostelium mycophagum* HI07-20L-IV	FW - habitat where strain was isolated not provided	1812	Y	Y	Shadwick et al. unpub. (2010)
FJ766466	*Protostelium mycophagum* QE07-15-1	FW - habitat where strain was isolated not provided	1814	Y	Y	Shadwick et al. unpub. (2010)
FJ766467	*Protostelium nocturnum* MCWTKF06-10L-2-1	FW - habitat where strain was isolated not provided	1810	Y	Y	Shadwick et al. unpub. (2010)
FJ766468	*Protostelium nocturnum* BADL04-A	FW - habitat where strain was isolated not provided	1821	Y	Y	Shadwick et al. unpub. (2010)

		GU290108	clone TKR07M.106	FW - Lake Tanganyika, metalimnion	1845	Y	Y	Tarbe et al. unpub. (2010)
V07	*Telaepolella*	EU273440	*Telaepolella tubasferens* ATCC 50593	FW - habitat where strain was isolated not provided	1934	Y	Y	Tekle et al. 2008; Lahr et al. 2012
		NEW	isolate SG-K190	this study		Y	Y	this study
V08	*Flamella*	EU186021	*Flamella aegyptia* A1-3	FW - Egypt, river Nile near Assuan	1858	Y	Y	Kudryavtsev et al. 2009
		EU186022	*Flamella arnhemensis* KWK - CCAP 1525/2	FW - Netherlands, cooling water circuit, Arnhem	1834	Y	Y	Kudryavtsev et al. 2009
		EU186023	*Flamella balnearia* WiII8a - CCAP 1525/3	FW - Germany, physiotherapy hospital pool, Wildbad	1832	Y	Y	Kudryavtsev et al. 2009
		EU186024	*Flamella fluviatilis*	FW - Australia, soil in the floodplain of the Murray river	1824	Y	Y	Kudryavtsev et al. 2009
		EU186025	*Flamella* sp. WBT	FW - Germany, drinking water treatment plant near Bonn	1845	Y	Y	Kudryavtsev et al. 2009
		NEW	isolate FN-414	this study		Y	Y	this study
		AF372778	clone LEMD267	FW - USA, anoxic lake sediment	1460	Y	Y	Dawson & Pace 2002
		AM114809	clone WIM81	FW - Netherlands, 1975 agricultural field sample	1869	Y	Y	Moon-van der Staay et al. 2006
		AY626163	clone Borok	FW - Russia, waste treatment plant, Borok	1824	Y	Y	Nikolaev et al. 2006
		DQ409131	clone VNP13	FW - France, hyper-eutrophic lake picoplankton	983	Y	N	Lepère et al. 2007
		FJ355431	clone NewOrleansYard3_YD3_032406_090	FW - USA, New Orleans yard, Louisiana	1135	Y	Y	Amaral-Zettler et al. 2008
		EF023373	clone Amb_18S_704 (chimera)	FW - USA, trembling aspen rhizosphere	1865/1393	Y	Y	Lesaulnier et al. 2008
		EF023606	clone Amb_18S_844 (chimera)	FW - USA, trembling aspen rhizosphere	1865/1393	Y	Y	Lesaulnier et al. 2008
		EF023634	clone Amb_18S_879 (chimera)	FW - USA, trembling aspen rhizosphere	1865/1393	Y	Y	Lesaulnier et al. 2008
		EF023656	clone Amb_18S_908 (chimera)	FW - USA, trembling aspen rhizosphere	1868/1393	Y	Y	Lesaulnier et al. 2008
		EF023685	clone Amb_18S_948 (chimera)	FW - USA, trembling aspen rhizosphere	1865/1393	Y	Y	Lesaulnier et al. 2008
		EF023712	clone Amb_18S_991 (chimera)	FW - USA, trembling aspen rhizosphere	1865/1393	Y	Y	Lesaulnier et al. 2008
		EF023441	clone Amb_18S_1015 (chimera)	FW - USA, trembling aspen rhizosphere	1865/1393	Y	Y	Lesaulnier et al. 2008
		EF023478	clone Amb_18S_1064 (chimera)	FW - USA, trembling aspen rhizosphere	1865/1393	Y	Y	Lesaulnier et al. 2008
		EF023545	clone Amb_18S_1147 (chimera)	FW - USA, trembling aspen rhizosphere	1864/1392	Y	Y	Lesaulnier et al. 2008
		EF023826	clone Amb_18S_1275 (chimera)	FW - USA, trembling aspen rhizosphere	1865/1393	Y	Y	Lesaulnier et al. 2008
		EF023984	clone Amb_18S_1455 (chimera)	FW - USA, trembling aspen rhizosphere	1865/1393	Y	Y	Lesaulnier et al. 2008

		EF024977	clone Elev_18S_1525	FW - USA, trembling aspen rhizosphere	1873	Y	Y	Lesaulnier et al. 2008
		AB425943	*Flamella* sp. H9a_3E	FW - Italy, rice field soil	1829	Y	Y	Murase & Frenzel 2008
		JF826388	clone North_Pole_SW170_108 (chimera)	MA - North Pole, 170 m-deep water under sea ice	1699/ 1457	Y	Y	Bachy et al. 2011
		FR874405	clone ws_96, clone 1802F11	MA - Norway, fjord coastal water, Bergen	1872	Y	Y	Newbold et al. 2012
		FJ153641	clone GoC1_G12	MA - Baltic Sea, anoxic water sample, Gotland Deep	1280	Y	Y	Stock et al. unpub. (2008)
V09	Schizo-plasmodiids	FJ544418	*Schizoplasmodium cavostelioides* ATCC PRA-197	FW - habitat where strain was isolated not provided	1920	Y	Y	Shadwick et al. 2009
		FJ544419	*Ceratiomyxella tahitiensis* HI04-93L-1	FW - habitat where strain was isolated not provided	1883	Y	Y	Shadwick et al. 2009
		FJ544420	*Nematostelium ovatum* JDS 6241	FW - habitat where strain was isolated not provided	1918	Y	Y	Shadwick et al. 2009
V10	Soliformoviids	FJ766479	*Soliformovum expulsum* ATCC 48083 (T)	FW - habitat where strain was isolated not provided	1878	Y	Y	Shadwick et al. 2009
		FJ766480	*Soliformovum irregulare* ATCC 26826 (T)	FW - Mexico, on purple bean pods	1881	Y	Y	Shadwick et al. 2009
		GQ438740	*Grellamoeba robusta* 4168 clone 111	FW - Czech Republic, gills of Sander lucioperca	1880	Y	Y	Dyková et al. 2010
		GQ438741	*Grellamoeba robusta* 4168 clone 122	FW - Czech Republic, gills of Sander lucioperca	1880	Y	Y	Dyková et al. 2010
		GQ438742	*Grellamoeba robusta* 4168 clone 841	FW - Czech Republic, gills of Sander lucioperca	1880	Y	Y	Dyková et al. 2010
		EF513181	*Soliformovum irregulare* ATCC 26826 (T)	FW - Mexico, on purple bean pods	1848	Y	Y	Fiore-Donno et al. 2010
		HE614594	*Soliformovum irregulare* ATCC 26826 (T)	FW - Mexico, on purple bean pods	1785	Y	Y	Nandipati et al. unpub. (2012)
V11	Cavosteliids	FJ766475	*Schizoplasmodiopsis pseudoendospora* ATCC PRA-195	FW - habitat where strain was isolated not provided	1786	Y	Y	Shadwick et al. 2009
		FJ766476	*Cavostelium apophysatum* ATCC 38567 (T)	FW - Granada, on plants	1777	Y	Y	Shadwick et al. 2009
		FJ766477	*Schizoplasmodiopsis amoeboidea* ATCC 46943 (T)	FW - Cook Islands, on pigeon pea pods	1927	Y	Y	Shadwick et al. 2009
		FJ766478	*Schizoplasmodiopsis amoeboidea* BG7A-12B	FW - habitat where strain was isolated not provided	1876	Y	Y	Shadwick et al. 2009
		FJ792703	*Tychosporium acutostipes* KEA-11A-L	FW - Kenya, more details not provided	1818	Y	Y	Shadwick et al. 2009
		FJ792704	*Tychosporium acutostipes* NZ05-15a-2	FW - New Zealand, more details not provided	1818	Y	Y	Shadwick et al. 2009
		EF513172	*Cavostelium apophysatum* ATCC 38567 (T)	FW - Granada, on plants	1732	Y	Y	Fiore-Donno et al. 2010
		EF513179	*Schizoplasmodiopsis amoeboidea* ATCC 46943 (T)	FW - Cook Islands, on pigeon pea pods	1909	Y	Y	Fiore-Donno et al. 2010
		EF513180	*Schizoplasmodiopsis vulgaris*	FW - Switzerland, on dead grass leaves near Geneva	1812	Y	Y	Fiore-Donno et al. 2010
		NEW	isolate SG-M071	this study		Y	Y	this study
		NEW	isolate SG-F003	this study		Y	Y	this study
V12	*"Ischnamoeba"*	NEW	*Ischnamoeba montis* Tib85 (T)	this study		Y	Y	this study
		NEW	*Ischnamoeba* sp. F4	this study		Y	Y	this study
		NEW	*Ischnamoeba* sp. FN352	this study		Y	Y	this study
		AB425950	undetermined amoeba Mb_5C	FW - Italy, rice field soil	1891	Y	Y	Murase & Frenzel 2008
		EF023260	clone Amb_18S_564 (chimera)	FW - USA, trembling aspen rhizosphere	2226/ 789	N	Y	Lesaulnier et al. 2008

		EF023267	clone Amb_18S_572 (chimera)	FW - USA, trembling aspen rhizosphere	1897/789	N	Y	Lesaulnier et al. 2008
		EF023369	clone Amb_18S_699 (chimera)	FW - USA, trembling aspen rhizosphere	1900/789	N	Y	Lesaulnier et al. 2008
		HE575399	uncultured amoeba	FW - Italy, water purification plant	1900	Y	Y	Chiellini et al. 2012
		AB695456	clone MPE1-13	FW - Antarctica, lake moss pillars	1923	Y	Y	Nakai et al. 2012
		AB695508	clone MPE2-13	FW - Antarctica, lake moss pillars	1923	Y	Y	Nakai et al. 2012
V13	*"Darbyshirella"*	NEW	*Darbyshirella terrestris* Tib177 (T)	this study		Y	Y	this study
		NEW	*Darbyshirella* sp. Esthw	this study		Y	Y	this study
		NEW	*Darbyshirella* sp. JDgam	this study		Y	Y	this study
		NEW	*Darbyshirella* sp. M68	this study		Y	Y	this study
		NEW	*Darbyshirella* sp. M77	this study		Y	Y	this study
		JN825696	clone Alchichica_AQ2_5E_20	FW - Mexico, alcaline lake microbialite	1272	Y	N	Couradeau et al. 2011
V14	*"Dictyamoeba"*	NEW	*Dictyamoeba vorax* WalEn (T)	this study		Y	Y	this study
		AB695458	clone MPE1-15	FW - Antarctica, lake moss pillars	1860	Y	Y	Nakai et al. 2012
V15	*"Heliamoeba"*	NEW	*Heliamoeba mirabilis* M134 (T)	this study		Y	Y	this study
V16	*"Arboramoeba"*	NEW	*Arboramoeba reticulata* Tib182 (T)	this study		Y	Y	this study
		AY969212	clone dfmo4344.099	FW - USA, mixed hardwood soil, North Carolina	734	Y	N	O'Brien et al. 2005
		EF023424	clone Amb_18S_765	FW - USA, trembling aspen rhizosphere	1862	Y	Y	Lesaulnier et al. 2008
V17	*"Angulamoeba"*	NEW	*Angulamoeba fungorum* 23-6A (T)	this study		Y	Y	this study
		NEW	*Angulamoeba* sp. F2	this study		Y	N	this study
		NEW	*Angulamoeba* sp. FN806	this study		Y	Y	this study
		DQ123626	undetermined amoeba CRIB-09	FW - Switzerland, hospital water network	568	Y	N	Thomas et al. 2006
		HM017143	uncultured amoeba clone A1WVB	FW - Belgium, hypertrophic urban pond	1714	Y	Y	Van Wichelen et al. 2010
		HM017144	uncultured amoeba clone A2WVB	FW - Belgium, hypertrophic urban pond	1475	Y	Y	Van Wichelen et al. 2010
		JN825704	clone Alchichica_AQ2w_5E_66	FW - Mexico, alcaline lake microbialite	1001	Y	N	Couradeau et al. 2011
		HM161754	"Cyanidioschyzon sp." Y16	FW - France, Phytophthora parasitica biofilm	1870	Y	Y	Galiana et al. 2011
		AB695473	clone MPE1-30	FW - Antarctica, lake moss pillars	1804	Y	Y	Nakai et al. 2012
		AB695474	clone MPE1-31	FW - Antarctica, lake moss pillars	1850	Y	Y	Nakai et al. 2012
		AB695529	clone MPE2-35	FW - Antarctica, lake moss pillars	1803	Y	Y	Nakai et al. 2012
		AB695530	clone MPE2-36	FW - Antarctica, lake moss pillars	1841	Y	Y	Nakai et al. 2012
		JN547325	clone S9-1	FW - France, Sep reservoir artificial lake	1576	Y	Y	Lepere et al. unpub. (2012)
V18	RT5	AY082976	clone RT5iin21	FW - Spain, green biofilm in the acidic and iron-rich Rio Tinto	1912	Y	Y	Amaral Zettler et al. 2002

		AY082989	clone RT5iin44	FW - Spain, green biofilm in the acidic and iron-rich Rio Tinto	1914	Y	Y	Amaral Zettler et al. 2002
		AM114808	clone WIM80	FW - Netherlands, 1975 agricultural field sample	1961	Y	Y	Moon-van der Staay et al. 2006
		EF441963	clone RT07C_2E_32	FW - Spain, endolithic community in the acidic Rio Tinto basin	1738	Y	Y	Lopez-Garcia et al. unpub. (2012)
		EF441972	clone RT07C_2E_9	FW - Spain, endolithic community in the acidic Rio Tinto basin	1732	Y	Y	Lopez-Garcia et al. unpub. (2012)
		EF441973	clone RT07C_2E_43	FW - Spain, endolithic community in the acidic Rio Tinto basin	1731	Y	Y	Lopez-Garcia et al. unpub. (2012)
V19	Mariager	AY605217	clone Sey088	FW - Switzerland, river sediment near Geneva	883	N	Y	Berney et al. 2004
		DQ103790	clone M1_18H06	MA - Denmark, anoxic Mariager Fjord	1656	Y	Y	Zuendorf et al. 2006
		DQ103816	clone M1_18G11	MA - Denmark, anoxic Mariager Fjord	1654	Y	Y	Zuendorf et al. 2006
		EF526901	clone SA1_4A12	MA - Norway, anoxic Framvaren Fjord	1367	Y	N	Behnke et al. 2010
		HQ868115	clone SHAO448 (chimera)	MA - Canada, micro-oxic water column near Vancouver	908/ 595	Y	N	Orsi et al. 2012
V20	WIM1	AM114811	clone WIM1	FW - Netherlands, 1975 agricultural field sample	1893	Y	Y	Moon-van der Staay et al. 2006
		AB695494	clone MPE1-51	FW - Antarctica, lake moss pillars	1839	Y	Y	Nakai et al. 2012
V21	WIM5	AM114801	clone WIM5	FW - Netherlands, 1975 agricultural field sample	1871	Y	Y	Moon-van der Staay et al. 2006
		AB695495	clone MPE1-52	FW - Antarctica, lake moss pillars	1833	Y	Y	Nakai et al. 2012
		AB695538	clone MPE2-44	FW - Antarctica, lake moss pillars	1833	Y	Y	Nakai et al. 2012
V22	Amb_18S_6341	EF024087	clone Amb_18S_6341 (chimera)	FW - USA, trembling aspen rhizosphere	1877/ 1668	Y	Y	Lesaulnier et al. 2008
V23	SAWY402	DQ423701	clone SAWY402	FW - USA, acid mine drainage biofilm, California	1163	Y	Y	Baker et al. 2009
		DQ423693	clone SAWY394	FW - USA, acid mine drainage biofilm, California	1146	Y	Y	Baker et al. 2009
		DQ423694	clone SAWY395	FW - USA, acid mine drainage biofilm, California	1155	Y	Y	Baker et al. 2009
		DQ423699	clone SAWY400	FW - USA, acid mine drainage biofilm, California	1146	Y	Y	Baker et al. 2009
		DQ423700	clone SAWY401	FW - USA, acid mine drainage biofilm, California	1157	Y	Y	Baker et al. 2009
		DQ423702	clone SAWY403	FW - USA, acid mine drainage biofilm, California	1089	Y	Y	Baker et al. 2009
		DQ423705	clone SAWY406	FW - USA, acid mine drainage biofilm, California	1155	Y	Y	Baker et al. 2009
		DQ423706	clone SAWY407	FW - USA, acid mine drainage biofilm, California	1172	Y	Y	Baker et al. 2009
		DQ423709	clone SAWY410	FW - USA, acid mine drainage biofilm, California	1160	Y	Y	Baker et al. 2009
		DQ423710	clone SAWY411	FW - USA, acid mine drainage biofilm, California	1022	Y	Y	Baker et al. 2009
		DQ423711	clone SAWY412	FW - USA, acid mine drainage biofilm, California	1097	Y	Y	Baker et al. 2009
		DQ423716	clone SAWY417	FW - USA, acid mine drainage biofilm, California	1151	Y	Y	Baker et al. 2009
		DQ423721	clone SAWY422	FW - USA, acid mine drainage biofilm, California	1150	Y	Y	Baker et al. 2009
		DQ423725	clone SAWY426	FW - USA, acid mine drainage biofilm, California	1091	Y	Y	Baker et al. 2009
		DQ423728	clone SAWY429	FW - USA, acid mine drainage biofilm, California	1028	Y	Y	Baker et al. 2009
		DQ423735	clone SAWY437	FW - USA, acid mine drainage biofilm, California	1164	Y	Y	Baker et al. 2009

		DQ423737	clone SAWY440	FW - USA, acid mine drainage biofilm, California	1180	Y	Y	Baker et al. 2009
		DQ423740	clone SAWY444	FW - USA, acid mine drainage biofilm, California	1168	Y	Y	Baker et al. 2009
		DQ423742	clone SAWY446	FW - USA, acid mine drainage biofilm, California	1074	Y	Y	Baker et al. 2009
		DQ423745	clone SAWY449	FW - USA, acid mine drainage biofilm, California	1158	Y	Y	Baker et al. 2009
		DQ423748	clone SAWY452	FW - USA, acid mine drainage biofilm, California	1130	Y	Y	Baker et al. 2009
		DQ423762	clone SAWY466	FW - USA, acid mine drainage biofilm, California	1135	Y	Y	Baker et al. 2009
		DQ423764	clone SAWY468	FW - USA, acid mine drainage biofilm, California	1161	Y	Y	Baker et al. 2009
		DQ423765	clone SAWY469	FW - USA, acid mine drainage biofilm, California	1148	Y	Y	Baker et al. 2009
		DQ423774	clone SAWY479	FW - USA, acid mine drainage biofilm, California	1040	Y	Y	Baker et al. 2009
		JN825703	clone Alchichica_AQ1B_5E_11	FW - Mexico, alcaline lake microbialite	1118	Y	N	Couradeau et al. 2011
V24	PRS2_4E_19	GU479959	clone PRS2_4E_19	FW - Switzerland, peat bog in the Jura mountains	1539	Y	Y	Lara et al. 2011
V25	MPE1-44	AB695487	clone MPE1-44	FW - Antarctica, lake moss pillars	1874	Y	Y	Nakai et al. 2012
		AB695493	clone MPE1-50 (chimera)	FW - Antarctica, lake moss pillars	1790/ 1217	Y	N	Nakai et al. 2012
V26	LP30ME5	FJ903024	clone LP30ME5	FW - Mexico, phreatic limestone sinkhole	857	Y	Y	Sahl et al. unpub. (2009)
V27	Elev603	EF024236	clone Elev_18S_603 (chimera)	FW - USA, trembling aspen rhizosphere	1957/ 643	N	Y	Lesaulnier et al. 2008
V28	9_69	EU087280	clone 9_69	MA - Korea, 9 cm deep core sediment in the East Sea	924	Y	N	Park et al. 2008
V29	9_174	EU545726	clone 9_174	MA - Korea, 9 cm deep core sediment in the East Sea	989	Y	N	Park et al. 2008
V30	Alchichica_31	JN825689	clone Alchichica_AQ2_5E_31	FW - Mexico, alcaline lake microbialite	1038	Y	N	Couradeau et al. 2011
V31	Alchichica_65	JN825698	clone Alchichica_AQ1_5E_65	FW - Mexico, alcaline lake microbialite	1119	Y	N	Couradeau et al. 2011
		JN825697	clone Alchichica_AQ1_5E_60 (chimera)	FW - Mexico, alcaline lake microbialite	1032/ 487	N	N	Couradeau et al. 2011

Part 1 – Chapter 4

Isolation of six new species of *Allovahlkampfia* and one new genus of the Vahlkampfiidae, Heterolobosea

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Abstract

Seven strains of amoebae were isolated from soil samples taken in Tibet (at high altitude), Sardinia and the Netherlands. They seemed to belong to one morphospecies. However, sequences of the small subunit rDNA and internal transcribed spacers, including the 5.8S rDNA, revealed that six represent different species of the genus *Allovahlkampfia* while one belongs to an unknown genus of which the closest relative is the genus *Fumarolamoeba*. Some unnamed strains of which the sequences had been published before are also given species status within the genus *Allovahlkampfia*.

Introduction

The name "Heterolobosea" was coined by Page and Blanton (1985) for amoebae with eruptive locomotion, often a differentiated flagellate stage and distinct ultrastructural features. Despite morphological similarities to organisms within the supergroup Amoebozoa, phylogenetic analyses place the Heterolobosea distantly in the supergroup Excavata sister to the morphologically different Euglenozoa (Adl et al. 2012). Identification of heterolobosean amoebae based on morphology is difficult. The flagellate stage and cyst structure have been used to enable species description (Page 1988), but due to the high morphological similarity amongst Heterolobosea, only molecular tools have made a breakthrough by detecting cryptic species and genera within morphospecies. Within the Heterolobosea (Vahlkampfiidae) the genus *Naegleria* has been investigated in most detail as *Naegleria fowleri* causes a fatal disease in humans, i.e. primary amoebic meningoencephalitis (De Jonckheere 2002, Visvesvara et al. 2007, De Jonckheere 2011). Many species within the genus *Naegleria* have been described based on the sequence of the internal transcribed spacers (ITS) (De Jonckheere 2004). In a similar way, other Vahlkampfiidae have been separated into different genera based on the small subunit (SSU) rDNA (Brown and De Jonckheere 1999) and ITS, including the 5.8S rDNA, (De Jonckheere and Brown 2005) sequences. Vahlkampfiid amoebae without a flagellate stage are morphologically very similar and were traditionally placed all together in a single genus *Vahlkampfia* (Page 1988). Only with the advent of DNA sequencing, this genus was found to be paraphyletic and was subsequently split into four different genera: *Tetramitus*, *Vahlkampfia*, *Neovahlkampfia*, and *Paravahlkampfia* (Brown and De Jonckheere 1999).

The diversity within the Heterolobosea is enormous despite that only about 150 species have so far been described; it contains organisms that either only adopt an amoeboid or a flagellate stage, others alter between both, while even multicellular forms exist (Page and Blanton 1985, Brown et al. 2012). Heteroloboseans seem to have an ubiquitous distribution, occupying also extreme environments (Amaral-Zettler et al. 2002, De Jonckheere 2006, De Jonckheere et al. 2011a, Park and Simpson 2011, Pánek et al. 2012).

We have investigated 7 new vahlkampfiid strains from diverse environments, four of which were cultivated from extremely high altitudes (> 4100 meters). As these strains are nearly indistinguishable morphologically, the work is focused primarily on molecular tools, i.e. the

ITS, including the 5.8S rDNA, and SSU rDNA sequences. Six strains from highly diverse soil conditions are closely related and branch in phylogenetic analyses together with *Solumitrus palustris* (Anderson et al. 2011) and *Allovahlkampfia spelaea* (Walochnik and Mulec, 2009), and we describe them as different species belonging to the genus *Allovahlkampfia*. Furthermore, we describe a new genus for a strain which clusters in phylogenetic trees with several environmental sequences, of which the closest relative is *Fumarolamoeba ceborucoi* (De Jonckheere et al. 2011b).

Materials and Methods

Site description, amoebae isolation and cultivation

The top 10 cm of soils were sampled from a pasture soil in the Netherlands, from high altitudes in Tibet and the Berchidda-Monti long term observatory, managed by the University of Sassari, Italy (Table 1).

Table 1. List of strains with their origin

Strain	Location	Location (Label)	GPS	Elevation (m)
A. sardiniensis Sar9	Italy	Sardinia	N 40°46′, E 9°10′	181
A. parasardiniensis Sar37	Italy	Sardinia	N 40°46′, E 9°10′	181
A. nederlandensis Nl64	The Netherlands	Veluwe	N 52°06′, E 6°00′	57
Pagea alta Tib23	Tibet	Mila East Slope	N 29°52′ E 92°33′	4149
A. tibetensis Tib32	Tibet	Mila Mountaintop	N 29°49′ E 92°20′	5033
A. paratibetensis Tib50	Tibet	Mila West Slope	N 29°42′ E 92°10′	4149
A. neotibetensis Tib191	Tibet	Mila East Slope	N 29°52′ E 92°33′	4149

50 g of each soil was suspended in 200 ml sterile ddH_2O and incubated on a shaker at room temperature for 10′. 200 µl of each suspension was subsequently transferred to ten 90 mm Petri dishes filled with autoclaved liquid wheat grass medium (WG), made by adding vacuum-dry wheat grass powder (Weizengras, Sanatur, Singen, Germany) to PJ medium (Prescott and James 1955) to the weight concentration 0.15 % to stimulate bacterial growth. Individual dishes were investigated with an inverted microscope (Nikon Eclipse TS100) after 14 and 28 days and individual amoebae were transferred and maintained in 60 mm Petri dishes containing fresh WG. Detailed light-microscopic analyses of each clonal culture and

measurements were performed using a Nikon Eclipse 90i with differential interference contrast (DIC) equipped with a Nikon DIGITAL SIGHT DS-fi1 camera. The strains were tested for growth at temperatures of 30 °C and 37 °C. Formation of flagellate stages was tested by flooding spores grown in a petri-dish with distilled water. Formation of fruiting forms were tested by adding autoclave-sterilized *Quercus alba* bark soaked in a sterile slurry of *Rhodotorula mucilaginosa* in H_20_{dest} as described in Brown et al. (2012). The isolates were feeding on the bacteria that grew with them upon isolation, but they were also tested to see whether they could grow when fed with yeast, by transferring isolates into a culture of the ascomycete *Saccharomyces cerevisiae* and the basidiomycete *R. mucilaginosa*. We subsequently investigated microscopically, whether amoebae actively take up yeast and use those as a food source.

DNA extraction, amplification and sequencing

Petri dishes with dense growth of amoebae were washed 3 times with fresh medium and replaced with 100 µl of guanidine isothiocyanate buffer. Cells were then scraped off the bottom using a sterile metal cell scraper and the buffer containing amoebae was transferred to an Eppendorf tube. After vortexing, the solution was heated to 72 °C for 10', cooled down to room temperature, 200 µl isopropanol was added and left at -20 °C overnight. After vortexing and centrifugation for 15' at 15,000 rpm the supernatant was removed and replaced by 100 µl 70 % EtOH. After another centrifugation step for 10' at 15,000 rpm, the supernatant was removed and DNA was resuspended in 50 µl ddH_2O. DNA was stored at -20 °C until further use (Maniatis et al. 1982).

Small subunit (SSU) ribosomal DNA was amplified using the universal eukaryotic primers EukA and EukB (Medlin et al. 1988), while the primers JITS-F and JITS-R were used to amplify and sequence the ITS region, including the 5.8S rDNA (De Jonckheere and Brown 2005). PCR reactions were run in 30 µl volume consisting of 0.6 µl of each primer (10 µM), 0.6 µl nucleotides (10 mM), 2 µl template DNA, 24.5 µl H_2O, 3 µl GreenTaq Buffer and 0.15 µl GreenTaq polymerase (5 U * μl^{-1}) (Fermentas, St. Leon-Rot, Germany). The cycling conditions included a 5' initial denaturation at 95 °C, followed by 30 cycles of 95 °C for 30'', 50 °C for 60'', and 72 °C for 120'', and a final extension at 72 °C for 5'. 8 µl of the PCR products were enzymatically purified by adding 0.15 µl Endonuclease I (20 U * μl^{-1}, Fermentas, St. Leon-Rot, Germany), 0.9 µl Shrimp Alkaline Phosphatase (1 U * μl^{-1}, Fermentas, St. Leon-Rot, Germany)

and 1.95 µl H_2O. The resulting mixture was incubated for 30’ at 37 °C, followed by 20’ at 85 °C. Cleaned PCR products were subsequently sequenced (GATC, Konstanz, Germany) using all amplification end primers and internal ones to obtain full length sequences of the SSU rDNA.

Phylogenetic analyses

Sequences obtained were subjected to BLAST searches to establish taxonomic affiliations. All sequences were aligned using Clustal Omega directly implemented in SEAVIEW v. 4.4.2 together with all closest BLAST hits of cultivated taxa, a subset of sequences of uncultivated taxa and sequences of other heterolobosean genera in order to obtain better resolved phylogenetic affinities of our strains. Two datasets including sequences of the SSU rDNA and the 5.8S rDNA were analyzed. 43 sequences were aligned in the SSU rDNA dataset resulting in 1,527 unambiguously aligned positions, while 23 sequences were aligned in the 5.8S rDNA dataset resulting in 145 unambiguously aligned positions. ITS1 and ITS2 could not reliably be aligned and were consequently excluded from phylogenetic analyses of all vahlkampfiids. To explain species differentiation of clones closely resembling *A. spelaea* we aligned the entire ITS region of *A. spelaea*, *S. palustris* and six new strains obtained in this study, increasing unambiguously aligned positions to 340.

A general time-reversible model with a proportion of invariable sites and γ distribution (GTR+I+gamma) as proposed by jModeltest v. 2.1.3 under the Akaike Information Criterion (Darriba et al. 2012). These were performed directly in SEAVIEW with 5 random starting trees using nearest-neighbor interchange and subtree pruning and regrafting algorithms for tree searching (Guindon and Gascuel 2003, Hordijk and Gascuel 2005). The stability of the clades was assessed using a non-parametric bootstrap with 100 pseudoreplicates using unambiguously aligned positions. Further, a Bayesian analysis was performed using Mr Bayes Version 3.1.2 (Huelsenbeck and Ronquist 2001) with the GTR+I+gamma model of substitution. Two runs of four simultaneous Markov chain Monte-Carlo analyses starting from different random trees were performed for 5,000,000 generations (default heating parameters), sampled every 100 generations. Convergence (average deviation of split frequencies < 0.01) was reached after 210,000 generations (SSU dataset) and 300,000 generations (5.8S dataset). All trees before convergence was reached were discarded as burnin and a consensus tree was built from the remaining trees.

Another focused phylogenetic analysis was performed on a clade containing *A. spelaea* to decipher species relations within that clade. Those sequences were aligned using Clustal Omega and subsequently modified manually. Most of the ITS regions could still not reliably be aligned and only the remaining reliable positions shared by all *Allovahlkampfia* spp. were used for subsequent phylogenetic analyses. Maximum likelihood and Bayesian analyses were performed as described above, but only running 2,000,000 generations in the Bayesian analysis as the runs converged after 70,000 generations, leaving sufficient numbers of trees after burning to construct a consensus tree. This focused alignment is uploaded to TreeBASE (treebase.org), submission ID 15118.

New nucleotide sequences from all Heterolobosea described in this study are available in GenBank under the accession numbers KF547907, KF547908, KF547909, KF547910, KF547911, KF547912 and KF547913 for the SSU-rDNA; KF547914, KF547915, KF547916, KF547917, KF547918, KF547919 and KF547920 for the ITS region including the 5.8S rDNA. Type cultures are deposited at the Culture Collection of Algae and Protozoa (CCAP) under the accession numbers CCAP 2502/1 to CCAP 2502/6.

Results

Cultivation and morphology

All isolated amoebae grew well at room temperature, as well as 30 °C, except for strain Nl64 (Table 2). Growth performance of Tib23 could not be tested as the strain went extinct before temperature tests (and any growth tests) could be completed (Table 2).

Table 2. Characteristics of strains

Strain	Morphology	Temp (max)°	Trophozoite length (µm)	Trophozoite width (µm)	Cyst diameter (µm)
A. sardiniensis Sar9	Mostly limax	< 37 °C	18.2 – 28.8 23.6 ± 3.4	3.6 – 5.2 4.6 ± 0.6	5.6 – 10.8 7.4 ± 1.2
A. parasardiniensis Sar37	Limax	< 37 °C	20.8 – 30.0 23.8 ± 2.4	3.4 – 6.0 4.6 ± 0.6	5.4 – 8.4 6.6 ± 0.8
A. nederlandensis Nl64	Mostly flabellate	< 30 °C	21.8 – 50.8 33.2 ± 10.0	3.6 – 8.6 5.8 ± 1.4	5.0 – 7.8 6.2 ± 0.8
Pagea alta Tib23	Limax	*	14.8 – 31.1 20.9 ± 4.8	3.9 – 13.4 7.9 ± 3.1	5.3 – 6.2 5.7 ± 0.4
A. tibetensis Tib32	Mostly flabellate	< 37 °C	21.2 – 31.2 25.2 ± 4.0	5.6 – 9.2 6.6 ± 1.2	5.0 – 8.2 6.6 ± 0.8
A. paratibetensis Tib50	Limax or flabellate	< 37 °C	27.6 – 36.0 30.0 ± 2.8	5.0 – 8.8 7.4 ± 1.4	5.8 – 9.2 8.0 ± 1.0
A. neotibetensis Tib191	Limax	< 37 °C	19.4 – 26.4 23.0 ± 2.2	4.2 – 7.6 6.2 ± 1.0	6.4 – 8.6 7.6 ± 0.6

*: could not be tested, culture extinct.
°: Temp (max) = maximum temperature tolerance.

None of all isolates actively took up and fed on the yeast species tested, i.e. *S. cerevisiae* and *R. mucilaginosa*. Sorocarp formation was not observed under any condition tested. Most of the isolates showed the typical morphology of vahlkampfiid amoebae including eruptive lobopodia and an elongated “limax” form during locomotion (Figure 1 and Figure 3), although flabellate forms were regularly seen in some. Further, all were uninucleate and often produced uroidal structures. However, the strains differed in morphology and could be classified in four groups; group I included organisms that mostly adopted an elongated “limax” shape when active (strains Sar37 [Figure 1B] and Tib191 [Figure 1F]), group II rarely moved in an elongated form most often adopting a flabellate shape (strains Nl64 [Figure 1C] and Tib32 [Figure 1D]), group III regularly switched between the two forms of locomotion (strains Sar9 [Figure 1A] and Tib50 [Figure 1E]) and group IV adopting a limax shape but morphologically differentiating it from all previously described strains by a more eruptive formation of pseudopodia and the presence of only a small, centrally located nucleolus (Tib23, Figure 3).

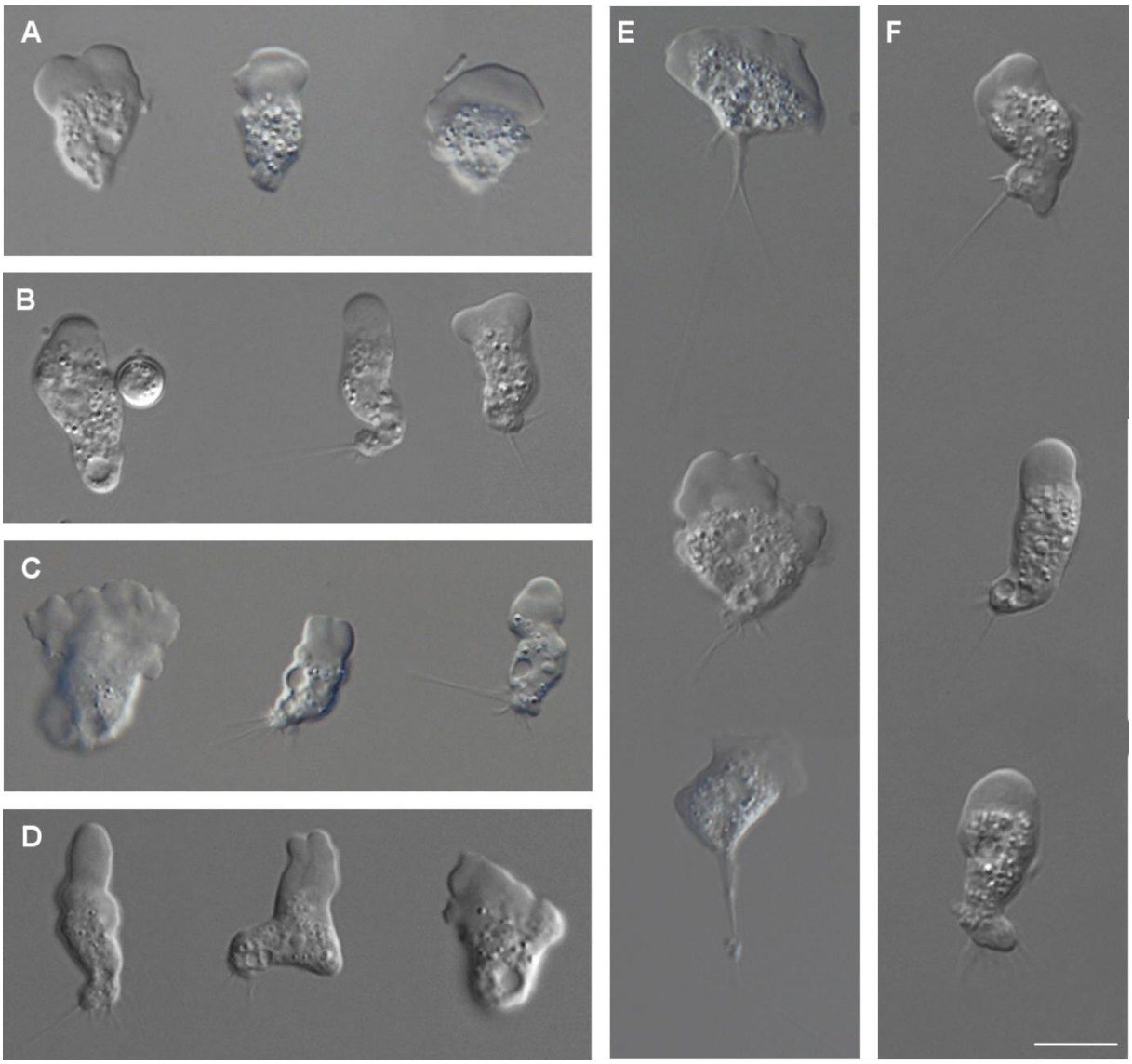


Figure 1. Differential interference contrast (DIC) pictures showing trophozoits in locomotive form of all new *Allovahlkampfia* strains described in this study, drawn at the same scale. **A.** *A. sardiniensis* Sar9. **B.** *A. parasardiniensis* Sar37. Note size difference between the trophozoite and cyst. **C.** *A. nederlandensis* Nl64. **D.** *A. tibetensis* Tib32. **E.** *A. paratibetensis* Tib50. Note pronounced length of uroidal filaments. **F.** *A. neotibetensis* Tib191. Scale bar: 10 µm.

All strains produced cysts (Figure 2), with Tib23 again differing strongly in cyst morphology by forming dimorphic cysts, but always of a similar size (Figure 3C). None of the cultures formed a flagellate stage. Size differences even within strains were usually profound so size differences between strains were negligible (Table 2).

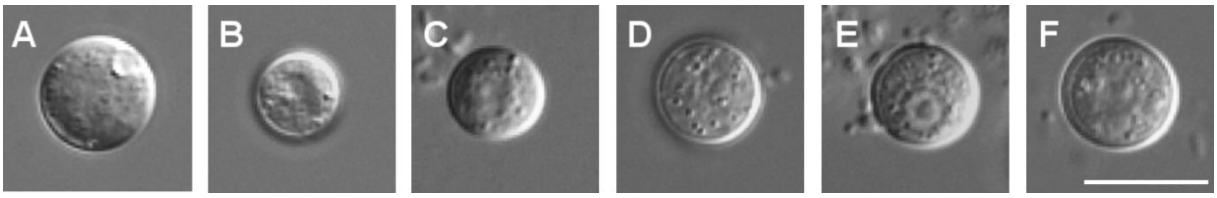


Figure 2. Differential interference contrast (DIC) pictures showing cysts of all new *Allovahlkampfia* strains described in this study, drawn at the same scale. **A.** *A. sardiniensis* Sar9. **B.** *A. parasardiniensis* Sar37. **C.** *A. nederlandensis* Nl64. **D.** *A. tibetensis* Tib32. **E.** *A. paratibetensis* Tib50. **F.** *A. neotibetensis* Tib191. Scale bar: 10 µm.

Despite some morphological and locomotive differences the lack of reliable uniform and constant characteristics, which is typical for Vahlkampfiidae, necessitate an identification based on molecular analyses (Brown and De Jonckheere 1999, De Jonckheere and Brown 2005).

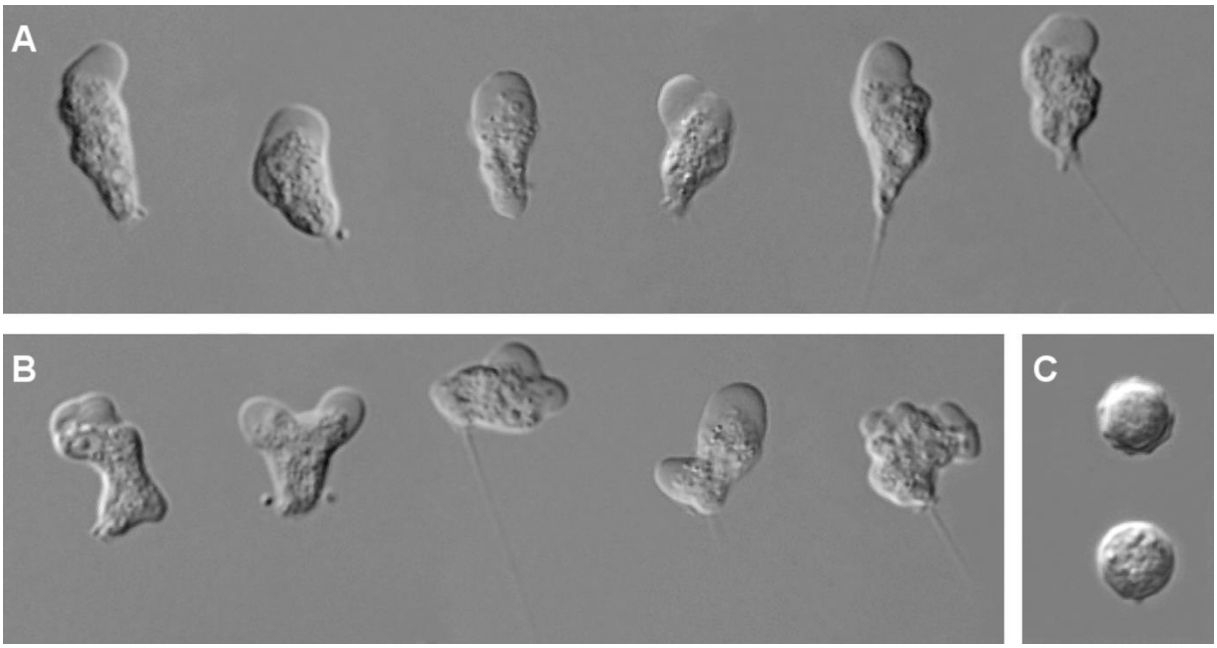


Figure 3. Differential interference contrast (DIC) pictures showing *Pagea alta* Tib23. **A.** Locomotive forms. Note pronounced uroidal filaments. **B.** Amoeba in indirect locomotion forming eruptive pseudopodia everywhere at cell body. **C.** Irregular cysts. Upper cyst wrinkled, lower cyst round.

Phylogenetic analyses

Full length sequences of the ITS region, including the 5.8S rDNA, were obtained for all strains. The sizes of ITS1 and ITS2 differed strongly between most strains, while the 5.8S rDNA were always 161 bp long, with the exception of Sar37 and Tib191, where it was 2 bp longer (Table 3).

Table 3. Length (bp) of the SSU rDNA available, presence and length of SSU group I intron, and length of ITS1, 5.8S and ITS2

Strain	SSU rDNA	Group I intron	ITS1	5.8S	ITS2	Origin
A. sardiniensis Sar9	2038	-	163	161	128	Italy
A. parasardiniensis Sar37	2026	-	117	163	130	Italy
A. nederlandensis Nl64	2060	1358	155	161	127	The Netherlands
A. tibetensis Tib32	1962	-	154	161	127	Tibet
A. paratibetensis Tib50	2032	-	153	161	125	Tibet
A. neotibetensis Tib191	2010	-	129	163	162	Tibet
A. palustris	671	-	339	161	152	USA
Soil amoeba AND12	2178	-	NA	NA	NA	Spain
A. canadensis BA	2125	1313	NA	NA	NA	Canada
A. paracanadensis OSA	2129	-	NA	NA	NA	Canada
A. spelaea	2138	-	166	161	108	Slovenia
Pagea alta Tib23	1738	-	163	157	162	Tibet
F. ceborucoi	2371	-	133	154	125	Mexico
P. ustiana	1903	-	129	158	316	Czech Republic
P. lenta	NA	-	120	159	397	UK
P. francinae	1880	-	129	158	270	USA
Paravahlkampfia sp. A1PW2	1921	-	NA	NA	NA	Germany
Paravahlkampfia sp. LA	1909	-	NA	NA	NA	Canada
Paravahlkampfia sp. li3	1876	-	NA	NA	NA	USA

-: absent
NA: Not available

Nearly full length sequences of the SSU rDNA, except for the very beginning and end, were obtained. Interestingly, one of our isolates (Nl64) contained a group I intron, which is only present in one other strain investigated in this group (Heterolobosea BA). The intron in strain Nl64 was located at the same position as in Heterolobosea BA, but sequence dissimilarity exist not only in the intron region (21.1 % dissimilarity), but also in the remaining sequence of the SSU rDNA (1.2 % dissimilarity) (Table 4). The group I intron in both strains contains an open reading frame, coding for a homing endonuclease with a His-Cys box (Wilmark et al. 2006).

Blast searches based on the SSU rDNA indicated closest affinities of strains Sar9, Sar37, Nl64, Tib32, Tib50 and Tib191 with *Allovahlkampfia spelaea* (Walochnik and Mulec, 2009), unnamed Heterolobosea BA and OSA (Shut and Gray, unpublished), and *Solumitrus palustris* (Anderson et al. 2011). Blast searches based on the entire ITS region including the 5.8S rDNA similarly detected closest affinities of Sar9, Sar37, Nl64, Tib32, Tib50 and Tib191 with *A. spelaea and S. palustris.* Blast hits of Tib23 based on the SSU rDNA matched best with several uncultured eukaryotes, followed by *Fumerolamoeba ceborucoi* (De Jonckheere et al. 2011). The latter, *F. ceborucoi,* represented the best hit when a Blast search was based on the ITS region.

Maximum likelihood and Bayesian phylogenetic analyses of the SSU rDNA alignments (Figure 4) confirmed a strongly supported group of our six isolates with *A. spelaea, S. palustris* and Heterolobosea BA and OSA. The *Allovahlkampfia* clade formed a strongly supported sister clade to species of the genus *Acrasis* with the uncultivated sequence "soil amoeba AND12" (Lara et al. 2007a) branching outside the *Allovahlkampfia* spp.

Strain Tib23 formed a clade with several uncultured sequences (Valster et al. 2009, Valster et al. 2010, Valster et al. 2011, Farhat et al. 2012), with *Fumarolamoeba* (De Jonckheere et al. 2011b) as the most closely related genus, while *Paravahlkampfia* formed a separate branch.

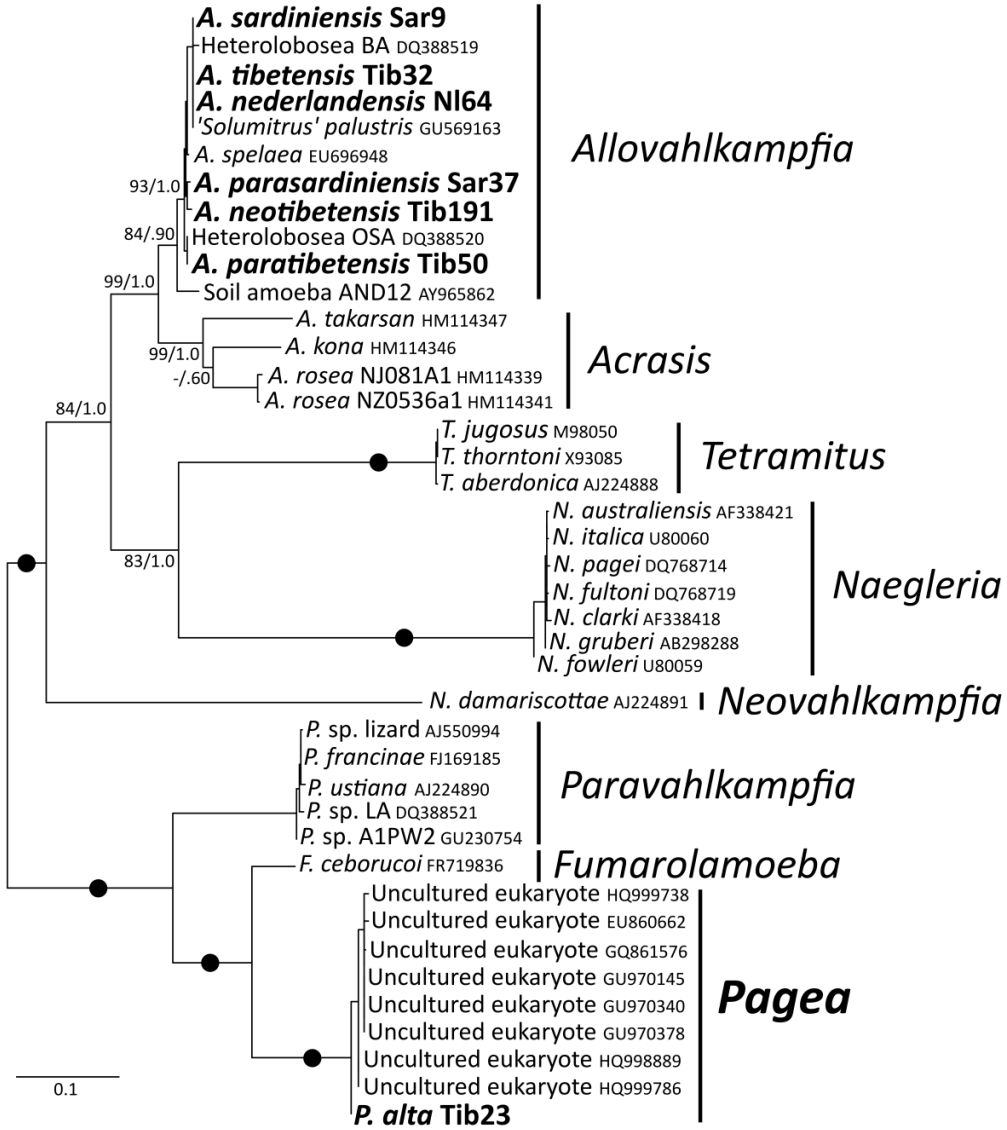


Figure 4. Maximum likelihood tree based on SSU rDNA sequences. Support values at each node presented for RAxML / BI. RAxML ML bootstrap values and BI posterior probabilities equal to 100 % / 1.00, respectively, are represented by a black dot, whereas – is placed for unrecovered topologies and support values < 50 % (RAxML) / 0.50 (BI).

Note: Phylogenetic relations within most genera omitted for convenience; relationships within *Allovahlkampfia* discussed in text. GenBank accession numbers of SSU sequences used in analyses listed next to taxon names. Scale bar represents evolutionary distance in changes per site.

Phylogenetic analyses based on the 5.8S rDNA resulted in a similar pattern (Figure 5); the *Fumarolamoeba* and *Paravahlkampfia* genera formed strongly supported branches with isolate Tib23. The six other new isolates formed a clade with *A. spelaea*, including *S. palustris.* The Sequence similarities for the SSU rDNA of our isolates most closely resembling *A. spelaea* in phylogenetic analyses ranged from 95.3 to 96.9 % (Table 4), indicating that they belong to the genus *Allovahlkampfia*, but that they should be treated as different species. The high sequence similarity of the 5.8S rDNA, between 95.4 and 99.4 % (Table 5), confirmed that they belong to the same genus *Allovahlkampfia*. Heterolobosea BA and OSA had similar high sequence similarities of the SSU rDNA compared to *A. spelaea*, 95.9 and 97.1 %, respectively. Therefore, these two should also be considered to be different species within the genus as well. On the other hand, *S. palustris* showed somehow lower sequence similarities for both molecules, 89.2 and 94.3 %respectively, but this could be due to the short sequence length of the SSU rDNA reported and the possibility of sequencing errors, as several mismatches were found in highly conserved regions in both genes. Strain AND12, with 90.3 % sequence similarity of the SSU rDNA compared to *A. spelaea*, and 89.2 to 91.2 % compared to our new isolates probably might represent a different genus other than *Allovahlkampfia* or a very basal species of *Allovahlkampfia*.

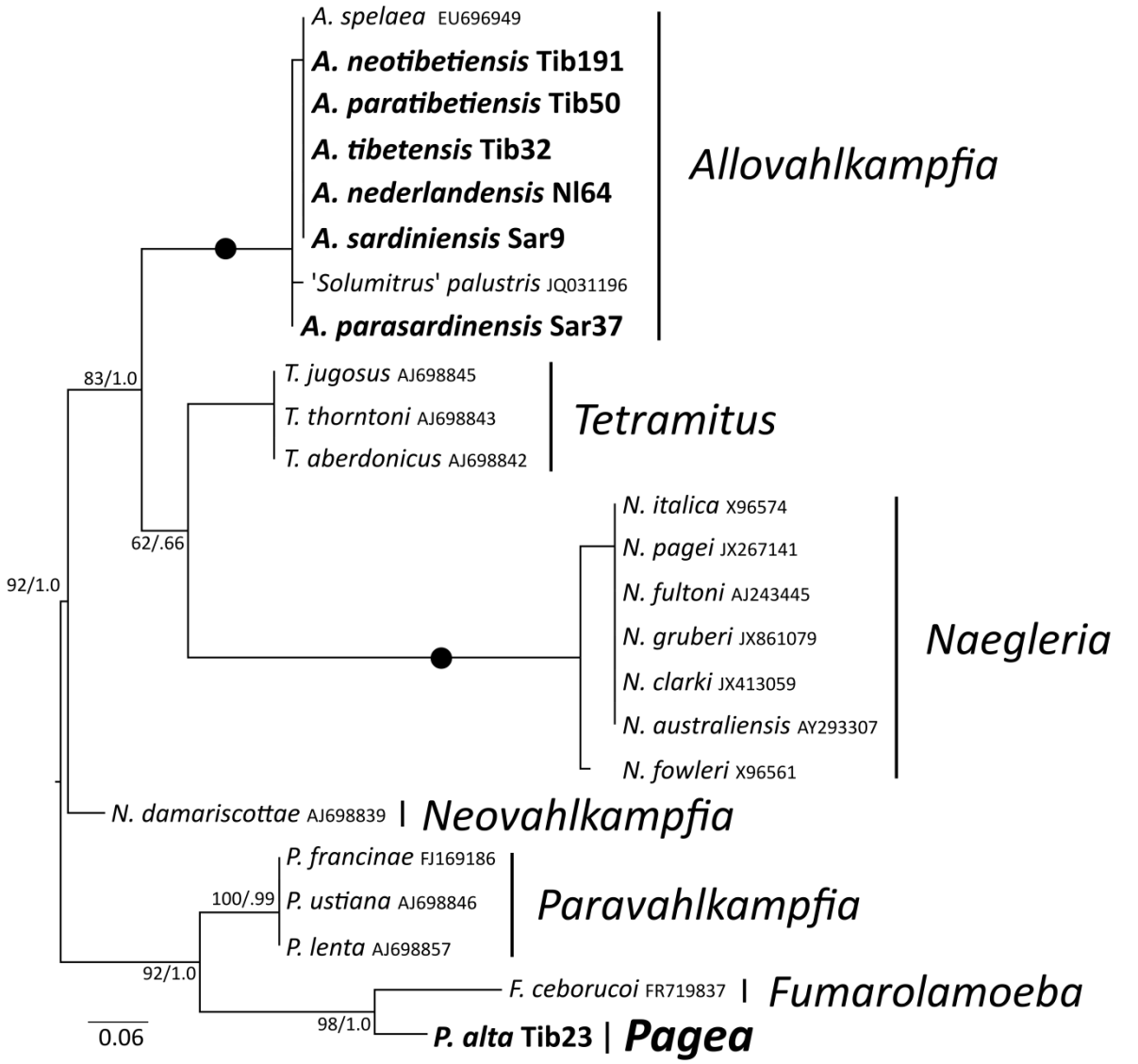


Figure 5. Maximum likelihood tree based on 5.8S rDNA sequences. Support values at each node presented for RAxML / BI. RAxML ML bootstrap values and BI posterior probabilities equal to 100 % / 1.00, respectively, are represented by a black dot, whereas – is placed for unrecovered topologies and support values < 50 % (RAxML) / 0.50 (BI).
Note: Phylogenetic relations within most genera omitted for convenience; relationships within *Allovahlkampfia* discussed in text. GenBank accession numbers of the sequences used in analyses listed next to taxon names. Scale bar represents evolutionary distance in changes per site.

Table 4. Percentage identity matrix obtained with manually modified Clustal Omega alignments of the SSU rDNA in the *Allovahlkampfia* clade

	Sar9	**Sar37**	**Nl64**	**Tib32**	**Tib50**	**Tib191**	***A. palustris***	**Soil amoeba AND12**	***A. canadensis* BA**	***A. paracanadensis* OSA**	***A. spelaea***
***A. sardiniensis* Sar9**	100										
***A. parasardiniensis* Sar37**	95.1	100									
***A. nederlandensis* Nl64**	98.4	94.9	100								
***A. tibetensis* Tib32**	98.4	94.9	99.9	100							
***A. paratibetensis* Tib50**	96.0	96.0	95.6	95.7	100						
***A. neotibetensis* Tib191**	95.4	98.3	95.0	94.9	96.0	100					
A. palustris	89.0	90.3	87.8	87.8	90.0	90.4	100				
Soil amoeba AND12	90.2	90.2	89.9	89.2	91.2	90.6	83.7	100			
***A. canadensis* BA**	99.1	95.5	88.8	89.1	95.8	95.1	97.2	90.3	100		
***A. paracanadensis* OSA**	96.0	96.0	95.5	95.6	99.8	96.0	89.8	90.6	95.9	100	
A. spelaea	96.1	96.6	95.3	95.3	97.0	96.9	89.2	90.3	95.9	97.1	100

Table 5. Percentage identity matrix obtained with manually modified Clustal Omega alignments of the 5.8S rDNA in the *Allovahlkampfia* clade

	Sar9	**Sar37**	**Nl64**	**Tib32**	**Tib50**	**Tib191**	***A. palustris***	***A. spelaea***
***A. sardiniensis* Sar9**	100							
***A. parasardiniensis* Sar37**	95.5	100						
***A. nederlandensis* Nl64**	99.4	94.6	100					
***A. tibetensis* Tib32**	100	95.0	99.4	100				
***A. paratibetensis* Tib50**	97.5	94.0	98.1	97.5	100			
***A. neotibetensis* Tib191**	96.3	98.8	95.7	96.3	95.0	100		
A. palustris	93.8	93.4	93.2	93.8	92.5	93.2	100	
A. spelaea	99.4	95.4	98.8	98.4	98.1	96.9	94.3	100

Strain Tib23 had sequence similarities of 85.9 % with *F. ceborucoi* and between 80.7 and 80.9 % with the *Paravahlkampfia* spp. for the SSU rDNA (Table 6) and even lower for the 5.8S rDNA, 80.7 and 70.4 %, respectively (Table 7). Therefore, strain Tib23 should be considered to represent a new genus.

Table 6. Percentage identity matrix obtained with manually modified Clustal Omega alignments of the SSU rDNA in the *Fumerolamoeba-Paravahlkampfia* clade

	***P. alta* Tib23**	***F. ceborucoi***	***P. ustiana***	***P. francinae***	***P.* sp. A1PW2**	***P.* sp. LA**	***P.* sp. li3**
***Pagea alta* Tib23**	100						
F. ceborucoi	85.9	100					
P. ustiana	80.7	83.1	100				
P. francinae	80.9	83.2	99.7	100			
***Paravahlkampfia* sp. A1PW2**	80.7	82.9	98.3	98.7	100		
***Paravahlkampfia* sp. LA**	80.8	82.6	98.7	98.9	98.0	100	
***Paravahlkampfia* sp. li3**	80.9	82.9	99.5	99.8	98.4	98.7	100

Table 7. Percentage identity matrix obtained with Clustal Omega alignments of the 5.8S rDNA in the *Fumerolamoeba-Paravahlkampfia* clade

	***P. alta* Tib23**	***F. ceborucoi***	***P. ustiana***	***P. francinae***	***P. lenta***
***Pagea alta* Tib23**	100				
F. ceborucoi	80.7	100			
P. ustiana	72.3	68.5	100		
P. francinae	71.6	67.8	99.3	100	
P. lenta	70.4	68.1	97.9	98.6	100

Generally, all strains showed distinct molecular sequence patterns in different parts of helix 23 of the SSU rDNA, i.e. the V4 region. Shared molecular signatures provided further evidence of the classification mentioned above; a clade separating the *Allovahlkampfia* clade including soil amoeba AND12 from *Acrasis* was supported by several molecular patterns, which are unique for the respective clades. For instance, the motif 5' - TACACTT - 3' in helix 8 of the SSU rDNA is specific for the *Allovahlkampfia* clade, whereas the motif in *Acrasis* is 5' - TACTCGT - '3. Another example is the addition of a C in helix 17 of the SSU rDNA specific in the *Allovahlkampfia* clade, where there is a gap in *Acrasis*.

Similar patterns were detected for all other clades denoted above, e.g. the separation of *Fumarolamoeba*, Tib23 and several uncultured environmental sequences from *Paravahlkampfia,* e.g. the profile 5' - AAGGTTTGG - 3' shared by *Fumarolamoeba*, Tib23 and the uncultured environmental sequences at the end of helix 17 is strikingly different from the pattern of all *Paravahlkampfia* spp., which share the sequence 5' – TCGGTTTGC - 3'.

More sequence patterns distinguishing *Fumarolamoeba,* Tib23, uncultured sequences and *Paravahlkampfia* are shown in Supplementary Table 1.

Discussion

New isolates

We provide molecular evidence that the genus *Allovahlkampfia* includes several distinct species as all SSU rDNA and ITS sequences obtained were dissimilar. Until now, *A. spelaea* was the only cultivated and described species in this genus (Walochnik and Mulec 2009). We propose the following species names for the new isolates: *A. sardiniensis* (Sar9), *A. parasardiniensis* (Sar37), *A. nederlandensis* (Nl64), *A. tibetensis* (Tib32), *A. paratibetensis* (Tib50) and *A. neotibetensis* (Tib191), all names indicating the origin of the type strains.

In vahlkampfiids, morphology is not a reliable character to differentiate species. As such, morphologically similar species formerly united in the genus *Vahlkampfia* are now being placed in several distinct genera based on phylogenetic information (Brown and De Jonckheere 1999, De Jonckheere and Brown 2005). Therefore, we base the description of the species largely on molecular information. We are, however, aware that some species have very similar sequences, such as *A. nederlandensis* and *A. tibetensis*. Nevertheless, we are confident that these sequence differences are reliable characters to distinguish species as both sequenced phylogenetic marker molecules, ITS and SSU contain differences. Another example of species differentiation based on small sequence differences are found in the related vahlkampfiid genus *Tetramitus* (De Jonckheere and Brown 2005, Robinson et al. 2007). Similar examples of (nearly) identical SSU with profound differences in ecological functioning are reported for chrysophytes and cercocoan flagellates, suggesting cryptic species (Bass et al. 2007, Boenigk et al. 2007). Some morphologically different species of vannellid amoebae located in the supergroup Amoebozoa vary by as little as 3 bp in the entire SSU (Smirnov et al. 2007), while even the independent genera *Rhizamoeba* and *Leptomyxa* have nearly identical sequences, despite differing strongly in morphology (Smirnov et al. 2009).

We have performed several independent PCRs and sequence reactions to build consensus sequences and they always supported the differences observed between species. Therefore,

we are confident that the sequence differences observed are reliable positions to differentiate species, and not errors introduced by PCR or sequencing. Furthermore, the most closely related species based on molecular information *A. nederlandensis* and *A. tibetensis* show differences in temperature tolerance, suggesting even functional differences between these two strains, reinforcing the need to treat both as separate species.

In addition to the *Allovahlkampfia* spp. we isolated a strain, which cannot be included in any described genus. It forms a clade with several environmental sequences and the most closely related genera are *Fumarolamoeba* and *Paravahlkampfia*. We propose the genus name *Pagea*, in honor of Frederick Page, who not only created the name Heterolobosea, but also described many genera and species; the species name *alta*, indicates the high altitude from where the type strain originates. The large numbers of similar published environmental sequences indicate that species related to *P. alta* Tib23 are common soil inhabitants. Having no remaining culture *P. alta* Tib23 undoubtedly is a disadvantage, but we obtained sequenced of the entire SSU and ITS region, have pictures of the morphology and have frozen DNA as a type material. Therefore, we conclude that a formal genus description of *Pagea* is useful in order to assign sequences obtained in the increasing number of environmental sequencing surveys (Lara et al. 2007a, Bates et al. 2013).

Published sequences

'*Solumitrus palustris*' has been recently been erected as a new genus closely related to *Allovahlkampfia* (Anderson et al. 2011). Brown et al. (2012) put '*S. palustris*' between inverted commas and placed it into a single clade together with the genus *Allovahlkampfia*. They suggested that it could eventually be considered as a species of *Allovahlkampfia*, which was further supported by phylogenetic analyses of another study (Harding et al. 2013). Also our phylogenetic analyses of both 5.8S and SSU rDNA, which included several other related species, consistently placed '*S. palustris*' within the *Allovahlkampfia* clade (Figure 4 and 5, respectively). Our new *Allovahlkampfia* isolates had 95.3 to 97.0 % sequence similarities with *A. spelaea*. '*S. palustris*' had lower sequence similarities of 89.2 % for the SSU rDNA (Table 4) and 94.3 % for the 5.8S rDNA (Table 5) compared to the type strain. As especially the beginning and end of the sequence of '*S. palustris*' showed several mismatches in highly conserved regions we strongly suspect that there are serious sequencing errors in both

molecules. Therefore, we suggest that the sequencing for '*S. palustris'* needs to be repeated. But we do suggest already renaming it *A. palustris*. Other authors already suggested that it should be included in *A. spelaea* (Brown et al. 2012, Harding et al. 2013).

In their trees Brown et al. (2012) and Harding et al. (2013) have also placed the Heterolobosea BA and OSA into *A. spelaea*. From our phylogenetic analyses we conclude that they have to be considered different species within the genus *Allovahlkampfia*, as the SSU rDNA sequences of strains BA and OSA showed sequence similarities of 95.9 and 97.1 %, respectively to *A. spelaea* (Table 4). Therefore, we propose species names for these two isolates: *A. canadensis* (strain BA) and *A. paracanadensis* (strain OSA), names which indicate the origin of the type strains. A sequence of the entire ITS region of these two strains is highly desirable in order to support the species relationship within the genus *Allovahlkampfia*.

Phylogenetic implications

Individual strains formed several distinct branches consistently in the SSU and 5.8S rDNA (and *Allovahlkampfia* focused ITS) phylogeny suggesting the existence of several distinct species. As mentioned above, even the most closely related strains in our study based on phylogeny, i.e. *A. tibetensis* Tib32 and *A. nederlandensis* Nl64 are distinct not only by the presence of a long group I intron in *A. nederlandensis* Nl64, but also in the differential growth ability at different temperatures.

One sorocarp formation in one *Allovahlkampfia* strain (the BA isolate) could be more a plesiomorphic artifact and does not necessarily lead to the inclusion of allovahlkampfiids into the family Acrasidae (Brown et al. 2012). Sorocarps were not formed by any of the new *Allovahlkampfia* strains in the media tested and has only been observed once (Brown et al. 2012). Therefore, taking into account clear phylogenetic differences we propose to keep these two genera separately without unifying both into a single family Acrasidae (Brown et al. 2012) until more information is obtained on other lineages that gap *Allovahlkampfia* and *Acrasis,* such as strain AND12. The formation of a simple sorocarp within the BA strain of *Allovahlkampfia* was also reported to be very distinct from the ones in the genus *Acrasis*. Taken together, a clear separation even at the family level can be inferred between acrasiids and allovahlkampfiids. ITS sequences of species within *Acrasis* and consequent phylogenetic

analysis of these are needed and will eventually clarify the designation on higher levels between acrasiids and allovahlkampfiids.

Our phylogenetic analyses also indicate that *Allovahlkampfia* is sister to the soil amoeba AND12 (Lara et al. 2007a). Due to significant sequence variations we agree with De Jonckheere et al. (2011b) and consider it as a separate genus. This organism is of specific interest as it bridges the genus *Allovahlkampfia*, where only once a form resembling an acrasid slime mold has been reported (Brown et al. 2012), with the purely acrasid slime mold forming genus *Acrasis* (Brown et al. 2012). No stage of soil amoeba AND12 that resembled an acrasid slime mold has been described yet. Therefore it is possible that the ability to form sorocarps might have been present in the progenitor of all organisms within that family, but has been lost in several species inside the genus *Allovahlkampfia*. It is also possible that individual *Allovahlkampfia* species only rarely form sorocarps and under highly specific conditions which are not yet known. But as currently sorocarp formation in *Allovahlkampfia* seems rare, at least under laboratory conditions, and detailed information on species that bridge the genera *Acrasis* and *Allovahlkampfia* are lacking, further research is needed in order to propose a sensible prediction on the higher level taxonomic affinities between both genera.

Pagea alta Tib23 undoubtedly represents a new genus based on phylogenetic information from both the 5.8S and SSU rDNA. Based on morphology, *P. alta* Tib23 shares several features with the closely related genera *Paravahlkampfia* and *Fumarolamoeba* such as high eruptive activity and the presence of both hyaline and granular regions (Figure 3). The presence of pronounced uroidal filaments, a single nucleus and a limax shape during locomotion differentiates *P. alta* Tib23 from *Fumarolamoeba* and makes it similar to *Paravahlkampfia*. Cyst sizes are, however, smaller than reported for any *Paravahlkampfia* species and in the lower range of cysts of *Fumarolamoeba*. Unfortunately, the strain went extinct before further morphological investigations and growths tests could be performed, so we are unable to compare specific features distinguishing it from other genera. Nevertheless, the phylogenetic information from both the SSU rDNA and 5.8S genes prove that *Fumarolamoeba* is the sister genus, and both are strongly different from the genus *Paravahlkampfia*.

The establishment of the genus *Fumarolamoeba* (De Jonckheere et al. 2011b) is appropriate and is supported by our analyses, and *P. alta* Tib23 proves to represent its closest relative. Considerable sequence dissimilarity suggests that *P. alta* Tib23 and several uncultured sequences (Valster et al. 2009, Valster et al. 2010, Valster et al. 2011, Farhat et al. 2012) should be considered as a separate genus, most likely containing several independent species.

Ecological implications

A. tibetensis Tib32, *A. paratibetensis* Tib50 and *A. neotibetensis* Tib191, and the new genus *P. alta*, were isolated from extreme altitudes in Tibet. Interestingly, De Jonckheere (2006) detected distinct species in the genus *Naegleria* that were identical in the arctic and sub-antarctic, but have not been detected anywhere else. We suspect that these particular *Naegleria* spp. might also be present at high altitude, but we were unable to isolate any *Naegleria* strain from the high altitude in Tibet. Maybe this is due to the isolation method used in our investigation, which is not the common procedure for isolating *Naegleria* strains. Also Robinson et al. (2007) described two closely related *Tetramitus* species both from cold and alpine sites.

Species of the class Heterolobosea have often been isolated from extreme environments such as low pH (Amaral-Zettler et al. 2002, Baumgartner et al. 2009), high salinity (Park and Simpson 2011) and extreme heat (De Jonckheere et al. 2011a) or cold (De Jonckheere 2006). In general it seems that strains of heteroloboseans can easily adapt to adverse conditions, as, in addition to those species isolated form high altitudes, *A. sardiniensis* Sar9 and *A. parasardiniesis* Sar37 came from hot and dry soil in Sardinia. Further, *T. thermoacidophilus* (Baumgartner et al. 2009) is an extreme thermophile (54 °C) and acidophile (pH 1 to 5), while most of the closely related *Tetramitus* spp. grow under 'normal' conditions. This suggests that a large variety of vahlkampfiid species are resistant to extreme environmental conditions, and might carry out important ecological functions in these environments. The environmental niche of closely related organisms seems also to be large within the genus *Allovahlkampfia* as the closely related strains Nl64 and Tib32 were isolated at very distant locations from soils, while '*S. palustris*' renamed here *A. palustris* (see above), was isolated from temperate freshwater. Therefore, tolerance of extreme conditions seems

not a characteristic of the genus, apparently many heterolobosean taxa show a marked tendency toward adaptation to extreme environments.

All genera within the family Allovahlkampfiidae seem to have a ubiquitous distribution and contain soil and freshwater species with a broad environmental niche.

Diagnosis

Genus *Allovahlkampfia*

Uninucleated amoebae, without a known flagellate stage, sharing eruptive pseudopodia formed during locomotion. Locomotive form limax or flabellate with eruptive pseudopodia. Uroidal filaments of different lengths often present during locomotion. Size and shape of amoebae strongly differing even within strains. Also the spherical cysts without pores produced in culture vary strongly in size. Usually no distinct multicellular stage formed.

No growth observed at 37 °C.

Food: mainly bacterivorous, some showing facultative feeding on yeast

Habitat: Soil, freshwater or tree bark

Molecular patterns: sequences of both SSU and 5.8S rDNA with > 95 % similarity between strains, with diverse molecular sequence patterns such as motif 5' - TACACTT - 3' in helix 8 only shared by members in this clade. Closest phylogenetic relationship based on the SSU rDNA with soil amoeba AND12 (~ 10 % bp difference) and the genus Acrasis (> 15 % bp differences). All species show sequence differences in the V4 region of the SSU rDNA.

Species *A. sardiniensis* (Sar9), *A. parasardiniensis* (Sar37), *A. nederlandensis* (Nl64), *A. tibetensis* (Tib32), *A. paratibetensis* (Tib50) and *A. neotibetensis* (Tib191)

***A. sardiniensis* Sar9**

Diagnosis: Length of trophozoites among the smallest in *Allovahlkampfia* ranging from 18.2 - 28.8 µm (average 23.6 µm) in length and 3.6 - 5.2 µm (average 4.6 µm) in width. Locomotion usually in a limax form, but often also flabellate. No pronounced uroidal filaments formed. Cyst diameter 5.6 µm - 10.8 µm (average 7.4 µm).

As the morphology is very similar to the type species of the genus *Allovahlkampfia*, the species can only be identified by ITS and / or SSU rDNA sequences (EBI accession N°s: KF547907 and KF547914, respectively). It has a specific length and sequence in both the ITS1 (163 bp) and ITS2 (128 bp) and the molecular sequence profile 5' - TTCAGCAATGGAGGAACGT - 3' in V4 of the SSU rDNA allowing its identification.

Type locality: the type strain *A. sardiniensis* Sar9 isolated on the island of Sardinia (N40°46', E9°104) at 181 m altitude from grassland soil under a cork oak silvo-pastoral system (Bagella et al. 2013).

Reference material: The culture is maintained in the cryopreserved state in liquid nitrogen at the Culture Collection of Algae and Protozoa (CCAP) accessioned as *Allovahlkampfia sardiniensis* strain Sar9 under number CCAP 2502/1.

***A. parasardiniensis* Sar37**

Diagnosis: Length of trophozoites ranging from 20.8 - 30.0 µm (average 23.8 µm) with very small width of 3.4 - 6.0 µm (average 4.6 µm). Locomotion almost exclusively in a limax shape. Uroidal filaments occasionally produced, sometimes as long as the cell body. Cyst diameter 5.4 - 8.4 µm (average 6.6 µm).

Only molecular sequences provide reliable evidence for the designation of this species (EBI accession N°: KF547908 and KF547915, respectively). Specific length and sequence in both the ITS1 (117 bp) and ITS2 (130 bp). Length of the 5.8s rDNA (163 bp in contrast to 161 bp in other allovahlkampfiids) and the molecular sequence profile 5' - TCGGTCTCCGGGTGG GTTAC - 3' only shared with *A. neotibetensis*. However, *A. parasardiniensis* possesses a unique sequence profile 5' - ATCTCCAGT - 3' in the beginning of V4.

Type locality: Type strain *A. parasardiniensis* Sar37 isolated on the island of Sardinia (N40°46', E9°104) at 181 m altitude from grassland soil under a cork oak silvo-pastoral system (Bagella et al. 2013).

Reference material: The culture is maintained in the cryopreserved state in liquid nitrogen at the CCAP accessioned as *Allovahlkampfia parasardiniensis* strain Sar37 under number CCAP 2502/2.

***A. nederlandensis* Nl64**

Diagnosis: Length of trophozoites is the longest found in the genus, in the range of 21.8 - 50.8 µm (average 33.2 µm), width 3.6 - 8.6 µm (average 5.8 µm). Despite large trophozoites, very small cysts formed (5.0 - 7.8 µm, average 6.2 µm). Locomotion mostly flabellate with long uroidal filaments, sometimes as long as the cell body. Cyst diameter 5.6 - 10.8 µm (average 7.4 µm). Very fast growth in liquid cultures, but the only species of the genus not growing at 30 °C.

Identification only possible with phylogenetic information. The molecular sequence profile 5' - CTCAGCAATGGGGAACGC - 3' in V4 specific for *A. nederlandensis*. (EBI accession N°: KF547909 and KF547916, respectively). The type strain has a long group I intron (1,358 bp) in the SSU rDNA.

Type locality: type strain *A. nederlandensis* Nl64 isolated from a pasture soil in the Netherlands (N52°06', E6°00') at 57 m altitude.

Reference material: The culture is maintained in the cryopreserved state in liquid nitrogen at the CCAP accessioned as *Allovahlkampfia* nederlandiensis strain Nl64 under number CCAP 2502/3.

***A. tibetensis* Tib32**

Diagnosis: Length of trophozoites 27.6 - 36.2 µm (average 30.0 µm), width 5.0 - 8.8 µm (average 7.4 µm). Locomotion mostly flabellate with less pronounced uroidal filaments. Cyst diameter 5.0 - 8.2 µm (average 6.6 µm).

Specific identification only possible with detailed molecular analyses, as sequences of both the ITS region and SSU rDNA are similar to those of *A. nederlandensis*. However, the molecular sequence profile 5' - CTCAGCGATGGGGAACGC - 3' in V4 is specific for *A. tibetensis*. (EBI accession N°: KF547910 and KF547917, respectively).

Type locality: type strain *A. tibetensis* Tib32 isolated from a high altitude soil in Tibet (N29°49', E92°20) at 5033 m altitude.

Reference material: The culture is maintained in the cryopreserved state in liquid nitrogen at the CCAP accessioned as *Allovahlkampfia tibetensis* strain Tib32 under number CCAP 2502/4.

***A. paratibetensis* Tib50**

Diagnosis: Length of trophozoites 21.2 - 31.2 µm (average 25.2 µm), width 5.6 - 9.2 µm (average 6.6 µm). Locomotion limax or flabellate with pronounced uroidal filaments, often much longer then the moving cell. Very fast production of cysts even in young cultures (~ 5 days) with diameters of 5.8 - 9.2 µm (average 8.0 µm).

Specific identification only possible based on sequence information of the ITS region and SSU rDNA. Close sequence similarity to Heterolobosea OSA but the molecular sequence profile 5' - GCCTTTTGGTCTCCAGGG - 3' in V4 specific for *A. paratibetensis*. (EBI accession N°: KF547911 and KF547918, respectively).

Type locality: type strain *A. paratibetensis* Tib50 isolated from a high altitude soil in Tibet (N29°42', E92°10) at 4149 m altitude.

Reference material: The culture is maintained in the cryopreserved state in liquid nitrogen at the CCAP accessioned as *Allovahlkampfia paratibetensis* strain Tib50 under number CCAP 2502/5.

***A. neotibetensis* Tib191**

Diagnosis: Length of trophozoites 19.4 - 26.4 µm (average 23.0 µm), width 4.2 - 7.6 µm (average 6.2 µm). Locomotion predominantly limax. Uroidal filaments mostly present, but shorter than moving cell. Cyst diameter 6.4 - 8.6 µm (average 7.6 µm). Fast growth at room temperature.

Characteristic 5.8S rDNA sequence length of 163 bp and the molecular sequence profile 5' - TCGGTCTCCGGGTGGGTTAC - 3' shared with *A. parasardiniensis*. Length of the ITS1 and ITS2 are, however, longer in *A. neotibetensis* and differ at several positions (EBI accession N°: KF547912 and KF547919, respectively). Furthermore, *A. neotibetensis* also has the unique profile 5' - ATTCCCAGT - 3' in the beginning of V4.

Type locality: the type strain *A. neotibetensis* Tib191 was isolated from a high altitude soil in Tibet (N29°52', E92°34) at 4149 m altitude.

Reference material: The culture is maintained in the cryopreserved state in liquid nitrogen at the CCAP accessioned as *Allovahlkampfia neotibetensis* strain Tib191 under number CCAP 2502/6.

Genus *Pagea*

Uninucleated amoebae, no known flagellate stage, exhibiting high eruptive activity while forming pseudopodia. Locomotive form limax. Uroidal filaments, sometimes branching, formed during locomotion, often longer than actual cell body. Cysts of almost equal diameter but of different shape varying from round to wrinkled forms.

Food: bacterivorous

Habitat: Soil

Only one species known. But environmental sequences might represent other species of the genus.

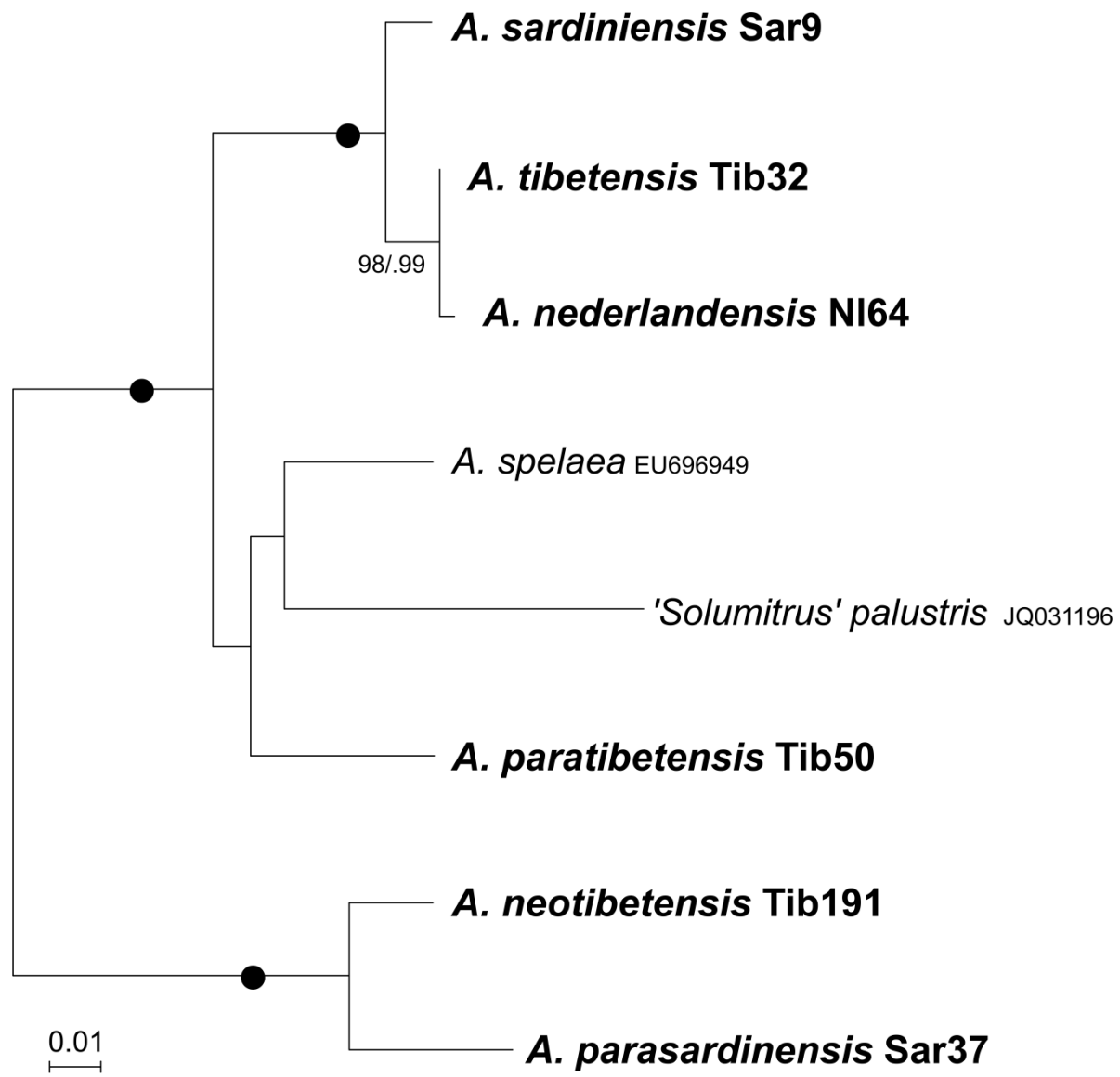
***Pagea alta* Tib23**

Length of trophozoites 14.8 - 31.1 µm (average 20.9 µm) in length and 3.9 - 13.4 µm (average 7.9 µm) in width. Locomotion with highly eruptive pseudopodia in limax form. Pronounced uroidal filaments formed. Cysts differing little in size (diameter 5.3 - 6.2 µm, average 5.7 µm) but differing in shape (wrinkled to round). Very distinct molecular sequencing profile both in the entire ITS region, including the 5.8S rDNA, and SSU rDNA (EBI accession N°: KF547913 and KF547920, respectively).

Type locality: type strain *P. alta* Tib23 isolated from a high altitude soil in Tibet (N29°52', E92°34) at 4149 m altitude.

Reference material: the culture went extinct, but frozen DNA as a type material is available upon request.

Supplementary



Supplementary Figure 1. Maximum likelihood tree based on the entire ITS region focusing on *Allovahlkampfia* spp. Support values at each node presented for RAxML / BI. RAxML ML bootstrap values and BI posterior probabilities equal to 100 % / 1.00, respectively, are represented by a black dot, whereas – is placed for unrecovered topologies and support values < 50 % (RAxML) / 0.50 (BI).

Supplementary table 1. SSU rDNA sequence motifs of *Pagea alta* Tib23, closely related uncultivated clones, *Fumarolamoeba ceborucoi,* and *Paravahlkampfia* spp.; - : SSU region not sequenced

Helix #	Starting position (in *F. ceborucoi*)	*Pagea alta* Tib23	Uncultured eukaryote (HQ998889, HQ999738, HQ999786, EU860662, GU970145, GU970340, GU970378)	Uncultured eukaryote GQ861576	*Fumarolamoeba ceborucoi*	*Paravahlkampfia* spp.
5	44	GTCTT	GTCTT	-	GCTAG	GCTCG
7	116	CTAGCTTCTTTTAT	CTAGCTTAAAGTTAA	-	CTAGCTTCTTTTAT	CTAGTTTWTCTTAC
9-10	165	GAACCAAAGCT	GAACCAAAGCT	-	GAATCAACGTC	GCTCAAAAGCC
11	268	GGA	GGA	-	GAA	GAG
12	319	GGCCGTCA	GGCCGTCA	-	GGCTATCA	GACACTTA
13	335	GAAAATTGGGGTTT	GAAAATTGGGGTTT	-	GGGAATCAGTGTTT	GGGAATCAGTGTTC
15	381	T	T	-	C	G
16	426	AT	AT	-	AT	AATT
17	454	TCCTTC	TCCTTC	-	TCCTTA	ACCTCA
18	505	CAAATT	CAAATT	-	CAAACT	TAAATC
18-19	523	ACA	ACA	-	ACA	TCG
32	2245	T	-	T	T	C
35-36	1733	GCGGGA	-	ACGGGA	GCGGGA	GCGGGG
36	1751	AT	-	AT	AC	TT
36-38	2047	G	-	G	G	T
37	1761	ATGAG	-	ATGAG	GTTAA	GGAGA
37	1780	ATATG	-	ATATG	ATTTG	GA
37	1799	TTTTGGAA	-	TTTTGGAA	CTCTGGAA	CTTTGATT
43	1882	TTAAC	-	TTAAC	TTAAC	CTAAT
43-44	1978	A	-	A	A	G
44	2010	A	-	A	A	G
45	2080	C	-	C	T	T
45-46	2084	G	-	G	C	A
47-48	2190	A	-	A	G	G
48-49	2234	T	-	T	T	C

Part 2

Diversity analyses characterizing soil protist communities with four different techniques

Part 2 – Chapter 5

Soil water availability strongly alters the abundance of soil protists with distinct impacts on individual genera of soil amoeba

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Abstract

Drought and heavy rainfall are opposite conditions expected to result from increasingly extreme weather conditions during climate change; and both scenarios will strongly affect the functioning of soil systems. However, little is known on the specific responses of soil microorganisms, whose functioning is intimately tied to the magnitude of the water-filled pore space in soil. Soil heterotrophic protists, being an important part of aquatic soil organisms are considered as key-regulators of microbial carbon and nutrient turnover. We investigated the responses of distinct protist taxa to changes in soil water availability using a modified enumeration technique that enabled quantification of protist taxa up to genus level. Our study revealed a non-linear shift of protist abundance with decreasing soil water availability and this became already apparent at a maximum water-filled pore size of ≤ 40 µm. Generally, taxa containing large specimen were more severely affected by drought, but responses to either drought or rewetting of soils were not uniform among taxa. Changes in water availability may thus affect the functioning of soil ecosystems long before aboveground “drought” effects become apparent

Introduction

Global temperature has increased and is expected to further increase in the coming century, with annual daily maximum temperature rising by about 3 °C by mid-21st century and by about 5 °C by the late 21st century, resulting in more frequent and extreme drought events in many parts of the world (IPCC 2012, Sherwood et al. 2013). The term 'drought' is generally associated with the damage of plants due to lack of soil water (Kramer 1983), but limited water availability can impair the function of soil ecosystems long before symptoms become visible aboveground. This is especially true for processes performed by microbial soil organisms, whose functions are intimately tied to the magnitude and connectivity of water films around soil particles. We are, however, still largely ignorant of specific responses of soil organisms to these global change phenomena (de Vries et al. 2012a, de Vries et al. 2012b, Bradford 2013).

Soil protists, due to their high biomass, and with estimated annual production rates of > 100 kg ha^{-1} (Bouwman and Zwart 1994) are considered to be key players in carbon (C) and nutrient cycling in soils (Schröter et al. 2003, Christensen et al. 2007, Crotty et al. 2012b). Protists are the most basal microbial consumers, being a fundamental source for C transfer to higher trophic levels in the soil food web (Crotty et al. 2012a). Direct effects of protists result from their high grazing impact on microbial communities, but more important appear indirect effects of protists that lead to a stimulation of microbial turnover and respiration (Clarholm 1985, Bonkowski 2004, Anderson 2008) and plant performance (Koller et al. 2013). For example laboratory experiments with planted soil have shown that consumption of microbial biomass by protists led to a 20 - 40 % increased microbial activity and CO_2-C release (Alphei et al. 1996, Rosenberg et al. 2009) and microbial liberation of CO_2 from decomposing plant litter increased up to 100 % in presence of protists (Bonkowski et al. 2000a).

Despite living in soil, protists are aquatic organisms, and their function ultimately depends on the availability of water in the three-dimensional pore space in soil availability (Anderson 2000, Griffiths et al. 2001). Decreasing soil water availability has been shown to reduce protist replication rates due to limited mobility of protist grazers in the microvolumes of soil water and hence reduced accessibility to bacterial prey (Darbyshire 1976). This can result in significantly negative effects on soil nutrient cycling and plant growth (Kuikman et al. 1991).

Protists, are extremely diverse and individual taxa differ fundamentally based on phylogenetic relatedness, morphology and behavior (Cavalier-Smith 1998, Adl et al. 2012). The size of individual taxa can differ by at least 3 orders of magnitude in soil (Foissner 1998, Finlay 2002, Glücksman et al. 2010). Consequently, taxon-specific dependencies on soil water levels are likely, simply because large, free-swimming taxa will be more vulnerable to desiccation than small, surface-associated forms. However, it is largely unknown, how complex, natural protist communities respond to altered soil moisture regimes.

Taxonomic studies on natural populations of soil protists have mainly been restricted to groups with larger specimens that dominate the upper humus layers and share fixed, readily determinable morphological characters, such as testate amoebae and ciliates (Bamforth 1971, Foissner 1987, 1999b, Bamforth 2007, Krashevska et al. 2007). In comparison, knowledge on the taxonomic composition of communities of flagellates and naked amoebae is extremely limited, despite these groups contain a huge diversity of soil species, and vastly outnumber other groups in the mineral soil horizons (Elliott and Coleman 1977, Finlay et al. 2000, Scharroba et al. 2012, Domonell et al. 2013). Unlike suspension and filter feeders such as many ciliated protists, naked amoebae and amoeboid flagellates can directly graze on the bacterial colonies and biofilms attached to substrates (Darbyshire et al. 1989, Parry et al. 2004), and their flexible bodies seem particularly suited to survive in the tiny water films around mineral particles. They still can access prey in water-filled soil pores of only 2 µm in diameter with help of their elongate pseudopodia (Elliott et al. 1980, Darbyshire 2005), but their overall activity is expected to decline. Profoundly fast changes in abundance of flagellates and naked amoebae were reported with increasing soil moisture using cultivation-based enumeration studies (Clarholm 1981, Anderson 2000, Bischoff 2002), but none of these studies aimed to resolve the taxonomic composition of the protist communities. Recent high-throughput soil surveys confirmed strong impacts of moisture on the community composition of soil protists (Baldwin et al. 2013, Bates et al. 2013), but these methods in turn failed to provide quantitative information on protist abundance (Medinger et al. 2010, Pawlowski et al. 2011, Weber and Pawlowski 2013, Stoeck et al. 2014). Further it is unclear if protist abundance will decline in a linear manner. Since protists are so extremely diverse, a linear decline of total protist abundance can be assumed. Therefore, detailed quantitative studies distinguishing the responses of specific protist taxa to changes in soil moisture regimes are needed.

The purpose of our study was to obtain quantitative estimates of the abundance of amoeboid soil protists with high taxonomic resolution, and to relate their expected changes in community composition to specific moisture conditions. The study was performed as part of a controlled semi-field experiment in Terrestrial Model Ecosystems (TME) (Knacker et al. 2004). We expected higher abundances of protists at higher soil water availability as habitat space and connectivity between particles would increase with increasing water films. Further, we hypothesized stronger changes in community composition and shifts towards smaller species at decreasing water availability.

Materials and Methods

Study site and experimental setup

A controlled semi-field experiment in terrestrial model ecosystems (TME) consisting of undisturbed soil cores was set up close to Flörsheim, Germany (N50°04'; E8°40') in order to manipulate soil moisture and evaluate effects on soil meso- and microfauna (www.bik-f.de). These soil cores (30 m diameter, 40 cm depth) were excavated from a meadow on alluvial clay. Soil texture was a silty clay with 9.9 % sand, 41.9 % clay and 28.2 % silt, that contained 2.93 % organic matter (pH ($CaCl_2$) = 6.9) and had a water holding capacity of 58.7 %.

After the TME had been extracted in the field they were incubated in plastic cylinders of corresponding size, which could be drained at the bottom on temperature-controlled carts under laboratory conditions (see Knacker *et al.* 2004 for details of the TME approach). The temperature of the soil cores was kept at 18 - 24 °C and 300 ± 50 µE m^{-2} s^{-1} light intensity. The volumetric soil water content of each TME was monitored in the upper 6 cm using hydra probes (accuracy ± 3 % v/v, ecotech Umwelt-Meßsysteme GmbH, Bonn, Germany). This monitoring enabled to adjust the daily irrigation volumes individually for each TME replicate. Depending on the watering volume either a pump disperser or "rain heads" (i.e. acrylic glass vessels with micropipettes at their bottom) were used for irrigation (Knacker et al. 2004). Moisture manipulation started at the beginning of the experiment (start: March 29th, 2013) in all TMEs, aiming for 30 %, 50 % and 70 % of the water holding capacity (WHC). The desired WHC levels were reached after 2, 9, and 31 days after starting the study, respectively. One week before harvest (harvest: July 19th, 2013), the irrigation scheme was changed in half of the TMEs in order to simulate heavy rain events and samples checked daily to retain the

targeted moisture values. Individual moisture treatments were replicated three times (Figure 1).

To estimate the diameter of water filled pores at a given moisture level, water holding capacities were converted into water potentials using the SPAW graphical interface (Saxton and Rawls 2006). The pore neck diameter of the largest water-filled pores (µm) was then calculated using the formula $D = 300 * P^{-1}$, where D is the pore neck diameter of the largest water-filled pores (µm), and P is the water potential (kPa) according to Carson et al. (2010). By calculating maximum water filled pore sizes at any given soil moisture level, it might be possible to infer the habitable pore space and to generalize results on protist performance at different soil moisture levels.

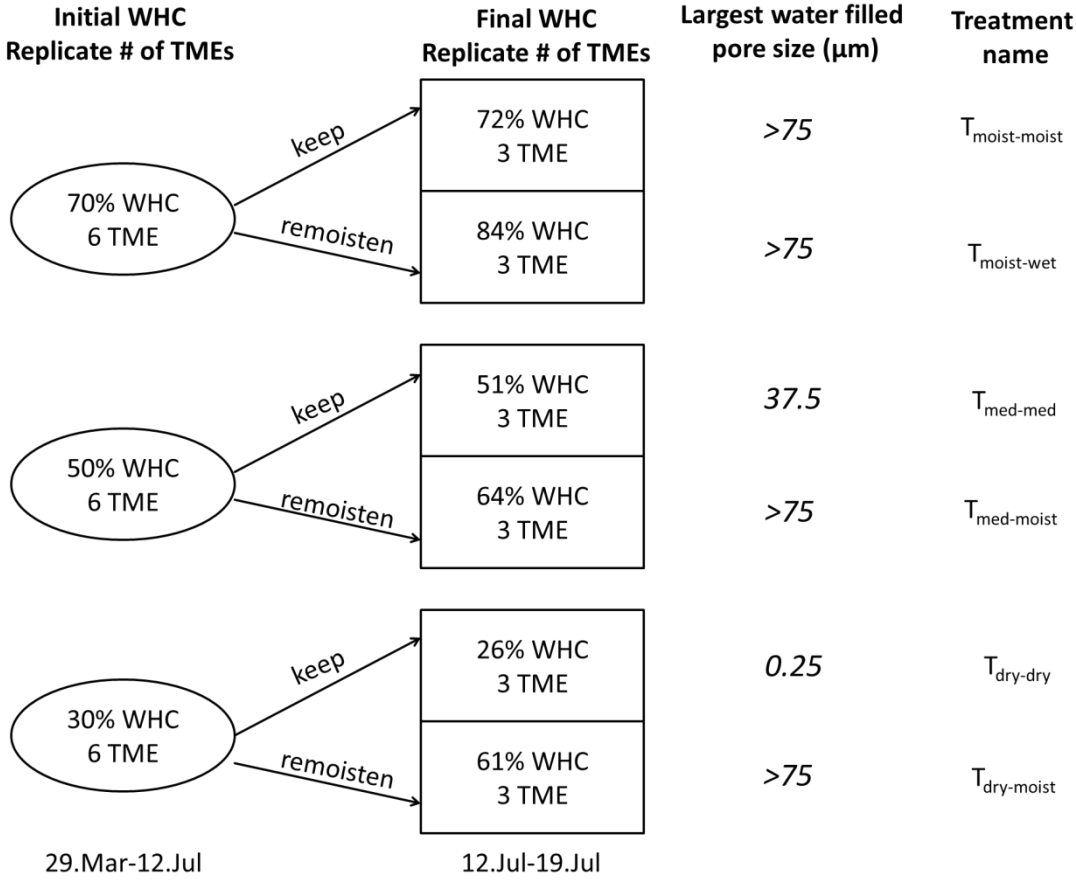

Figure 1. Left: Schematic diagram of the experimental design (TME: Terrestrial Model Ecosystem; WHC: Water Holding Capacity); largest water filled pore sizes (µm) estimated according to SPAW graphical interface; Treatment names as they are used in the present manuscript; they reflect the aspired soil moisture during the first 15 weeks and during the last week of the experiment.

After destructive sampling the TME's, soil samples were stored at 5 °C for up to one week. Soil water content was determined after oven drying of a small subsample for 2 days at 80 °C. The mean values are depicted in Figure 1.

Enumeration of protists

Protist numbers were determined by a liquid aliquot method (LAM) according to Butler and Rogerson (1995) with slight modifications to simplify the procedure. Briefly, 20 g fresh weight soil of each replicate was homogenized by vigorous shaking in a plastic bag for 10''. A subsample of 1 g dry wt was suspended in 200 ml Neff's Modified Amoeba Saline (NMAS; Page 1976) by vigorous shaking at 100 rpm, on an orbital shaker for 10' (Köttermann, Germany) followed by inversing the suspension and shaking vigorously for 10'' to detach protists from soil particles. The homogenized samples were left to settle for 5', before 5 µl aliquots from the centre of the suspension were inoculated each to one of 144 wells of two 96 well microtitre plates (flat bottom, Sarstedt, Germany) which were filled each with 195 µl 0.15 % wheat grass medium (WGM) (Weizengras, Sanatur, Singen, Germany). Plates were sealed with Parafilm and incubated at 15 °C in the dark. Each well was checked twice after 14 and 28 days for presence of protists with an inverted microscope (Nikon Eclipse TS100) at 100 x and 200 x magnification.

Protists were determined and grouped to morpho-group level according to Lee et al. (2000), Smirnov and Brown (2004), Smirnov et al. (2011b) and Jeuck and Arndt (2013). Naked amoebae were identified to genus level whenever possible. The most recent phylogeny of amoebae was then used to combine individual genera together into higher taxonomic levels (Smirnov et al. 2011b). Figure 2 gives an overview of all genera of Amoebozoa identified with their respective phylogenetic classification. Small amoebae (< 7 µm) could not be reliably identified by light microscopy and were grouped together as "Nanoamoebae", which consequently represents an artificial taxonomic assembly of amoeba.

Finally, total numbers of flagellates and amoebae were calculated from the cumulative abundances in the 96 well-plates, the relative proportions of individual groups were determined and corrected using a Poisson distribution.

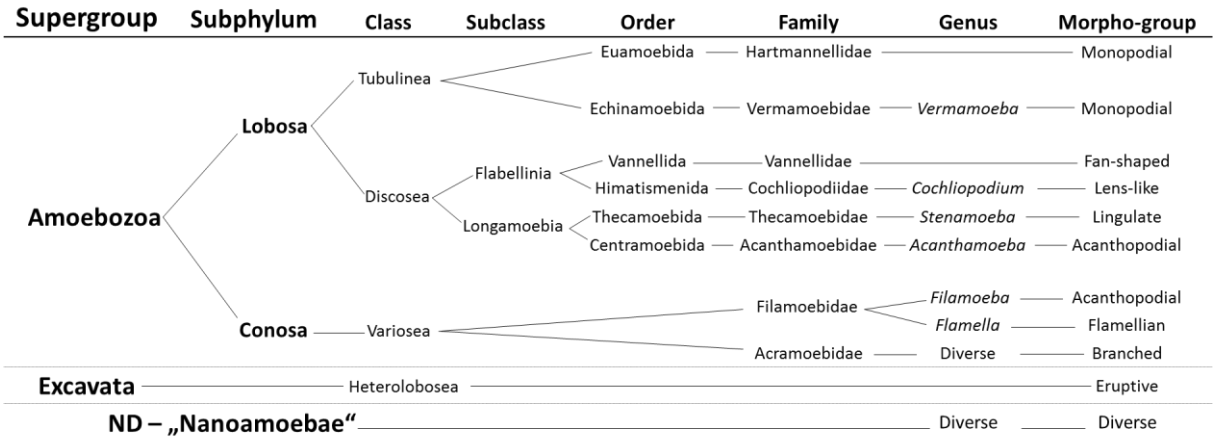

Figure 2. Phylogenetic classification of the identified amoebae according to Smirnov et al. (2011b) and morpho-groups according to Smirnov and Brown (2004).

Statistical analyses

Because relative ratios of protist taxa were not independent of each other, the data were analysed by multivariate analysis of variance (MANOVA, Roy's Greatest Root). In case of significant MANOVA followed a one-way analyses of variance (ANOVA), i.e. 'protected ANOVA' (Scheiner and Gurevitch 2001).

Relative contributions of individual protist taxa were arcsinus transformed to approximate homogeneity of variance. All ANOVAs were performed in R version 3.0.2 (R foundation for statistical computing; available at http://www.R-project.org). Spearman rank correlations between protist taxa were performed to investigate if taxa responded similar to changes in moisture levels. Correlations were tested for significance using GraphPad Prism software, version 5 (GraphPad Software, San Diego, CA, USA). The Shannon–Weaver index was applied to compare protist diversity between treatments (Shannon and Weaver 1949),

$$H = -\sum_{i} p_i * \ln p_i$$

where p_i is the proportion of the *i*th morpho-group.

The Simpson index (D) was applied to determine the evenness of protist communities (Simpson 1949)

$$D = 1 - \sum_{i=1}^{s} \frac{n_i(n_i - 1)}{n(n-1)}$$

where n_i is the number of individuals of one morpho-group and n is the total number of individuals.

Results

Abundance

As expected, total protist numbers were significantly affected by soil moisture levels ($F_{[5, 12]} = 3.39$, $p < 0.05$). Total protist abundance differed 8-fold between the two most extreme moisture treatments, i.e. $T_{dry\text{-}dry}$ and $T_{moist\text{-}wet}$ (Figure 3). Total flagellate numbers tended to increase with increasing soil moisture ($F_{[5, 12]} = 2.64$, $p = 0.08$), but the higher number of total protists was mainly caused by an increased abundance of amoebae ($F_{[5, 12]} = 3.59$, $p < 0.05$), which reached 7.2-fold higher numbers in $T_{moist\text{-}wet}$ compared to $T_{dry\text{-}dry}$ (Figure 3).

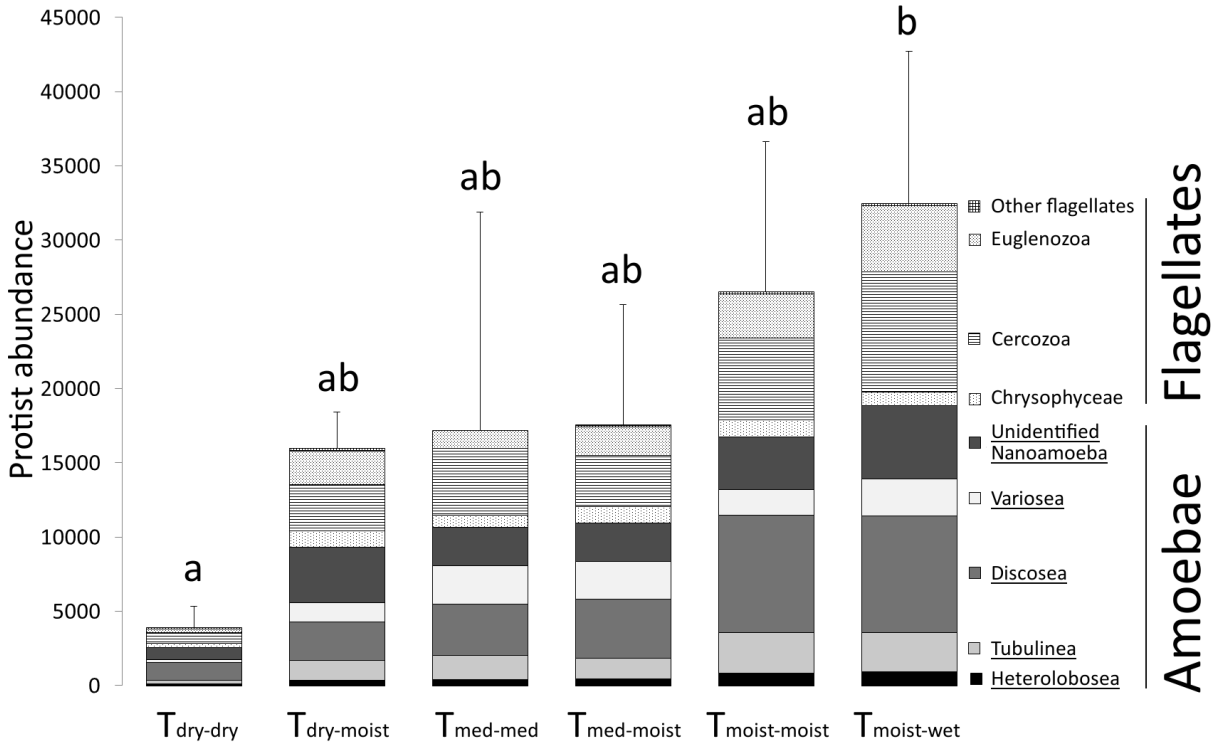

Figure 3. Total abundance of protists (individuals g^{-1} soil dry weight) showing the abundance of major clades of flagellates and amoebae with increasing soil moisture levels. For an explanation of moisture treatments see Figure 1. Underlined: Classes of amoebae; Means ± SD. Bars with different letter are statistically significant (Tukey's HSD test; $p \leq 0.05$).

Generally, protist numbers responded positively with increasing soil moisture contents. Overall both, flagellates and amoebae increased, but numbers of amoebae increased relatively stronger than flagellates as seen by the lower than 1:1 slope in Figure 4.

Although a general uniform positive response of protist abundance to increasing soil water availability was observed, correlations revealed two distinct clusters of protists that responded more uniformly to each other. The Spearman's rho revealed a statistically positive relationship between the amoeba classes the Discosea with both Tubulinea ($\rho = 0.79$, $R^2 = 0.70$, $p < 0.001$) and Variosea ($\rho = 0.69$, $R^2 = 0.46$, $p < 0.001$). Similarly Heterolobosea abundance changed parallel with the flagellate genera Euglenozoa ($\rho = 0.7$, $R^2 = 0.45$, $p < 0.001$), Cercozoa ($\rho = 0.6$, $R^2 = 0.38$, $p < 0.01$), and Chrysophyceae ($\rho = 0.5$, $R^2 = 0.26$, $p < 0.01$).

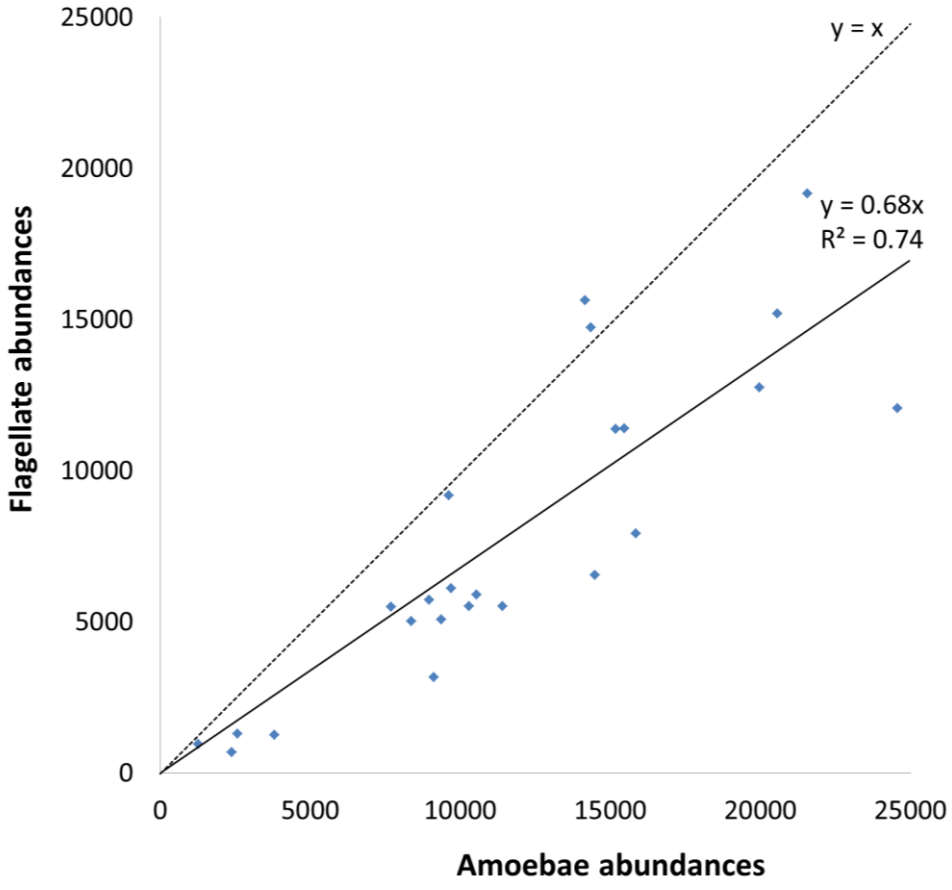

Figure 4. Correlation of the abundances of flagellates to amoebae; dotted line: y = x.

Almost all protist taxa could still be detected even in the extreme moisture treatments, but significant shifts in relative abundance of specific clades were detected in specific clades of

amoebae. For instance, amoebae of the class Discosea were most abundant in moist soils ($T_{moist\text{-}wet}$ and $T_{moist\text{-}moist}$) averaging 7,900 ind g^{-1} dry wt soil. Numbers decreased to 33 % and 15 % in $T_{dry\text{-}moist}$ and $T_{dry\text{-}dry}$, respectively ($F_{[5, 12]} = 6.49$, $p < 0.01$). Within Discosea, numbers of Longamoebia decreased most strongly with decreasing soil moisture ($F_{[5, 12]} = 5.02$, $p < 0.05$), and among Longamoebia the genus *Stenamoeba* responded most sensitive to decreasing soil moisture ($F_{[5, 12]} = 13.92$, $p < 0.001$; Figure 3), while the genus *Acanthamoeba* decreased only marginally at the lowest soil moisture treatment ($F_{[5, 12]} = 2.39$, $p = 0.09$) (Figure 5). Heterolobosea increased in $T_{moist\text{-}wet}$ and decreased in $T_{dry\text{-}dry}$ compared with $T_{med\text{-}med}$, but the overall effect of soil moisture on Heterolobosea was only marginal ($F_{[5, 12]} = 2.74$, $p < 0.07$).

Among flagellates, both Cercozoa and Euglenozoa tended to increase in $T_{moist\text{-}wet}$ with 1.8- and 3.6-fold numbers, respectively compared to $T_{med\text{-}med}$, while decreasing to 15 % and 22 % of $T_{med\text{-}med}$ in $T_{dry\text{-}dry}$, respectively, albeit with marginal significance (Cercozoa: $F_{[5, 12]} = 2.63$, $p = 0.08$; Euglenozoa: $F_{[5, 12]} = 2.55$, $p = 0.09$).

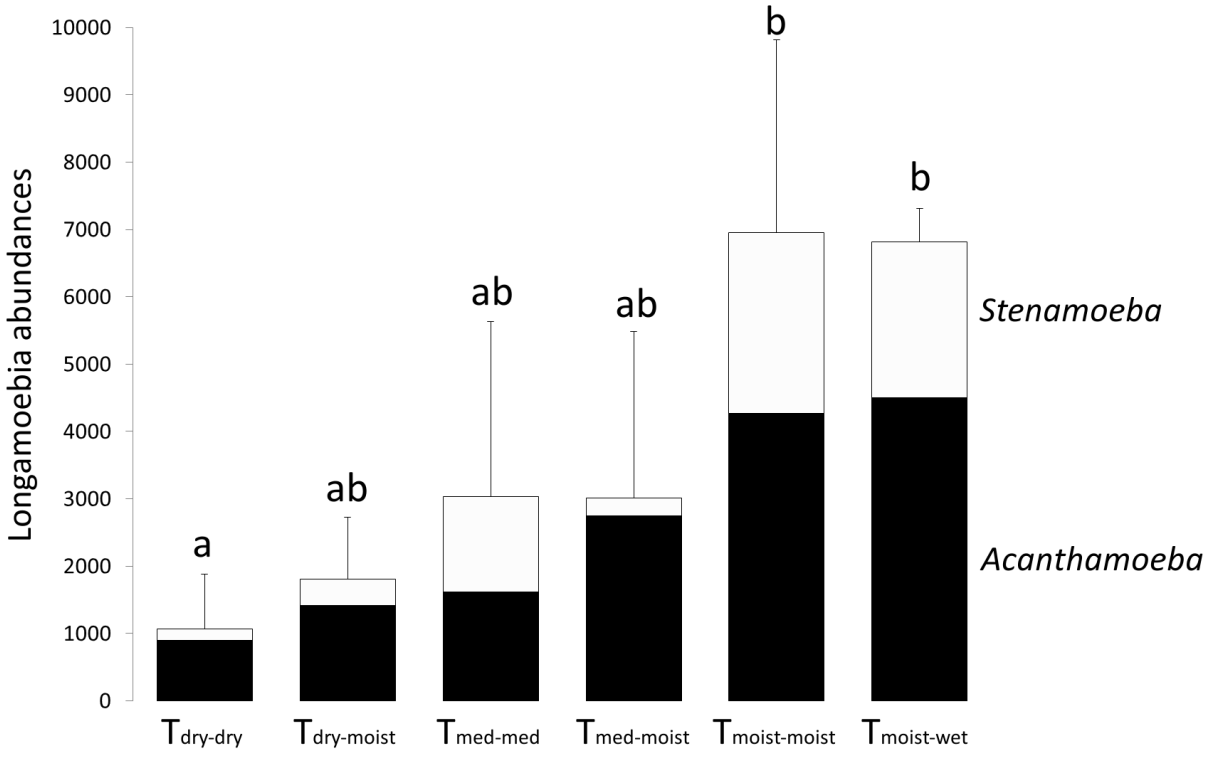

Figure 5. Abundance of the genera *Stenamoeba* and *Acanthamoeba* within the order Longamoebia; Means ± SD. Bars with different letter are statistically significant (Tukey's HSD test; $p \leq 0.05$).

Community structure

Amoebae always slightly dominated the protist community comprising between 59 and 69 % of total protist abundance. Within amoebae, the class Discosea represented always the numerically dominant group (28 - 49 % of all amoebae [OA], 16 - 31 % of all protists [OP]) with the exception of unidentified Nanoamoebae, which were dominant in $T_{dry\text{-}moist}$ (44 % OA, 26 % OP) and $T_{med\text{-}moist}$ (36 % OA, 24 % OP). Nanoamoebae represented the second most abundant clade comprising 21 - 44 % OA (15 - 26 % OP). The classes Variosea (6 - 29 % OA, 4 - 19 % OP), Tubulinea (10 - 21 % OA, 6 - 9 % OP,) and Heterolobosea (3 - 5 % OA, 2 - 4 % OP) were less abundant. At the genus level, *Acanthamoeba* represented always the most dominant genus of all identified amoebae comprising between 11 to 40 % of all amoebae, while the genera *Cochliopodium* and *Flamella* were always rare (≤ 3 %).

Flagellates comprised 33 - 43 % OP. The dominant flagellate taxa were Cercozoa (39 - 79 % of all flagellates [OF], 19 - 24 % OP), followed in descending order by Euglenozoa (12 - 33 % OF, 5 - 13 % OP), Chrysophycea (7 - 21 % OF, 3 - 7 % OP) and others (0 - 12 % OF, 0 - 4 % OP). On a finer taxonomic resolution, cercomonads were most abundant (32 - 77 % OF, 16 - 24 % OP), followed by bodonids (11 - 30 %, 4 - 12 % OP) and chrysomonads (7 - 21 % OF, 3 - 7 % OP).

Diversity

Neither overall protist diversity nor evenness were altered by moisture treatments (Figure 6). Only the diversity of flagellates was reduced in $T_{dry\text{-}dry}$ compared to $T_{med\text{-}med}$ from 0.92 to 0.61 ($p < 0.05$).

Similarly, the overall ratio of amoebae to flagellate numbers was not affected by soil moisture. Some individual groups, however, responded with a proportional shift in abundance to soil moisture changes. The contribution of the class Variosea to the protist community peaked their maximum contribution peaked in $T_{med\text{-}med}$ and reached its minimum in $T_{dry\text{-}dry}$ ($F_{[5,\ 12]} = 3.11$, $p < 0.05$), which was caused by a reduction of larger, branched amoebae ($F_{[5,\ 12]} = 4.47$, $p < 0.05$). The genus *Stenamoeba* was strongly reduced to 1 % OP (2 % OA) in $T_{med\text{-}moist}$ and a to 3 % OP (4 % OA) in $T_{dry\text{-}dry,}$ while *Stenamoeba* contributed most to the abundance of protists (12 %) and amoebae (18 %) in $T_{moist\text{-}moist}$ ($F_{[5,\ 12]} = 4.26$, $p < 0.05$). None of the flagellate groups were affected by soil moisture.

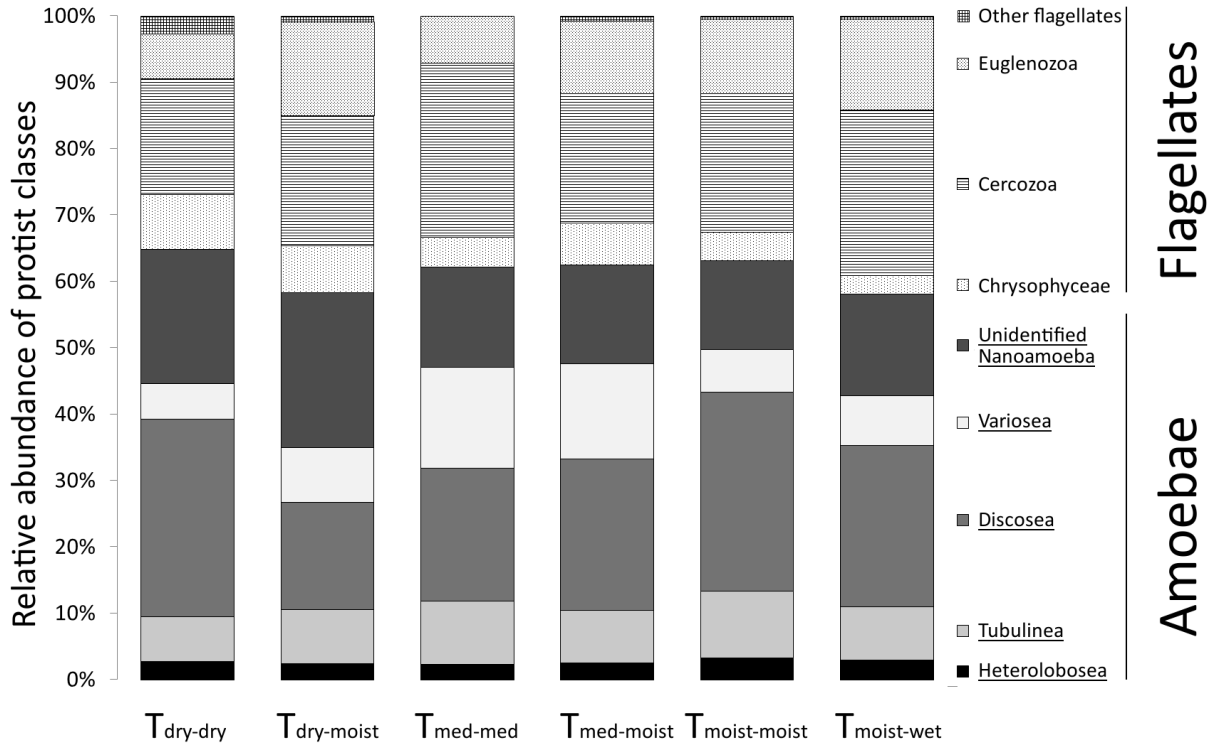

Figure 6. Relative abundance of major classes of flagellates and amoebae. Underlined: Classes of amoebae.

Habitable pore space and protist performance

As expected, reduced water availability reduced protist numbers, but surprisingly, strong reductions in protist abundance occurred only at the most extreme drying treatment when the maximum size of water filled pores (Pmax) had dropped to ~ 0.25 µm. Protist community composition was extremely resilient, and did almost not change even at this extreme dryness. Moistening the dry soil for one week to Pmax > 75 µm increased total protist numbers fourfold to exactly the same level as in $T_{med\text{-}med}$ where Pmax was ~ 38 µm. Moistening $T_{med\text{-}med}$ to Pmax > 75 µm for one week, however, did not affect protist abundance at all. Significantly higher protist numbers were only observed at an initial Pmax ≥ 75 µm, as in $T_{moist\text{-}moist}$ and $T_{moist\text{-}wet}$ (Figure 2). Using these rough estimates on habitable pore space, we propose the following model:

Highest protist abundance was observed when all soil pores larger than 100 µm were water-filled. When the habitable pores space became smaller (~ 75 µm), the abundance of the protist community slightly decreased, but dropped almost by half, when the size of water-filled pores became lower than ~ 50 µm. Extrapolating these data to protist abundance at Pmax of ~ 1 µm, where the protist abundance had 10-fold decreased and most likely only

few cysts remained, indicates that the protist community might have already sharply declined at a Pmax of ~ 10 µm (Figure 7). However, individual taxa reacted differently to differences in water availability. As expected, the largest protists decreased with increasing soil dryness, but in particular Nanoamoebae dominated in dry soils and not flagellates as we initially expected. Other taxa reached highest numbers only in fully water-saturated soils and readily decreased when the habitable pore space became smaller, e.g. *Stenamoeba* (Figure 7-2). Other taxa appeared to be more resistant to decreasing water and only decreased at a later stage of drought (Pmax < 60 µm), e.g. the abundant genus *Acanthamoeba* (Figure 7-1). Finally, some clades such as Variosea became relatively more abundant when Pmax was still ~ 30 - 40 µm when relative numbers of *Stenamoeba* and *Acanthamoeba* were already in decline (Figure 7-3).

Interesting, individual responses of protist taxa to increased soil water availability at the end of the experiment strongly differed from their responses to decreasing soil water availability. After rewetting soils for one week, Variosea showed maximum numbers at a small Pmax ~ 10 - 40 µm, but did not further increase with increasing soil moisture, while *Acanthamoeba* showed a constant increase in abundance with increasing water availability. Numbers of *Stenamoeba* on the other hand only started to increase when Pmax was larger than ~ 50 - 80 µm.

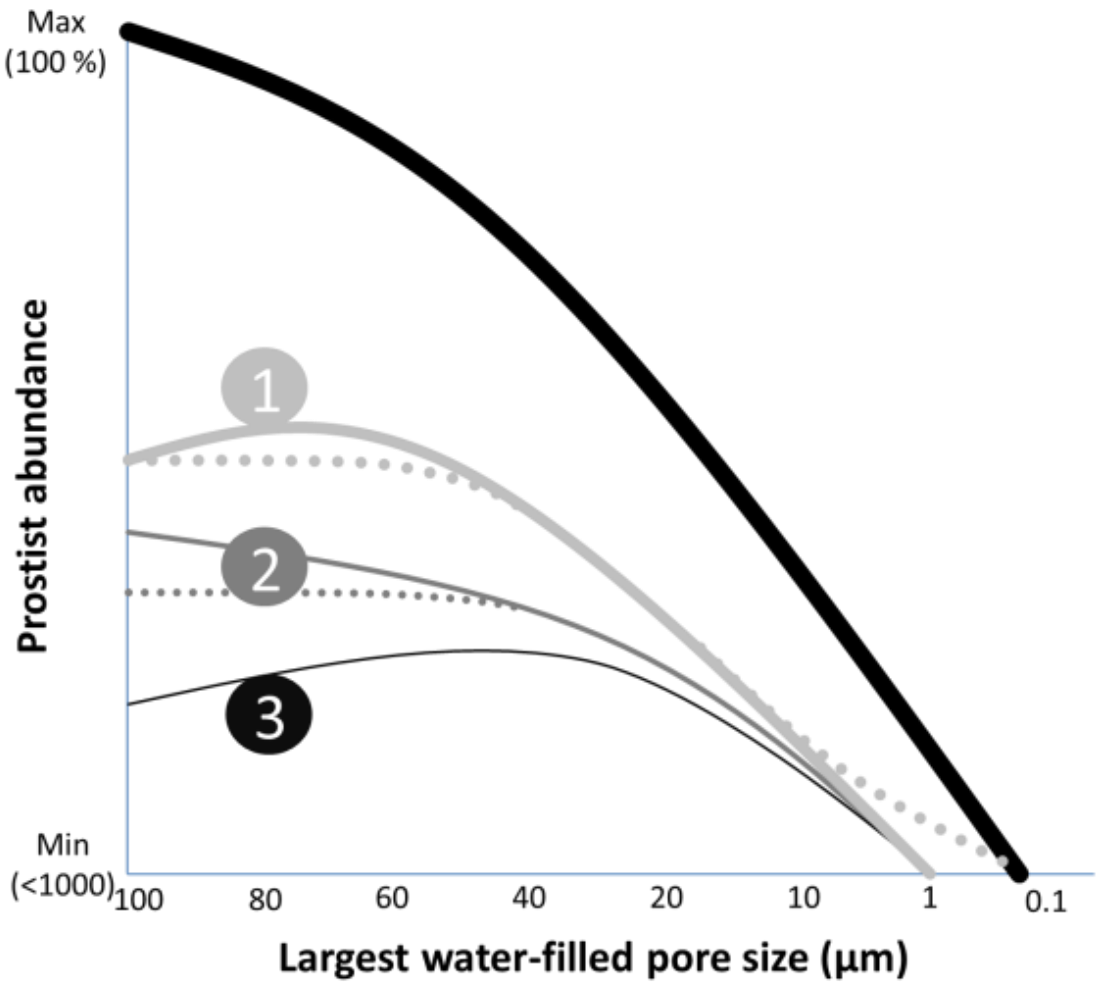

Figure 7. Conceptual graph of drying and re-wetting on relative abundance of three different taxa of amoeboid protists: *Acanthamoeba* (1), *Stenamoeba* (2) and Variosea (3) at different soil moisture levels, expressed as largest water-filled pore size (LWPS). *Acanthamoeba* peaked below 70 LWPS, Variosea at ~ 30 µm LWPS. *Stenamoeba* had highest relative numbers at highest LWPS, and gradually decreased with decreasing LWPS. All Amoebae strongly declined below ~ 25 µm LWPS. Active trophozoits could still be detected at 0.1 µm LWPS.

Discussion

Total protists

Protist densities in soil will mainly be regulated by the availability of water and food. As expected, protist numbers strongly decreased on average from 32,500 to 4,000 ind. g^{-1} soil dry wt in wet to dry soil, respectively. This is well within the range reported for soil protists (Darbyshire 1994, Finlay et al. 2000, Domonell et al. 2013). However, measures of protist community composition, such as diversity and evenness did barely change within the range of 84 % to 26 % WHC of our soils. Amoebae were slightly more numerous than flagellates in all treatments, and amoeba did also benefit stronger from increased soil water content.

As protists essentially depend on the water layer connecting soil pores to move, feed and multiply, the habitat size of these organisms will increase or shrink with changing soil water contents. Consequently, maximum protist numbers are often found during moist seasons and after rainfall (Anderson 2000, Bass and Bischoff 2001, Rodriguez-Zaragoza et al. 2005). Interestingly, protist abundance did not increase in our study, when soils were kept at 50 % WHC or brought from 50 % to 70 % WHC ($T_{med\text{-}med}$ and $T_{med\text{-}moist}$). Foissner (1987) explained this effect with the accumulation of microbial antibiotics in moist soils, and meanwhile a strong chemical warfare of bacteria against protist grazers has been well confirmed (Matz and Kjelleberg 2005, Bonkowski and Clarholm 2012, Jousset 2012). Only when soil moisture levels were suddenly increased drastically, more than in our experiment, inhibitory substances are diluted and maximum protist numbers are observed. In addition, the quick recovery of protist abundances after drought may be explained by fast growth and recovery of other (prey) microorganisms few days after drought (Meisner et al. 2013, Fuchslueger et al. 2014).

Due to their small size, protists can rapidly respond to increasing soil moisture regimes after drought. As mentioned above, it is well known that rewetting of air-dried soils will lead to strongly enhanced protist abundance (Foissner 1987). In a long-term study Anderson (2000) found precipitation, but not soil moisture, to be the most important environmental factor influencing the abundance of amoebae. Similarly, precipitation after a prolonged drought led to a 20-fold enhanced abundance of naked amoebae four days after rainfall (Clarholm 1981), and also Finlay et al. (2000) reported the period of 4 - 6 days after rewetting of air-dried soils to determine maximum numbers of flagellates. These findings support our results where protist numbers essentially recovered within one week in the $T_{dry\text{-}moist}$ treatment, but the increase was not uniform. Individual taxa responded with different proportion than during decreased water availability and also the increase of protist abundance was not uniform with increasing soil water availability in the different treatments.

Individual groups

This is the first soil ecological study classifying individual amoebae according to recent phylogenetic classifications (Smirnov et al. 2011b, Adl et al. 2012). The individual distinguished clades of amoebae responded differently to changes in soil water availability. Since even closely related protist taxa exert different feeding strategies (Page 1977, Boenigk

and Arndt 2002, Glücksman et al. 2010), drought-induced shifts in the relative abundance of specific protist taxa can be expected to influence the composition and functioning of microbial communities (Saleem et al. 2012).

In line with the classic note of Page (1988) that *Acanthamoeba* is "the most frequently isolated and probably the most common genus of gymnamoebae, possibly even the most common free-living protozoon", *Acanthamoeba* was also the most abundant genus in our study. *Acanthamoeba* have also been found to represent the most abundant group in deserts (Rodriguez-Zaragoza et al. 2005) and grassland soils (Elliott and Coleman 1977, Brown and Smirnov 2004). Our results indicate that significant shifts in protist community composition at decreasing soil water availability may occur well before these extreme values are reached. Surprisingly, the activity of Acanthamoebae in our study rapidly decreased below a Pmax of 40 µm. The limiting pore size for protist activity in soil has been estimated to be 3 - 6 µm, which may be reached at a water potential of -0.15 MPa, corresponding to a pF-value of 3.2 (Alabouvette et al. 1981), but more recent investigations have set this threshold of Pmax even to 2 µm (Darbyshire 2005).

The functions of *Acanthamoeba* in soil systems have been intensively studied and it was shown that these amoebae can strongly control bacterial abundance, shape the bacterial community composition and positively influence plant growth (Bonkowski 2004, Kreuzer et al. 2006, Rosenberg et al. 2009, Bonkowski and Clarholm 2012, Koller et al. 2013). *Acanthamoeba* can tolerate long periods of dehydration (Rodriguez-Zaragoza et al. 2005) and correspondingly, we found that *Acanthamoebae* made up the highest proportions among protists in the driest soils, indicating their superior resistance to environmental stress. This is important since several *Acanthamoeba* strains can cause human infections or harbour pathogenic bacteria (Schuster and Visvesvara 2004, Khan 2006, Visvesvara et al. 2007), revealing that targeted analyses of this genus are essentially needed to evaluate in more detail their functional role and niches of survival in soils.

The group most strongly affected by moisture was the genus *Stenamoeba*. Its abundance peaked in treatments with the highest average soil water availability, indicating that these amoebae fundamentally depend on high water contents. This genus was long considered as being a single species within the genus *Platyamoeba (*now *Vannella*), and only recently it became an independent genus within a completely different subclass in Amoebozoa

(Smirnov et al. 2007). Meanwhile four new species of *Stenamoeba* have been described, two were amphizoic species isolated from fish organs (Dyková et al. 2010b) and two more from soils (Geisen et al. 2014), indicating that *Stenamoeba* is a species-rich genus. Characteristics of *Stenamoeba* in cultures are rapid growth and a low attachment to the substratum. The latter might therefore explain the changes caused by decreasing soil water availability.

Since both *Stenamoeba* and *Acanthamoeba* responded strongly to moisture, the subclass and class combining both genera, i.e. Longamoebia and Discosea were affected by soil moisture availability. However, the detailed phylogenetic information retrieved in our study allowed us to clearly separate the responses of *Stenamoeba* and *Acanthamoeba,* which became only apparent at the higher taxonomic resolution used here. These results show that the way of grouping protist taxa can fundamentally alter the outcome of a study, and it is advisable to identify protists to the deepest taxonomic level possible.

It is possible to differentiate the smallest taxa using light microscopy and grouped them together as Nanoamoebae, representing a variety of different families, among them most likely heteroboseans, *Echinamoeba*, *Nolandella* and most likely currently unknown amoebae, such as the recently described *Micriamoeba* (Atlan et al. 2012). Nanoamoebae generally comprised the majority of amoebae which is in line with other studies that reported highest numbers of small amoebae in soil (Elliott and Coleman 1977, Anderson 2000). Due to their slow motion compared to flagellates and extremely small size Nanoamoebae are easily overlooked in cultivation-based enumeration techniques. Their share was disproportionately high in the rewetted treatments suggesting that these small amoebae have faster recovery rates than larger amoebae. Their proportion increased particularly in the rewetted dry soil where their fast reproduction rates might have provided them with a decisive head start compared to larger protists such as *Acanthamoeba* (~ 3 - 5 x in biomass) and Variosea (~ 5 - 100 x in biomass). The latter, being the largest protist in this study, seem to be desiccation resistant and significantly increased in proportion among amoebae at intermediate soil moisture and were most abundant in soils with medium and high initial moisture.

In conclusion, our detailed analysis of amoebae and flagellates revealed that amoebae and amoeboflagellates dominated the protist community. As expected, protist abundance was lowest in the driest conditions and highest in soils with highest moisture, while diversity was

decreased in the driest soil. Our study shows that most protist groups uniformly increase with higher soil moisture, but that some groups differ from these general patterns showing different responses to changes in soil moisture contents. We identified three strategies of amoebae on changes in soil moisture that will be essential for further studies evaluating the impact of climate change on soil organisms. As protists represent important components within soil ecosystems playing fundamental roles in nutrient cycling in soil food webs, it is essential to distinguish protist taxa in order to evaluate individual roles in nutrient cycling and to estimate the impact of potentially altered soil protist communities induced by the global change.

Part 2 – Chapter 6

Acanthamoeba everywhere: High diversity of *Acanthamoeba* in soils

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Abstract

Acanthamoeba, a genus within the supergroup Amoebozoa, are very abundant soil protists with fundamental importance in nutrient cycling, but several strains can also act as human pathogens. The systematics of the genus is still unclear: currently 18 small-subunit (SSU or 18S) ribosomal RNA sequence types (T1-T18) are recognized, which sometimes contain several different morphotypes; on the other hand, some morphological identical strains belong to different sequence types, sometimes appearing in paraphyletic positions. Here we cultivated 65 *Acanthamoeba* clones from soil samples collected under grassland at three separate locations in the Netherlands, in Sardinia and at high altitude mountains in Tibet. We obtained 24 distinct partial sequences, which predominantly grouped within sequence type T4 followed by T2, T13, T16 and "OX-1" (in the T2/T6 clade). Our sequences were 98 - 99 % similar, but none was identical to already known *Acanthamoeba* sequences. The community composition of *Acanthamoeba* strains differed between locations, T4 being the dominant sequence type in Sardinia and Tibet, but represented only half of the clones from soils in the Netherlands. The other half of clones from the Dutch soils was made up by T2, T16 and "OX-1", while T13 was only found in Sardinia and Tibet. None of the sequences was identical between localities. Several T4 clones from all three localities and all T13 clones grew at 37 °C while one T4 clone was highly cytopathogenic.

Introduction

The amoebozoan genus *Acanthamoeba* shows an ubiquitous worldwide distribution ranging from aquatic to terrestrial environments, and it might even be one of the most dominant protists in soil (Page 1988, Rodríguez-Zaragoza 1994). In these habitats, acanthamoebae are important grazers of the bacterial biomass, thereby not only controlling the abundance and turnover, but also the diversity of bacterial communities in soil and plant rhizospheres (Griffiths et al. 1999, Rønn et al. 2002b, Kreuzer et al. 2006, Rosenberg et al. 2009). In the soil microbial loop *Acanthamoeba* liberates nutrients bound in the microbial biomass, ultimately benefiting plant growth (Bonkowski and Clarholm 2012).

In the past decades special attention was also given to *Acanthamoeba* from a medical point of view as several species can cause human diseases such as *Acanthamoeba* keratitis and granulomatous amoebic encephalitis (Rodríguez-Zaragoza 1994, Schuster 2002, Schuster and Visvesvara 2004).

Acanthamoeba belongs to the phylum Amoebozoa (Cavalier-Smith 1998) and the family Acanthamoebidae (Sawyer and Griffin 1975, Pussard and Pons 1977). Molecular phylogenies based on the small-subunit (SSU) ribosomal RNA gene provided further evidence for the monophyly of the genus *Acanthamoeba* with *Balamuthia* as sister genus (Amaral-Zettler et al. 2000, Smirnov et al. 2011b). Traditionally, species of *Acanthamoeba* were classified into three groups based on cyst morphology (Pussard and Pons 1977). Investigations based on molecular data confirmed the monophyly of group I (with the apomorphy of stellate endocysts) but not of the remaining two groups (group II: various endocysts, ectocyst wrinkled; group III: round endocyst, smooth ectocyst) (Pussard and Pons 1977). Although group II and III are related, sufficient divergence within these groups and of both groups to group I led to the progressive recognition of 18 SSU sequence types (T1 – T18), based on ≥ 5 % sequence dissimilarity between sequence types (Gast et al. 1996, Stothard et al. 1998, Horn et al. 1999, Hewett et al. 2003, Corsaro and Venditti 2010, Nuprasert et al. 2010, Qvarnstrom et al. 2013). Current systematics within *Acanthamoeba* is obscured by several polyphyletic species, such as *Acanthamoeba castellanii* and *A. polyphaga* and an unavailability of type strains for the described species (Gast et al. 1996, Qvarnstrom et al. 2013). Furthermore, T16 has independently been erected twice (Łanocha et al. 2009,

Corsaro and Venditti 2010), with both isolates described as T16 being in fact unrelated (Corsaro and Venditti 2011), but none has been abandoned yet.

Lastly, neither morphological groups nor sequence types are indicators of pathogenicity, as pathogenic *Acanthamoeba* have been detected in all morphological groups and most sequence types (Qvarnstrom et al. 2013, Risler et al. 2013). The majority of human infections have been caused by amoebae belonging to the most prevalent sequence type T4 that is however intermingled with non-pathogenic ones (Gast et al. 1996, Stothard et al. 1998, Booton et al. 2002, Maciver et al. 2013, Risler et al. 2013).

The main objective of this study was to assess the occurrence and diversity of the dominant cultivable *Acanthamoeba* spp. in soil samples from three spatially distinct locations, i.e. the Netherlands, Sardinia and Tibet. In total, we cultivated 65 strains of *Acanthamoeba* and obtained 24 distinct partial SSU rDNA sequences. We discuss the phylogenetic results in the light of ecology, biogeography and potential medical relevance.

Materials and Methods

Establishing cultures

The top 20 cm of mineral soil from were collected from grassland sites in the Netherlands (Hedlund et al. 2003), in the Berchidda-Monti long term observatory on Sardinia, Italy (Bagella et al. 2013), and in mountain meadows at high altitudes of > 4100 m from Mila mountain in Tibet (Table 1). After 2 mm sieving the soil samples were transferred in thermo-isolated containers to the laboratory. A soil suspension was prepared by mixing 50 g of dry wt soil with 50 ml of sterile distilled water. After gently shaking the soil suspension for 20', soil particles were allowed to settle for 15'. From each site, 20 enrichment cultures were established by transferring 100 µl of the soil suspension each, into 10 standard Petri dishes (9 cm) filled with Prescott-James (PJ) medium (Page 1991), enriched with 0.15 % wheat grass (WG) (Weizengras, Sanatur GmbH, Germany), and into 10 Petri dishes filled with 1.5 % agarised PJ medium, respectively. Each dish was carefully examined twice (at days 10 - 14 and 24 - 28) with an inverted Nikon Diaphot phase contrast microscope at 100 x and 400 x magnifications. To establish clonal cultures, single amoebae were transferred with a glass pipette to new 6 cm Petri dishes filled with PJ medium enriched with WG (WG medium).

Clonal cultures of amoebae were morphologically identified according to Pussard and Pons (1977), Page (1988), Smirnov and Brown (2004) and Smirnov et al. (2011b).

Table 1. Description of soil samples used in this study

Code used in this study	Locality	Soil origin and land use	Geographic coordinates	Altitude (m)
NlMY	The Netherlands, Veluuwe	Ex arable field; 2 years abandoned	N52°21′, E5°82′	16
NlMM	The Netherlands, Veluuwe	Ex arable field; 9 years abandoned	N52°01′, E5°99′	47
NlMO	The Netherlands, Veluuwe	Ex arable field; 22 years abandoned	N52°03′, E5°80′	18
NlLui	The Netherlands, Veluuwe	Ex arable field; 34 years abandoned	N52°06′, E6°00′	57
Sar	Italy, Sardinia, Berchidda-Monti	Grassland	N40°46′, E9°10′	181
TibE	Tibet, Mila mountain east slope	Meadow	N29°52′ E92°33′	4149
TibT	Tibet, Mila mountaintop	Meadow	N29°49′ E92°20′	5033
TibW	Tibet, Mila mountain west slope	Meadow	N29°42′ E92°10′	4149

Testing for pathogenicity-related characters

Thermophily was tested by growing *Acanthamoeba* strains at temperatures of 34 °C, 37 °C and 42 °C. 50 amoebae were inoculated in each well of a 24 well-plate containing 1 ml of WG medium. Growth of trophozoites was estimated every 24 hours for one week. Further, we tested temperature tolerance of all *Acanthamoeba* clones that multiplied at 37 °C by incubating them at 42 °C for one day followed by an incubation at 37 °C for one week using the same settings as described above.

All strains that showed growth ≥ 34 °C (temperature of the human cornea) were subjected to cytopathogenicity testing as described earlier (Walochnik et al. 2000). In brief, cysts were harvested from plate cultures, washed in sterile saline solution, and incubated in 3 % HCl overnight to eliminate co-existing bacteria. After centrifugation (500 g, 10′) the pellet was washed once again in sterile saline solution and transferred into sterile-filtrated PYG medium (proteose peptone-yeast extract-glucose medium in a 4:2:1 mixture) (axenised suspension). In parallel, HEp-2 cells were cultured in a 1:1 mixture of PC-1 and CO_2-independent medium (Life Technologies, Ltd., Paisley, Scotland) supplemented with L-glutamine (2 mM) in 75 cm^2 tissue culture flasks (Corning / Costar, Bodenheim, Germany) at 37 °C under sterile conditions. Subsequently, 1 ml of a 10^5-cell * ml^{-1} axenised suspension

of each isolate was inoculated into a culture flask containing a HEp-2 cell monolayer. Co-cultures were incubated at 34 °C and monitored for 72 hours. All experiments were carried out in duplicates and repeated in an independent set-up.

DNA extraction, amplification and sequencing

Genomic DNA was isolated from fresh cell cultures using the guanidine isothiocyanate protocol (Maniatis et al. 1982). Briefly, WG medium was discarded, Petri dishes washed twice with sterile WG medium, which was subsequently replaced by 100 µl guanidine isothiocyanate. Amoebae were scraped off using a sterile metal cell scraper, and transferred to 2 ml centrifuge tubes. Subsequent stages were performed according to the cited protocol.

The complete SSU rDNA was amplified from all strains using the universal eukaryotic primers RibA (all primers sequences written in the order 5' - 3': ACC TGG TTG ATC CTG CCA GT) and RibB (TGA TCC ATC TGC AGG TTC ACC TAC) (Cavalier-Smith and Chao 1995, Pawlowski 2000). PCR settings consisted of an initial denaturation at 95 °C for 5', followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 45 s and elongation at 72 °C for 90'' with a final 5' elongation at 72 °C. PCR products were enzymatically purified by adding 0.15 µl Endonuclease I (20 U * μl^{-1}, Fermentas GmbH, St. Leon-Rot, Germany), 0.9 µl Shrimp Alkaline Phosphatase (1 U * μl^{-1}, Fermentas, Germany) and 1.95 µl H_2O. The mixture was incubated for 30' at 37 °C, followed by 20' at 85 °to stop the reaction. Sequencing was carried out at GATC (Konstanz, Germany) using the following sequencing primers: RibB, 12r (AAC GGC CAT GCA CCA CC) and JDP2 (CTC ACA AGC TGC TAG GGG AGT CA) (Dyková et al. 1999).

Alignments and phylogenetic analyses

We obtained 65 sequences of which 24 were unique. These were deposited in GenBank under the accession numbers KF928933 to KF928956. Unique sequences were manually aligned in Seaview 4 (Gouy et al. 2010) together with representatives of the 18 currently described *Acanthamoeba* sequence types (Gast et al. 1996, Stothard et al. 1998, Horn et al. 1999, Hewett et al. 2003, Corsaro and Venditti 2010, Nuprasert et al. 2010, Qvarnstrom et al. 2013). We also used our new sequences to perform a BLASTn search against the NCBI nucleotide database online (http://blast.ncbi.nlm.nih.gov/; last accessed October 24th 2013). Five best hits for each sequence were added to our alignment. Five sequences of

Balamuthia spp. and two of *Protacanthamoeba* spp. were added as outgroups resulting in a total of 145 sequences.

For phylogenetic analyses, 1,771 unambiguously aligned positions were retained, excluding ambiguous positions and several positions in variable regions especially in V2 (helices 9-11), V4 (helix E23), V5 (helix 29), V7 (helix 43), V8 (helices E45-1 and 46) and V9 (helix 49). Maximum likelihood phylogenetic analyses were run using RAxML v. 7.2.6 (Stamatakis 2006) with the GTR+γ+I model of evolution, as proposed by jModeltest v. 2.1.3 under the Akaike Information Criterion (Darriba et al. 2012), the γ approximated by 25 categories. 1,000 non-parametric bootstrap pseudoreplicates were run. Bayesian phylogenetic analyses were run using Mr Bayes v. 3.2.1 with GTR+γ+I model of evolution and 8 categories (Huelsenbeck and Ronquist 2001). Two runs of four simultaneous Markov chains were performed for 4,000,000 generations (with the default heating parameters) and sampled every 100 generations; convergence of the two runs (average deviation of split frequencies < 0.01) was not reached. Analyses of the two independent consensus trees revealed that both trees diverged in tree regions that were also unresolved in the maximum likelihood analysis. Therefore we decided to discard the first 30,000 trees and built a consensus tree from the remaining 10,000 trees, where the average deviation of split frequencies between runs was < 0.02. In order to improve the resolution between closely related sequences, we performed another phylogenetic analysis using the same settings as above, focusing on the *Acanthamoeba* clones obtained in this study. In total, 116 sequences with 1,947 sequences unambiguously aligned positions were included in maximum likelihood and Bayesian analyses as described above. Convergence of the two Bayesian runs was reached after 2,610,000 generations. The remaining 1,390,000 trees were used to build a consensus tree.

Results

Cultures

In total, 65 *Acanthamoeba* clones were retrieved from the enrichment cultures of all eight soils (32 clones from the Netherlands, 18 clones from Tibet and 15 clones from Sardinia; Table 2). Most cultures of *Acanthamoeba* (80 %) were obtained from agar medium, the others from liquid culture. Our clones belonged to morphogroups II and III, and none to morphogroup I.

The tests on pathogenicity-related characters of 18 of the 24 *Acanthamoeba* clones that differed in their SSU rDNA sequences showed that all isolates grew well at 34 °C, half of the isolates at 37 °C, but none at 42 °C (Table 2). More specifically, several clones from each location grew at 37 °C, i.e. four from the Netherlands (clones Nl4, Nl123, Nl134 and Nl135), three from Sardinia (Sar44, Sar48 and Sar63) and two from Tibet (clones Tib1 and Tib122). Six clones (Nl4, Nl123, Nl134, Nl135, Sar63 and Tib122) tolerated 42 °C for 1 day and subsequently grew at 37 °C (Table 2). Only clone Nl123 rapidly multiplied on HEp-2 cell monolayers that were entirely destroyed within 48 hours. No other clone tested showed cytoplasmic effects on cell layers. However, morphological features of Nl123 did not reveal any morphological characters that distinguished this clone from the others.

Sequences and phylogenetic analyses

We obtained 24 different SSU rDNA sequences from the 65 clones. None of our sequences was identical to already published sequences, the similarities with each of the best hit using BLASTn ranged from 98 % to 99 %. Phylogenetic analyses of an alignment of 145 sequences and 1,771 positions resulted in several strongly supported clusters with a clade containing T7-T8-T9-T17-T18 in the most basal position within the genus *Acanthamoeba* (Supplementary Figure 1). This clade comprised species assigned to the morphogroup I. T5 containing *A. lenticulata* formed a strongly supported clade, while the other clades were not highly supported using a Centramoebida-wide (*Acanthamoeba*, *Protacanthamoeba* and *Balamuthia*) phylogenetic analysis (Supplementary Figure 1).

The second analysis aimed to assign the phylogenetic positions of our new sequences more precisely. Therefore outgroups and the two most basal clades were omitted, and the

remaining group was resolved into distinct clades with a deep dichotomy (Figure 1, Supplementary Figure 2). The first clade, T1-T2-T10-T12-T13-T14-T15-T16, contained two described T16 sequence types (Łanocha et al. 2009, Corsaro and Venditti 2010), accordingly we added the initials of the respective authors for clarity, i.e. T16 (C&V) for the type described by Corsaro and Venditti (2010) and "T16" (Ł) for that described by Łanocha et al. (2009). However, we place the latter in quotation marks to indicate that this sequence type has been described based on a partial sequence only, leading Corsaro and Venditti to abandon "T16" (Ł) as a sequence type (Corsaro and Venditti 2011).

The other clade showed a dichotomy between T3-T4-T11 and T2-T6. The latter further split into different groups, named "OX-1", "pol", T2 (containing *A. palestinensis* "Reich") and T6, as previously suggested (Corsaro and Venditti 2010, Corsaro and Venditti 2011, Risler et al. 2013).

All of our sequences branched into groups composed of known sequence types. The majority of our clones was placed across the largest sequence type T4. Three consensus sequences retrieved from 9 clones grouped in T16 (C&V), 3 consensus sequences from 5 clones in T13, one consensus sequence grouped in T2 (3 clones) and one consensus sequence grouped with "OX-1".

Growth performance varied between and within clades. Most *Acanthamoeba* clones from T4 (75 %) and all T13 grew at 37 °C, while T16 (C&V) and "OX-1" did not grow at high temperatures. T13 clones did not tolerate 42 °C while all except one of the T4 clones growing at 37 °C tolerated a 24 hour incubation at this high temperature (Table 2).

Comparison of the Acanthamoeba community composition in soils

Despite the high number of isolated clones from each site, we never found a similar clone from distinct locations. Instead, a diverse community of at least five different sequences occurred at each location, i.e. the Netherlands, Sardinia and Tibet (Table 2). Almost all isolated clones from Sardinian and Tibetan belonged to T4 (93 % and 78 %, respectively), the remaining to T13 (7 % and 22 %, respectively; Table 2).

The dominant community of Acanthamoebae from soils in the Netherlands was richer in distinct isolates and differed in composition from the other locations with only

approximately half of the clones belonging to T4 (56 %), followed by T16 (C&V) (28 %), T2 (9 %) and "OX-1" (6 %), while T13 was not recovered (Table 2).

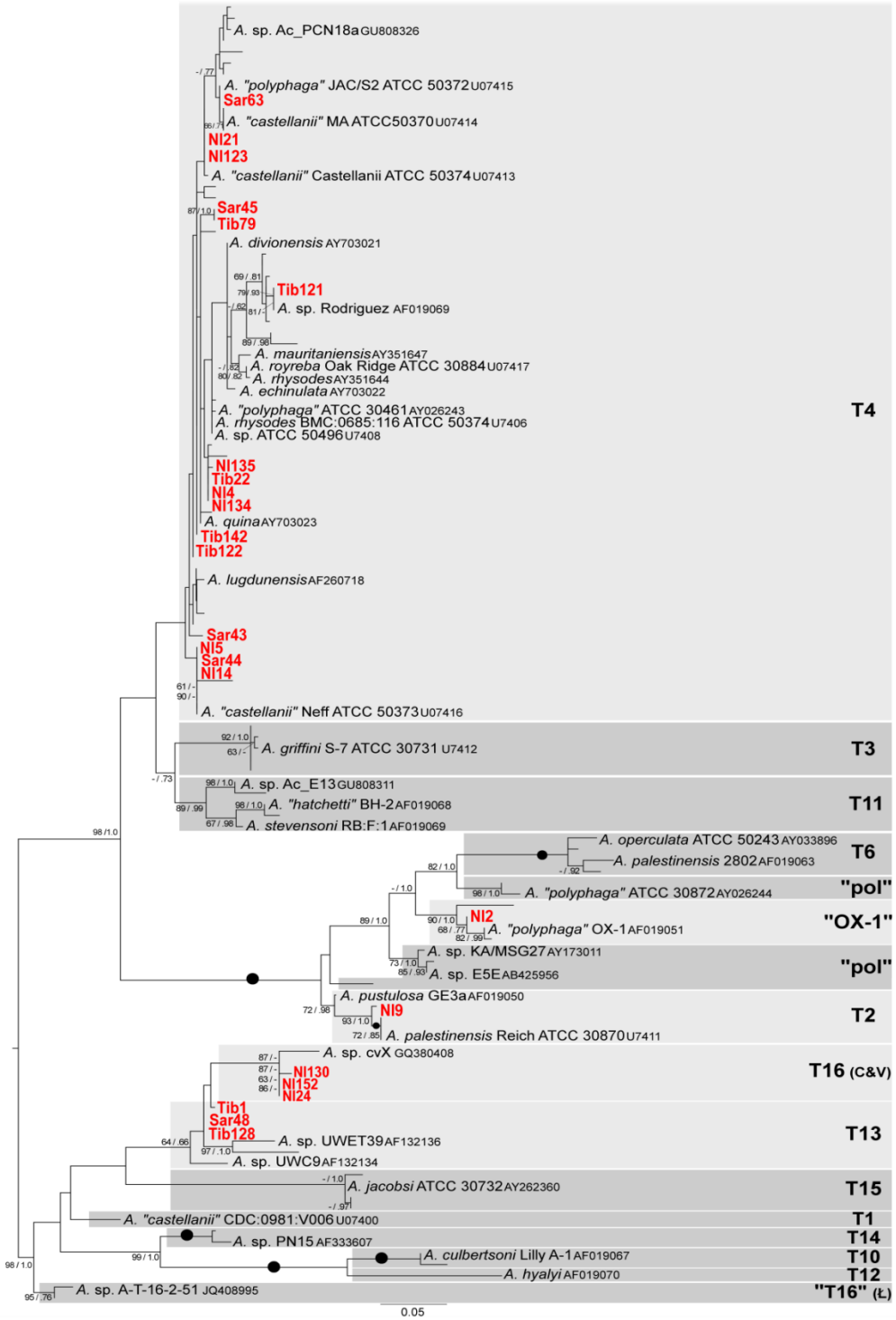


Figure 1. Phylogenetic analysis focusing on sequence types and sequences associated with the new sequences obtained in this study. In total, 116 sequences with 1,947 unambiguously aligned positions were used, with only single representative sequences shown for individual sequence types and strains; the tree is unrooted; values higher than 60 for maximum likelihood analyses (left) and 0.60 for Bayesian analyses (right) are shown. Black circles represent full support; red: strains obtained in this study; boxes: associated sequence types with bright boxes containing clones obtained in this study; the entire figure showing all strains included in the analysis is shown in Supplementary Figure 2.

Table 2. Name, origin, SSU type, primers used, length of partial sequence obtained, temperature tolerance and cytopathogenicity of the *Acanthamoeba* strains obtained in this study; ND = not determined

Strain	Additional clones (Strain)	Soil Origin	SSU type	Sequencing primers	Sequence length obtained (bp)	Growth @ 37 °C	Growth @ 37 °C after 24h @ 42 °C	Cytopathogenic on HEp-2 cells
Nl2	1 (Nl76)	NlMM,NlMO	"OX1"	RibB, 12r, JDP2	1869	No	No	No
Nl4	1 (Nl8)	NlO	T4	RibB, 12r	1709	Yes	Yes	No
Nl5	0	NlY	T4	RibB	912	ND	ND	ND
Nl9	2 (Nl41, Nl72)	NlMY	T2	RibB, 12r	1559	ND	ND	ND
Nl14	0	NlM	T4	RibB, 12r	1473	No	No	No
Nl21	2 (Nl35, Nl49)	NlY,NlMM	T4	RibB, 12r	1371	ND	ND	ND
Nl24	1 (Nl70)	NlMY	T16 (C&V)	RibB, 12r	1269	No	No	No
Nl123	1 (Nl150)	NlLui	T4	RibB, 12r	1591	Yes	Yes	Yes
Nl130	5 (Nl141, Nl142, Nl145, Nl151, Nl156)	NlLui	T16 (C&V)	RibB, 12r	1441	No	No	No
Nl134	0	NlLui	T4	RibB, 12r	1515	Yes	Yes	No
Nl135	7 (Nl144, Nl149, Nl153, Nl154, Nl166, Nl172, Nl180)	NlLui	T4	RibB, 12r	1759	Yes	Yes	No
Nl152	0	NlLui	T16 (C&V)	12r	699	ND	ND	ND
Sar43	5 (Sar46, Sar60, Sar65, Sar73, Sar91)	Sar	T4	RibB, 12r, JDP2	1939	No	No	No
Sar44	3 (Sar47, Sar55, Sar84)	Sar	T4	RibB, 12r	1338	Yes	No	No
Sar45	0	Sar	T4	RibB, 12r, JDP2	1941	No	No	No
Sar48	0	Sar	T13	RibB, 12r	1501	Yes	No	No
Sar63	2 (Sar76, SarSC2)	Sar	T4	RibB, 12r	1290	Yes	Yes	No
Tib1	2 (Tib75, Tib170)	TibE, TibW	T13	RibB, 12r	2195	Yes	No	No
Tib22	4 (Tib29, Tib 116, Tib125, Tib186)	TibT,TibE,TibW	T4	RibB, 12r	1331	No	No	No
Tib79	1 (Tib157)	TibW	T4	12r	656	ND	ND	ND
Tib121	1 (Tib185)	TibE	T4	RibB, 12r	1264	No	No	No
Tib122	2 (Tib127, Tib171)	TibE,TibW	T4	12r	676	Yes	Yes	No
Tib128	0	TibE	T13	12r, JDP2	1041	No	No	No
Tib142	1 (Tib160)	TibE	T4	12r	632	ND	ND	ND

Discussion

We retrieved a wide variety of different *Acanthamoeba* clones from all samples, confirming the potential ubiquity, but also a significant diversity of these common amoebae in soils (Page 1988, Rodríguez-Zaragoza 1994). The phylogenetic analyses using the Centramoebida-wide alignment revealed well-supported branches especially in basal *Acanthamoeba* sequence types (Supplementary Figure 1), while the topology of the more derived SSU types was only recovered in more focused phylogenetic analyses (Figure 1, Supplementary Figure 2). Combined information from the two phylogenetic analyses show stable clades such as T10-T12-T14, T13-T16 (C&V) and T2-T6 (Corsaro and Venditti 2010, Corsaro and Venditti 2011, Risler et al. 2013). As previously described, T2-T6 was split into independent lineages (Corsaro and Venditti 2010, Corsaro and Venditti 2011, Risler et al. 2013). The two T16 SSU types T16 (C&V) and T16 (Ł) represent distinct sequence types as suggested by Corsaro and Venditti (2011) and T13-T16 (C&V) formed a separate clade, but without bootstrap support. Perhaps the clade was destabilized by the inclusion of shorter sequences Tib1, Sar48 and Tib128, which branched intermediate between T13 and T16 (C&V); in an analysis including more sites aiming at placing Tib1, Sar48 and Tib128 revealed their unambiguous placement in T16 (C&V) (data not shown). Full sequences would likely have increased the phylogenetic resolution within T13-T16 (C&V) when more diverent sequence types are included in phylogenetic analyses, as shown by Corsaro and Venditti (2011).

Our results confirm the predominance of the T4 sequence type, both in clinical and environmental samples (Gast et al. 1996, Stothard et al. 1998, Booton et al. 2002, Maciver et al. 2013, Risler et al. 2013). Several formally described species based on morphological features have been located in T4 such as *A. royreba*, *A. hatchetti*, *A. divionensis*, *A. echinulata*, *A. polyphaga*, *A. rhysodes*, *A. lugdunensis* and *A. castellanii,* making a subdivision of this genotype desirable as suggested by Booton et al. (2002), Maciver et al. (2013) and Risler et al. (2013). Several of our T4 clones showed clear differences in pathogenicity-related characters, indicating diverse environmental adaptations within this clade (Maciver et al. 2013) Similarly, the T2-T6 clade contains several named species, i.e. *A. operculata*, *A. hatchetti*, *A. polyphag*a, *A. pustulosa* and *A. palestinensis.* This clade has also been divided based on sequence information (Corsaro and Venditti 2010, Corsaro and Venditti 2011, Risler et al. 2013). This subdivision is confirmed by our isolates Nl9 resembling

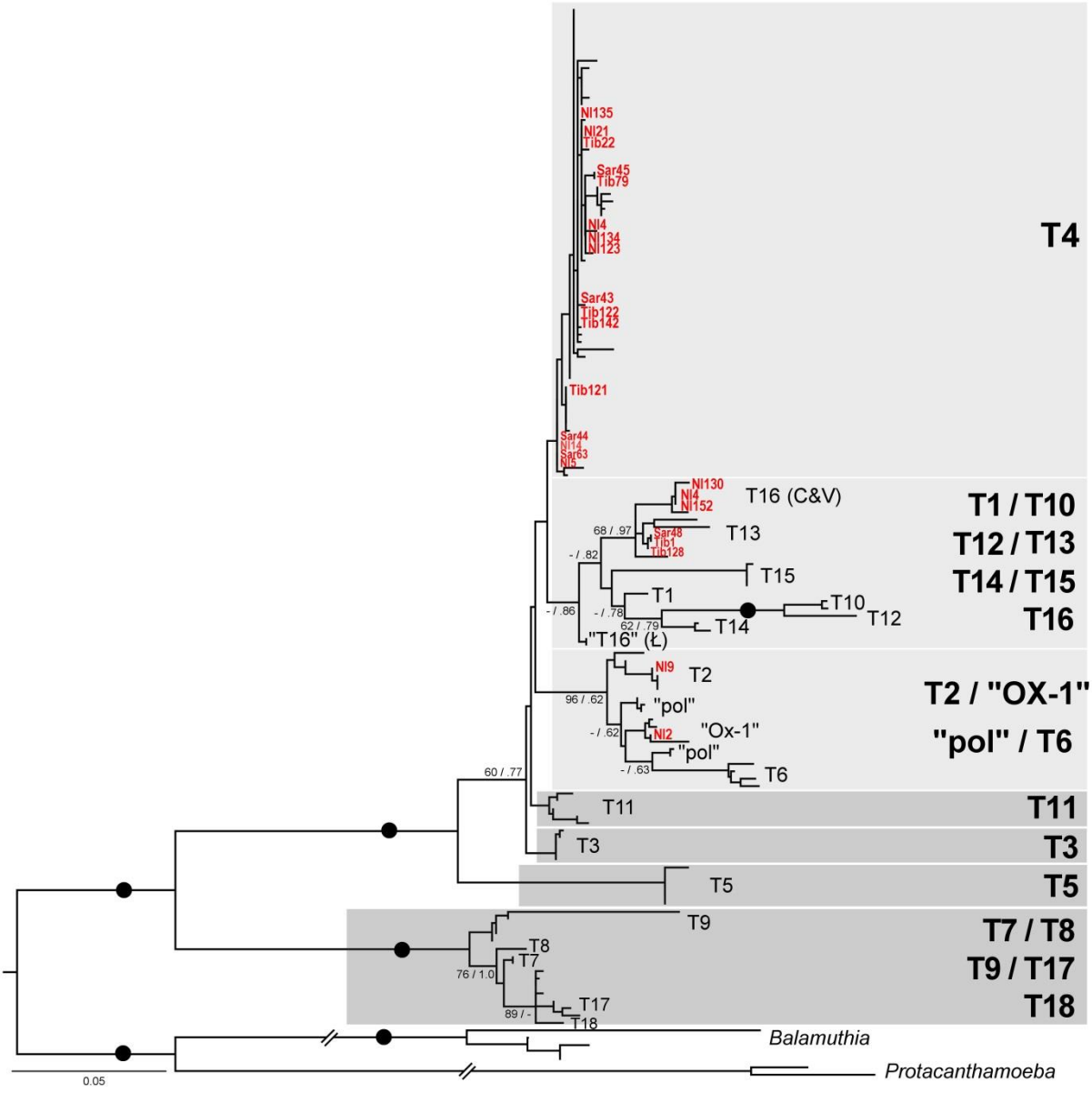
T2 and NI2 resembling “OX-1”, both not matching any sequences in GenBank. These two examples suggest that a more precise definition of *Acanthamoeba* species and subspecies is needed based on a combination of morphological features, multi-gene phylogenies and information on ecological functions or potential pathogenicity.

Several *Acanthamoeba* clones of the T4 and T13 sequence types were isolated from soils at high altitudes in Tibet. These environments are characterized by harsh, often extremely cold conditions, which are in strong contrast to temperatures in the human body. Nevertheless, strain Tib1 proliferated at 37 °C revealing a broad ecological tolerance. As several other strains of *Acanthamoeba* from all locations grew at high temperatures and several exhibited cytopathogenicity on human cell monolayers, soils may act as a major source for potentially pathogenic *Acanthamoeba*. Generally, *Acanthamoeba* is known to have a broad ecological niche and has been found to dominate protist communities even in extreme environments such as deserts (Rodriguez-Zaragoza et al. 2005) or polluted soils (Lara et al. 2007a) and our findings of different Acanthamoeba isolates from high altitude soils add to this spectrum. The generally high numbers of Acanthamoeba in soils, the robustness of its cysts and their ease of spread with dust particles, and its growth on various substrates explains why potentially pathogenic *Acanthamoeba* strains are so widely distributed in the environment.

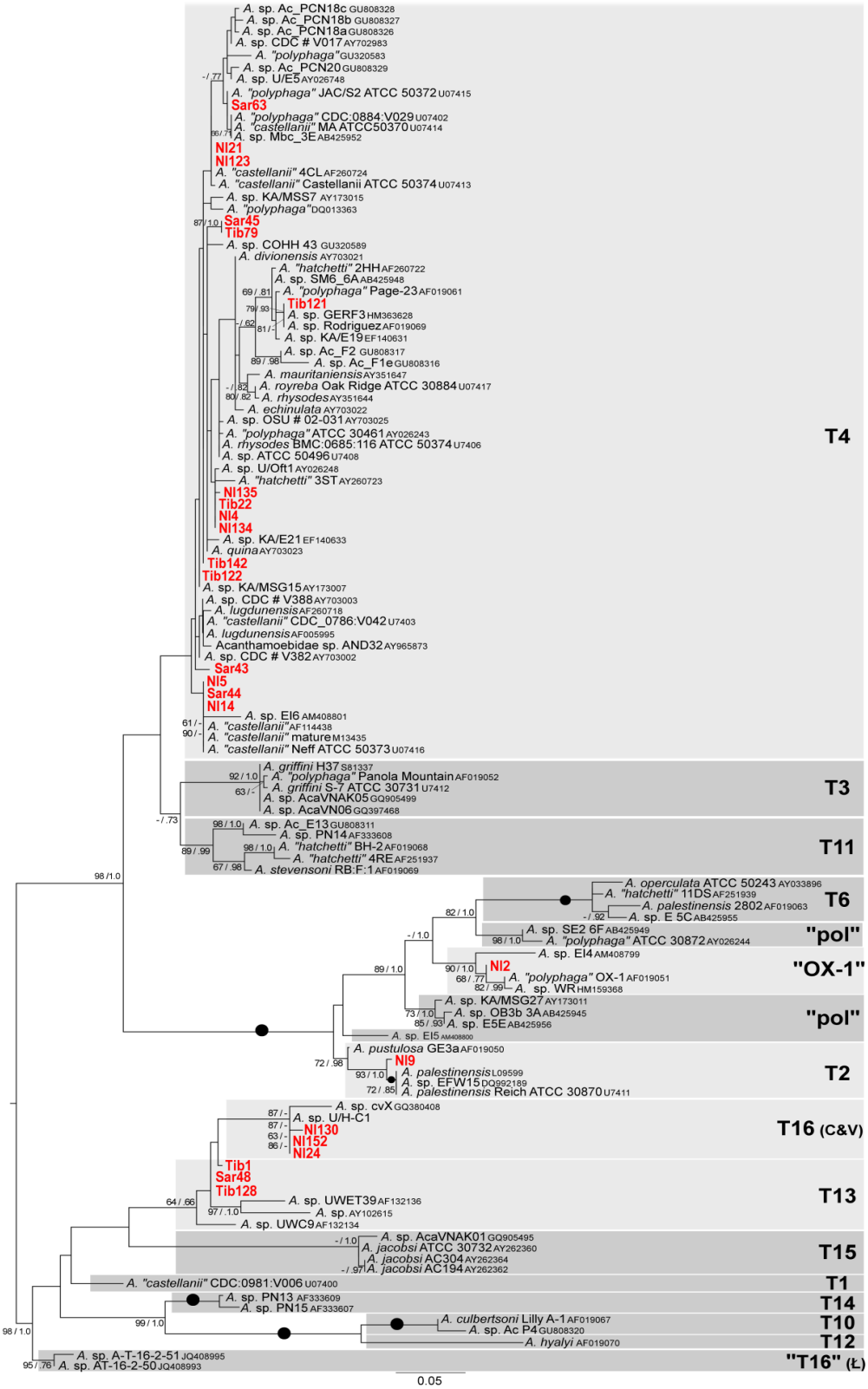
The Dutch soils appeared to contain a different community of *Acanthamoeba* sequence types compared with soils from Sardinia and Tibet, including the only cytopathogenic clone NI123. As none of the individual sequences were identical between locations the real *Acanthamoeba* species diversity and their functional adaptations are likely underestimated. Although we cannot deliver an ultimate proof for the population structure of *Acanthamoeba* due to a too shallow sequencing depth to allow reliable statistical analyses, our study suggests that individual soil sites may harbour distinct communities of *Acanthamoeba*. Future studies are needed to investigate environmental niches, community structures and determine potential (a)biotic factors that shape *Acanthamoeba* populations in soils, such as recently conducted for other groups of soil protists (Heger et al. 2013, Vannini et al. 2013).

Taken together, our study indicates that different soil types may harbour distinct communities of *Acanthamoeba*. Further, potentially pathogenic strains might be present in all soils, even in extreme altitudes.

Supplementary



Supplementary Figure 1. Phylogenetic analysis of Centramoebida based on 145 SSU sequences (all 18 described SSU types of *Acanthamoeba* represented) and 1,771 positions, with *Balamuthia* and *Prothacanthamoeba* as outgroups. Major clades and T4 are boxed. Only sequence types are named, while individual sequences were omitted for clarity, except for new sequences obtained in this study (in red). Support values > 50 % (maximum likelihood, left) or > 0.5 (posterior probability, right) are shown for branches leading to major clades. Filled circles represent full support in both analyses; boxes: clades of sequence types with bright boxes containing clones obtained in this study.



Supplementary Figure 2. Unrooted phylogenetic analysis focusing on sequence types and sequences associated with the new sequences obtained in this study. In total, 116 sequences with 1,947 unambiguously aligned positions were used; the tree is unrooted; only values higher than 60 for maximum likelihood analyses (left) and 0.60 for Bayesian analyses (right) are shown. Black circles represent full support; red: strains obtained in this study; boxes: associated sequence types with bright boxes containing clones obtained in this study.

Part 2 – Chapter 7

Profound effects of geographic location and use intensity on cercozoan communities in European soils

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Abstract

Cercozoa are among the most abundant and diverse groups of soil protists. However, their diversity, community composition and the influence of environmental shaping that shape those remain largely unknown. We applied a high-throughput sequencing (HTS) approach using primers optimized to select for Cercozoa to evaluate potential differences in soil cercozoan communities and decipher factors that shape those. Cercozoan communities in a total of 122 samples from at least fourfold replicates of two land use intensity (LUI) treatments in five geographically distant sites across Europe were analysed and compared. All geographically distant sites differed profoundly in cercozoan community composition and LUI also influenced cercozoan diversity in some cases. However, the differences observed depended on the taxonomic level of analysis, with slight differences shown at high taxonomic resolution (class) and most profound differences at the lowest taxonomic level (OTU). Distinct OTUs showed strong association to treatment or site that were driving overall differences in the community composition of Cercozoa. The strong biogeographic patterns of soil cercozoan communities underline urgently needed follow-up studies to determine environmental factors that shape biogeographic patterns of soil cercozoan communities and those of protists in general.

Introduction

Soil single-celled heterotrophic protists are key components in terrestrial soil food webs due to their high abundance, turnover, diversity and their functional roles as dominant bacterivores (Clarholm 1985, Ekelund and Rønn 1994, Adl et al. 2012). Cercozoa are among the most abundant soil protists are Cercozoa, confirmed both by traditional (Ekelund et al. 2001, Bass and Cavalier-Smith 2004, Bass et al. 2009b, Howe et al. 2009, Scharroba et al. 2012, Domonell et al. 2013) and recent high-throughput sequencing (HTS) studies (Urich et al. 2008, Baldwin et al. 2013, Bates et al. 2013). The phylum Cercozoa was only erected in 1998 being the first phylum described based solely on molecular phylogenetic information (Cavalier-Smith 1998, Bass and Cavalier-Smith 2004). Several studies have now proven the tremendous diversity of taxa in the phylum Cercozoa, which include naked (e.g. vampyrellids) and testate amoebae (e.g. euglyphids), amoeboflagellates (e.g. cercomonads), flagellates (e.g. glissomonads), and plant / stramenopile parasitic protists (e.g. plasmodiophorids), with total estimated species numbers considerably exceeding a thousand (Bass and Cavalier-Smith 2004, Bass et al. 2007, Bass et al. 2009b, Howe et al. 2009, Howe et al. 2011a, Adl et al. 2012, Berney et al. 2013, Neuhauser et al. 2014). Little, however, is known about the overall composition of soil Cercozoa and if communities differ between soils.

Similarly, knowledge on biogeographic patterns of protists in general is scarce and has been debated intensely, especially in the last decade (Baas-Becking 1934, Finlay 2002, Foissner 2006, Martiny et al. 2006). Recent evidence supports a wide distribution of dominant morphospecies of protists, while some species display a clearly more limited distribution (Foissner 2006, Bass et al. 2007, Heger et al. 2013). Undebated is, however, the high abundance and enormous diversity of protists. Especially soil protists show nearly a continuum of morphologically defined species, with molecular information often introducing a finer continuum within morphologically indistinguishable taxa (Bass et al. 2007, Epstein and López-García 2008, Adl et al. 2012, Bachy et al. 2013). The functional importance of this vast diversity of soil protists remains, however, largely unknown; the high diversity of bacteria, for instance, is suggested to act as an insurance for soil functioning to long term changes (Nielsen et al. 2011, Reich et al. 2012), and increasing resistance to pathogen invasion (van Elsas et al. 2012). Likewise, protist diversity might be important for the

resilience of soil functions, e.g. due to differential feeding on bacterial species (Glücksman et al. 2010, Saleem et al. 2012, Saleem et al. 2013), which in turn might alter plant growth and other ecosystem services (Clarholm 1985, Bonkowski 2004, Bonkowski and Clarholm 2012).

Molecular tools, such as HTS techniques, enable the analysis of high sample numbers at the same time. Since many protists cannot be cultivated, molecular methods targeting DNA has resulted in the discovery of many novel protist lineages and avoids laborious cultivation (Moon-van der Staay et al. 2001, Dawson and Hagen 2009, Lejzerowicz et al. 2010, Bates et al. 2013).

Until today, the few studies targeting soil protists with HTS techniques have focused on the broad taxonomic patterns of soil protist community composition, but detailed studies of specific taxonomic groups of free-living soil protists are virtually lacking with the exception of plant parasitic *Phytophthora* spp. (Vannini et al. 2013).

In this study we developed new specific primers targeting protists of the phylum Cercozoa in a HTS approach. We hypothesised that the community composition of Cercozoa would differ between geographically distinct sites and that change in LUI further influence the cercozoan community structure.

Materials and Methods

Soil locations, sampling and DNA extraction

Five well-characterized long term observatories (LTOs) were appointed within the EU project EcoFINDERS, which were located at distant sites across Europe and represented a variety of typical land management types of those regions (Table 1). Each LTO contained two contrasting types of LUI to discriminate local effects of land management form regional differences in species diversity. The following treatments were differentiated: Grasslands were sampled in England; one treatment was improved by application of fertilizers (Ei), the other unimproved (Eu), both represented by 15-fold replicatedsamples. Permanent grasslands (Fg) and grassland in a permanent agricultural rotation (Fc) were sampled in France, each fourfold replicated. Three treatments were differentiated in Italy, with intensively managed grassland (Ii), samples from grass patches (Ig) and under trees (It) taken from wooded land, all three replicated ninefold. Recently abandoned fields, two-five years

after agricultural use (Nl) were differentiated from long-term abandoned (20 - 25 years) fields in the Netherlands (Nh), replicated 9- and 12-fold, respectively. The last treatments were taken from fertilized (Sf) and unfertilized coniferous forests (Su) in Sweden, both being replicated 18-fold. A summary of these site and treatments characteristics are shown in Table 1.

Sampling was conducted between March and June 2011. The upper 10 cm of the organic soil horizon were sieved, roots and stones removed and DNA was extracted according to a standardized ISO 11063 protocol (Plassart et al. 2012).

Table 1. Site and treatment characteristics; $\bar{X}$ = mean value

Site location	Sampling time	H_2O (g/kg)	C_{org} (g/kg)	Total N (g/kg)	C/N	Organic matter (g/kg)	pH	P (P_2O_5 OLSEN) (g/kg)	Land use (Label; sample # before /after quality filtering])
Lusignan, France	March 2011	NA	9 – 12; $\bar{X}$ = 10.7	1.0 – 1.3; $\bar{X}$ = 1.2	8.9 – 9.6; $\bar{X}$ = 9.3	16 – 21; $\bar{X}$ = 18.5	NA	NA	Permanent agriculture (Fc; 4 / 4) Permanent grassland (Fg; 4 / 4)
Lancester, England	June 2011	21 – 46; $\bar{X}$ = 33.1	43 – 95; $\bar{X}$ = 59.3	2.8 – 8.7; $\bar{X}$ = 5.3	9.8 – 15.1; $\bar{X}$ = 11.3	74 – 165; $\bar{X}$ = 102.7	4.8 – 6.6; $\bar{X}$ = 5.4	0.009 – 0.170; $\bar{X}$ = 0.040	improved grassland (Ei; 14 / 15) unimproved grassland (Eu; 13 / 15)
Sardinia, Italy	May 2011	9 – 23; $\bar{X}$ = 15.1	16 – 35; $\bar{X}$ = 24.0	0.9 – 2.1; $\bar{X}$ = 1.5	12.5 – 21.5; $\bar{X}$ = 15.9	28 – 60; $\bar{X}$ = 41.4	5.2 – 6.4; $\bar{X}$ = 5.9	0.005 – 0.045; $\bar{X}$ = 0.015	intensive grassland (Ii; 8 / 9) wooded land grass patch (Ig; 9 / 9) wooded land under tree (It; 8 / 9)
Veluwe, Netherlands	July 2011	7 – 15; $\bar{X}$ = 11.2	17 – 48; $\bar{X}$ = 29.3	1.0 – 2.3; $\bar{X}$ = 1.4	16.0 – 25.5; $\bar{X}$ = 21.5	30 – 83; $\bar{X}$ = 50.7	4.1 – 6.0; $\bar{X}$ = 5.4	0.090 – 0.360; $\bar{X}$ = 0.227	recently abandoned fields (Nl; 5 / 9) long abandoned fields (Nh; 8 / 12)
Lamborn, Sweden	July 2011	5 – 42; $\bar{X}$ = 22.3	21 – 178; $\bar{X}$ = 68.6	0.6 – 4.7; $\bar{X}$ = 1.9	31.3 – 49.8; $\bar{X}$ = 36.8	36 – 308; $\bar{X}$ = 118.7	4.0 – 4.9; $\bar{X}$ = 4.6	0.005 – 0.042; $\bar{X}$ = 0.017	fertilized forest (Sf; 11 / 18) unfertilized forest (Su; 12 / 18)

Primer design and amplicon preparation of 18S rRNA gene

Amplicons of ~ 1,200 bp were generated from each sample using the cerocozan-specific primer combination 25F (5' - CAT ATG CTT GTC TCA AAG ATT AAG CCA - 3') and 1256R (5' - GCA CCA CCA CCC AYA GAA TCA AGA AAG AWC TTC - 3'; Bass and Cavalier-Smith 2004) in a first round (94 °C for 1', 35 cycles of 94 °C for 30'', 70 °C for 60'' and 72 °C for 2' with a final extension for 5' at 72 °C). PCR reactions were carried out in 31 µl volume consisting of 0.6 µl of each primer (10 µM), 0.6 µl nucleotides (10 mM), 1.0 µl template DNA, 24.5 µl H_2O, 3 µl GreenTaq Buffer and 0.15 µl GreenTaq polymerase (5 U * μl^{-1}) (Fermentas, St. Leon-Rot, Germany). 1.0 µl aliquots of the resulting PCR products were used as template for a hemi-nested PCR step using the same reverse primer with the new forward primer "PreV4" (5' - GYT GCA GTT AAA AAG CTC GTA GTT G - 3'; this study) at the 5' end of the SSU V4 region, judged to be the most informative SSU barcoding region, because of its high variable nature and sufficient length for phylogenetic analyses (Pawlowski et al. 2012), while producing an amplicon of ~ 500 bp, appropriate for 454 sequencing. PreV4 was designed *in silico* and tested for specificity by sequencing environmental DNA from 14 clones extracted from Dutch soil. All sequences obtained were of cercozoan taxa (data not shown).

PCR conditions of the second step were the same, except an annealing temperature of 66 °C and elongation for 90'' were used. A five bp long MID-identifier was incorporated onto the 5' ends of both PreV4 and 1256R before the nested PCR step. Both PCRs were replicated twice for each sample to increase product yield and to reduce PCR errors. Duplicates from each sample were pooled, purified by gel extraction using the Agarose GelExtract Mini Kit (5PRIME, Hilden, Germany) and quantified by NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, USA).

All 122 samples were devided into eight batches by equimolar pooling PCR products of 15 - 16 random samples. Pooled libraries were sent for pyrosequencing using the standard protocol (titanium chemistry) on a Genome Sequencer FLX system (Beckman Coulter, Fullerton, USA).

Bioinformatics analyses

Sequences obtained were first demultiplexed according to their multiplex identifier (MID) using the sffinfo command of Mothur v.1.22.2 (Schloss et al. 2009), allowing one mismatch

per MID. Fasta and quality files were converted into fastq file using the faqual2fastq.py script of Usearch v7.0.1001 (Edgar 2013). All sequences were then labelled with a sample name and pooled.

The raw sequences were filtered and trimmed using the fastq_filter command of Usearch with the option fastq_truncqual 10, such as sequences are truncated at the first position having a quality score ≤ 10. Sequences were then truncated to the length of 300 bp, with shorter sequences discarded using the option fastq_trunclen. Sequences from forward and reverse primers were sorted according to their primer sequences using the trim.seqs and split.groups commands of Mothur, allowing two mismatches. Sequences from reverse primers were removed from the analysis. Trimmed sequences were dereplicated to remove duplicated sequences using derep_fulllength command of Usearch. Dereplicated sequences were sorted by decreasing abundance and singletons were discarded using sortbysize command of Usearch. Operational taxonomic units (OTUs) were generated from abundance-sorted sequences using the cluster_otus command of Usearch for 99 %, 98 %, 97 %, 96 %, 95 % and 90 % similarity thresholds. For each similarity threshold considered, trimmed sequences (including singletons) were mapped against the OTU representative sequences using usearch_global of Usearch. Based on these mapping results, matrices containing the sequence abundances of different OTUs in each soil sample were generated using uc2otutab.py script of Usearch. To make comparable samples with different number of sequences, matrices were subsampled with an identical sequences number (n = 500) for each soil sample using the sub.sample command of Mothur. This sequence number was determined to conserve a minimum of four independent replicates for each treatment considered in the study.

Taxonomic assignation was determined for each OTU representative sequences using the Basic Local Alignment Search Tool (BLAST) algorithm v 2.2.23 (Altschul et al. 1990) against the Protist Ribosomal Reference Database PR2 (Guillou et al. 2013). All assignations were determined using an e-value cut-off of $1e^{-5}$, an identity cut-off of 90 % and a coverage cut-off of 80 % of the query sequence covered in the alignments.

Only sequences assigned to sequences specific for the phylum Cercozoa were subject to downstream comparisons and statistical analyses. The cercozoan OTUs were assigned to the different taxonomic levels class, order, family, genus, species and OTU level.

Statistics

Statistical analyses were performed as suggested by Anderson and Willis (2003) including unconstrained ordination using principal coordinate analyses with Bray-Curtis distance matrices (Gower 2005), constrained analyses using canonical analysis of principal coordinates (Anderson and Willis 2003), statistical tests using analysis of similarities (ANOSIM) (Clarke 1993) and permutational multivariate analyses of variance (PERMANOVA) (Anderson 2001) and characterization of OTUs that shape the multivariate analyses using detrended correspondence analysis (DCA) and linear discriminant analyses (LDA). Further, two clustering methods (paired group (UPGMA, using Bray-Curtis similarity index) and Ward's method (Euclidian similarity index) were applied. Global beta richness was assessed by combining all samples from each of the five distinct geographic origins. All analyses were performed in PAST (Hammer et al. 2001). Differences were considered as significant when a threshold of $p < 0.05$ was reached.

After the initial taxonomic binning of 966 cercozoan OTUs several verification / refinement steps were carried out. A custom database was built incorporating as many taxonomically assigned cercozoan sequences as possible: the PR2 database (Guillou et al. 2013), unpublished cercomonad and glissomonad sequences (David Bass laboratory), and sequences from recent publications not yet incorporated into PR2. The 957 OTUs were locally BLASTn-searched against the custom database. All query sequences returning a BLASTn sequence identity result > 95 % were accepted as belonging to the genus indicated. The remaining 536 were aligned with a representative pared-down cercozoan alignment to identify sequence clusters in the OTUs and their broad taxonomic affiliations. Representative OTUs from the clusters were re-aligned with a comprehensive sequence dataset of the group to which they belonged. The genus-level affiliation of all the OTUs in the cluster was inferred from this. Where a genus-level affiliation was not possible due to the divergence of the sequence, order level was used instead, or a clear definition of an environmental clade. Our convention for reporting the taxonomic affiliations is to associate a genus with the OTU number that blasts to it (e.g. Cercomonas_[OTU]12), as robust affiliation to the species level is difficult to prove (possibly apart from the rare cases where the blast sequence identity match is 100 %); we have devised an approach that can be consistently applied across the dataset as a whole.

Results

Amplicon analyses

A total of 224,179 sequences were obtained from 122 sites after quality filtering. 28 samples with sequence numbers lower than 500 were discarded leaving 94 samples with an average of 2,343 sequences (546 - 7,994 sequences). OTU clustering at different levels revealed an exponential increase of assigned OTUs up to a similarity threshold of 98 % ($r^2 = 0.975$). Clustering to 99 % strongly outreached exponential growth and reducing the r^2 to 0.87 (Figure 1). Similarly, reads were remapped confidently up to an OTU clustering of 98 % assuming an exponential decrease of assignable OTUs ($r^2 = 0.98$), while when including clustering at 99 % strongly deflated the successful re-assignment of reads ($r^2 = 0.83$, Figure 1). Therefore, subsequent analyses focused on an OTU clustering level of 98 %.

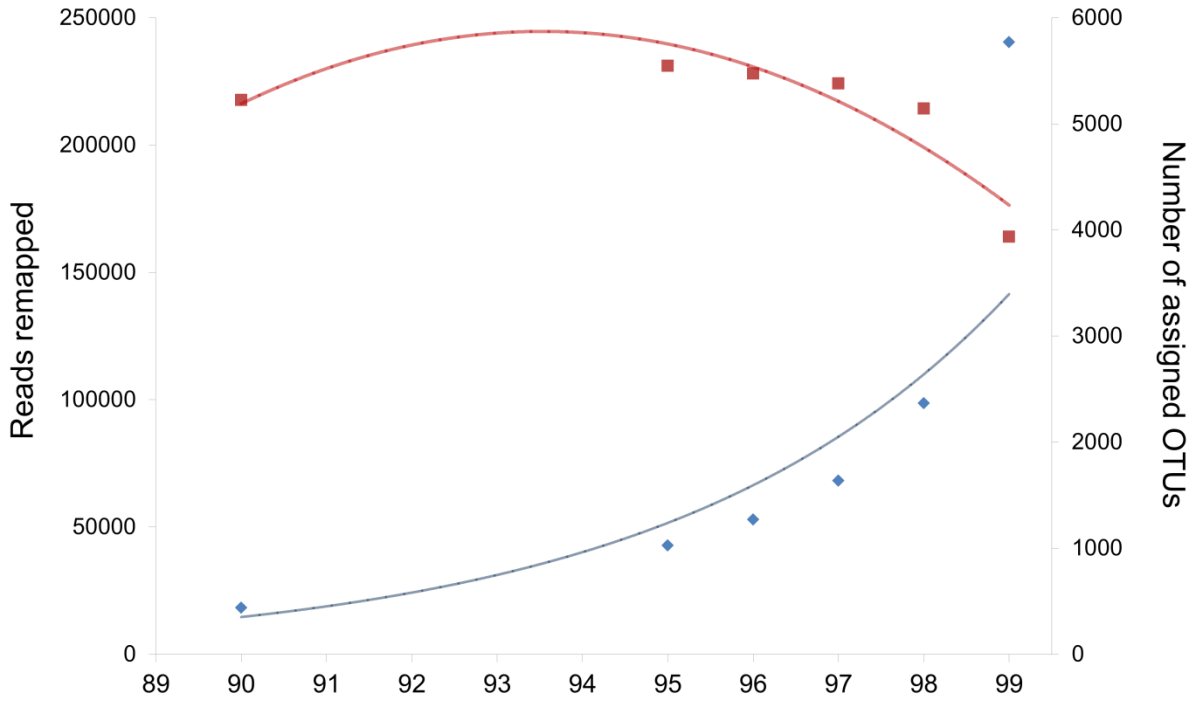


Figure 1. Number of OTUs (diamonds and line in blue; right y-axis) and reads remapped (squares and polynomial line in red, left y-axis) at OTU assignment of 90 % and 95 - 99 %. Exponential fitting best explained increasing numbers of OTU (blue, $r^2 = 0.87$) and decreasing numbers of reads remapped (red, $r^2 = 0.83$) with increasing OTU clustering levels.

The primers proved to be highly cercozoan specific with 81.9 % (1,919 sequences per sample) assigned as Cercozoa. 11.7 % of the sequences remained unassigned, 4.2 % were fungi and 1.7 % streptophytes. Other assigned phyla were negligible (> 0.4 %). OTU

clustering at 98 % sequence similarity resulted in a total of 1,636 OTUs of which 957 were cercozoan-specific, while 559 could not be assigned against the protist PR2 database. OTU numbers levelled off, but did not reach saturation either at sites (Figure 2) and only rarely in individual samples (Supplementary Figure 1).

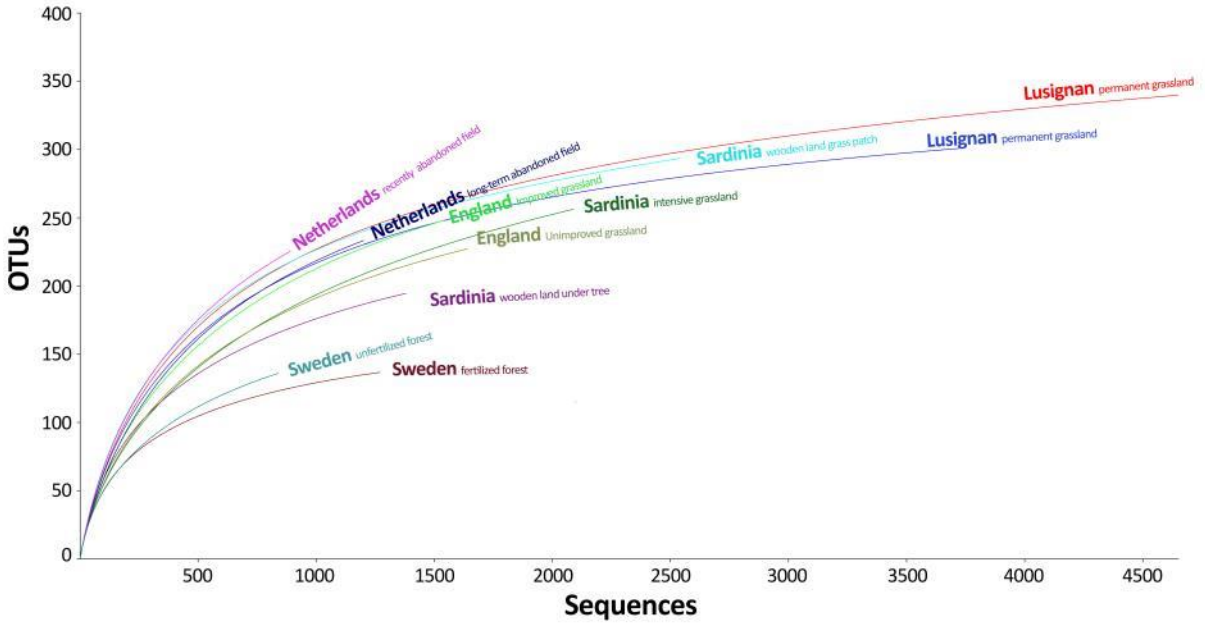


Figure 2. Rarefaction curves of amplicon data sets from all combined OTUs combining all samples of respective treatments.

The 957 cercozoan sequences were assigned to 9 classes, 24 orders, 42 families, 65 genera and 110 species. Re-blasting using our own database revealed that most sequences could not reliably be assigned on the species level, often not even on genus or family level (Supplementary Table 2). The majority of OTUs showed identities < 99 % on the species level, strongly suggesting that these sequences were not necessarily equivalent to the assigned species. A subset of 47 OTUs is shown as an example for focused phylogenetic analyses of cercomonads and glissomonads in Supplementary Figures 2 and 3.

We therefore focus our respective analyses on the lowest (i.e. OTU) taxonomic level and compare those to highest (i.e. class) levels.

Cercozoan community structure and site comparisons

We found strongly differing cercozoan community compositions (CCCs) between sites and treatments. The CCCs in coniferous forest sites in Sweden differed most strongly from grassland and arable field sites, suggesting that coniferous forests contain distinct CCCs. The level of taxonomic classification had a profound impact on the results. When CCCs were

analysed on the deepest level (OTU level), CCCs differed between all sites revealing similarities between 0.24 (Sweden and France) to 0.51 (England and France; Figure 3 and 4). The differences in CCCs were less pronounced when analysing the cercozoan communities at higher taxonomic levels, especially class level. Here, similarities ranged from 0.74 (England and Sweden) to 0.97 (Netherlands and Sweden; Supplementary Figure 4 and 5). However, multivariate analyses demonstrated that the CCCs clearly differed between locations irrespective of phylogenetic resolution (class: $R = 0.43$, $p < 0.001$ (ANOSIM) and $F = 14.9$, $p < 0.001$ (PERMANOVA); OTU: $R = 0.63$, $p < 0.001$ (ANOSIM) and $F = 8.7$, $p < 0.001$ (PERMANOVA); Table 2).

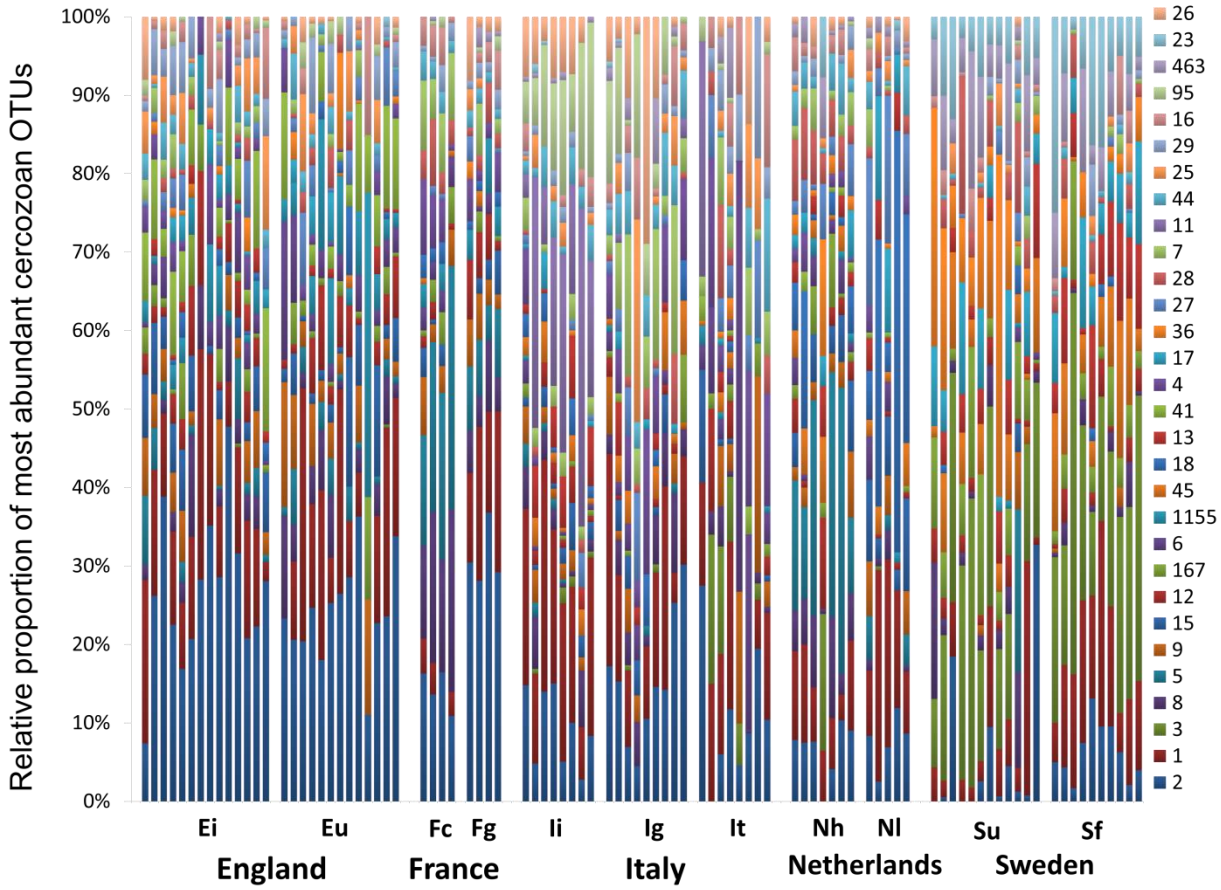


Figure 3. Rarified relative abundances of the 30 most abundant cercozoan OTUs in all samples at respective sites and treatments (x-axis); (see Table 1 for details on soil characteristics and treatment abbreviations).

In general, LUI of specific land management types had a lower influence on CCCs than geographic location, indicating the existence of specific CCCs in different European soils. In line, the majority of treatments from respective sites clustered together, especially at lower taxonomic resolution (Figure 4; Supplementary Figure 4). Most similar were both English grassland treatments (class level: 0.97; OTU level: 0.71), while Swedish fertilized forest

samples were least similar to those of French culture at OTU level (similarity = 0.14; Figure 4), while the lower resolution on CCC differences on class level revealed largest differences from intensive grassland in Italy with culture in France (similarity of 0.63; Supplementary Figure 4). Despite being grasslands, the CCCs in the respective treatments in England, France, the Netherlands and Italy clearly differed. Interestingly, the CCCs of recently abandoned agricultural fields in the Netherlands clustered with agricultural fields in France, suggesting that agricultural management provokes distinct and long-lasting changes in protist communities. Both multivariate statistical analyses (ANOSIM and PERMANOVA) proved that cercozoan communities differed between treatments (class: $R = 0.49$, $p < 0.001$ (ANOSIM) and $F = 10.9$, $p < 0.001$ (PERMANOVA); OTU: $R = 0.67$, $p < 0.001$ (ANOSIM) and $F = 5.2$, $p < 0.001$ (PERMANOVA); Table 3).

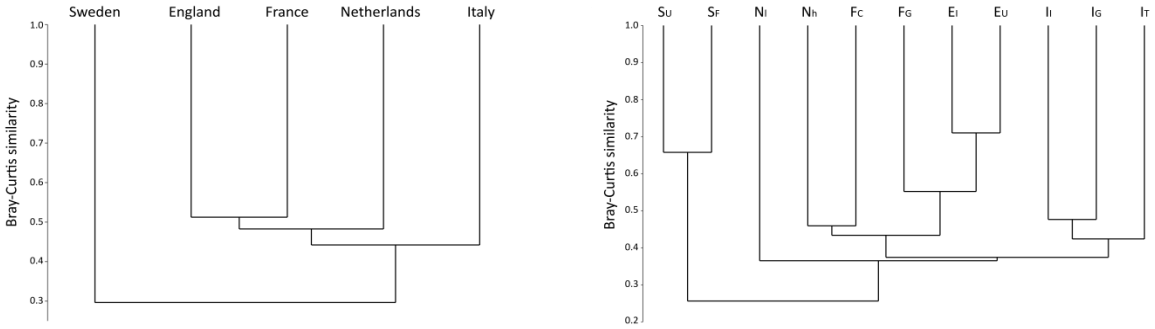


Figure 4. Similarity of cercozoan communities between sites (left) and treatments (right) based on OTU level using the paired group (UPGMA) analyses with Bray Curtis similarity; see Table 1 for details on soil characteristics and treatment abbreviations.

Table 2. Differences of Cercozoan communities on the OTU level between geographic sites based on ANOSIM (ASIM) and PERMANOVA (POA)

	England		**France**		**Italy**		**Netherlands**		**Sweden**	
	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA
England			0.0012	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
France	0.0012	0.0001			0.1505	0.0001	0.0016	0.0002	0.0001	0.0001
Italy	0.0001	0.0001	0.1505	0.0001			0.0036	0.0001	0.0001	0.0001
Netherlands	0.0001	0.0001	0.0016	0.0002	0.0036	0.0001			0.0001	0.0001
Sweden	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001		

Table 3. Differences between treatments based on OTU level using the multivariate statistical analyses ANOSIM (ASIM) and PERMANOVA (POA); see Table 1 for details on soil characteristics and treatment abbreviations

	England (Ei)		**England (Eu)**		**France (Fc)**		**France (Fc)**		**Italy (Ii)**		**Italy (Ig)**		**Italy (It)**		**Netherlands (Nh)**		**Netherlands (Nl)**		**Sweden (Su)**		**Sweden (Sf)**	
	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA	ASIM	POA
Ei			0,242	0,4334	0,0002	0,0007	0,1044	0,0034	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0002	0,0005	0,0002	0,0001	0,0001	0,0001	0,0001
Eu	0,242	0,4334			0,0008	0,0007	0,2114	0,0005	0,0001	0,0001	0,0001	0,0002	0,0001	0,0001	0,0002	0,0001	0,0005	0,0001	0,0001	0,0001	0,0001	0,0001
Fc	0,0002	0,0007	0,0008	0,0007			0,0303	0,0287	0,0021	0,003	0,0043	0,0018	0,1536	0,0016	0,0493	0,0063	0,014	0,0068	0,0003	0,0003	0,0011	0,0008
Fg	0,1044	0,0034	0,2114	0,0005	0,0303	0,0287			0,0022	0,0017	0,0525	0,0014	0,488	0,0019	0,0025	0,0039	0,0162	0,0067	0,0005	0,0008	0,0014	0,0008
Ii	0,0001	0,0001	0,0001	0,0001	0,0021	0,003	0,0022	0,0017			0,0002	0,0002	0,0002	0,0003	0,0002	0,0005	0,0007	0,0008	0,0001	0,0001	0,0001	0,0001
Ig	0,0001	0,0001	0,0001	0,0002	0,0043	0,0018	0,0525	0,0014	0,0002	0,0002			0,0001	0,0001	0,0003	0,0001	0,0008	0,0012	0,0001	0,0001	0,0001	0,0001
It	0,0001	0,0001	0,0001	0,0001	0,1536	0,0016	0,488	0,0019	0,0002	0,0003	0,0001	0,0001			0,0018	0,0001	0,0127	0,0007	0,0002	0,0002	0,0002	0,0001
Nh	0,0001	0,0002	0,0002	0,0001	0,0493	0,0063	0,0025	0,0039	0,0002	0,0005	0,0003	0,0001	0,0018	0,0001			0,0095	0,009	0,0012	0,0019	0,0004	0,0004
Nl	0,0005	0,0002	0,0005	0,0001	0,014	0,0068	0,0162	0,0067	0,0007	0,0008	0,0008	0,0012	0,0127	0,0007	0,0095	0,009			0,0006	0,0002	0,0005	0,0003
Su	0,0001	0,0001	0,0001	0,0001	0,0003	0,0003	0,0005	0,0008	0,0001	0,0001	0,0001	0,0001	0,0002	0,0002	0,0012	0,0019	0,0006	0,0002			0,0311	0,0006
Sf	0,0001	0,0001	0,0001	0,0001	0,0011	0,0008	0,0014	0,0008	0,0001	0,0001	0,0001	0,0001	0,0002	0,0001	0,0004	0,0004	0,0005	0,0003	0,0311	0,0006		

For convenience restrain our analysis to the OTU level in the analysis of individual soil samples. Cercozoan communities followed the general trends observed on combined site and treatment analyses, with CCCs largely being separated between sites and treatments (Figure 5). All Swedish samples clustered distantly from other samples (brown symbols), English grassland sites grouped together (red symbols) and French agriculture (black circles) grouped with recently abandoned agricultural fields in the Netherlands (blue triangles; Figure 5). The most divergent communities according to LUI were found in the abandoned agricultural fields in the Netherlands (Figure 5).

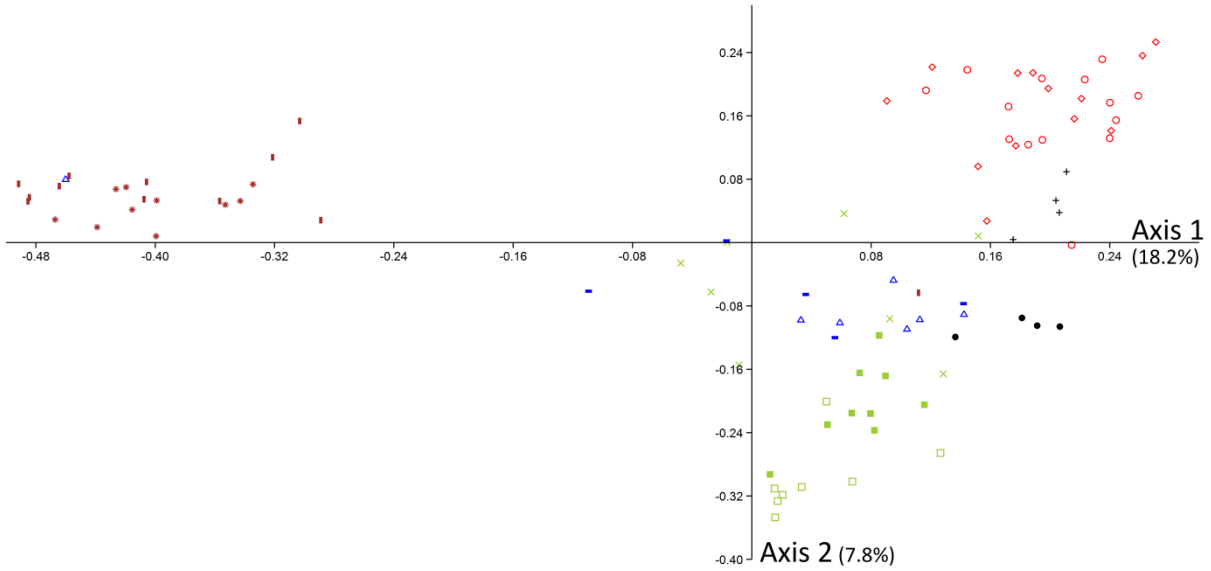


Figure 5. PCoA showing sample specific differences in the cercozoan communities on the OTU level; geographically distant sites are illustrated with different colours, treatments with different symbols; black dots: French culture (Fc); black plus: French grassland (Fg); green empty squares: Italian intensive grassland (Ii); green filled squares: Italian wooden land, grass patch (Ig); green X: Italian wooden land under tree (It); red circle: English improved grassland (Ei); red diamond: English unimproved grassland (Eu); blue triangle: Dutch recently abandoned fields (Nh); blue dash: Dutch long-term abandoned fields (Nl); brown bars: Swedish unfertilized forest; brown stars: Swedish fertilized forest.

Taxon-specific patterns

On the class level, differences in cercozoan communities were largely caused by changes in Thecofilosea. This class comprised the highest relative abundance of 40.7 % in English grasslands and lowest in Swedish forests (14.3 %), but a general dependence on land management, such as an association with grasslands could not be detected (Supplementary Figure 5; Supplementary Table 1). The dominant class Sarcomonadea was with 64.3 % of all cercozoan sequences significantly higher represented in France compared to Italy (45.8 %) and England (43.6 %; Supplementary Figure 5; Supplementary Table 1), while Imbricatea was

higher in Italy (27.0 %) and Sweden (21.7 %) compared to England (10.2 %) and France (8.8 %; Supplementary Figure 5; Supplementary Table 1).

Especially on the OTU level the Swedish forests were clearly different to the other sites (Figure 3; Supplementary Table 3). Striking examples were *Corythion* OTU3 and Glissomonad_Z OTU 36 being highly dominant in Swedish sites unlike at other sites (Figure 3; Supplementary Table 3). *Rhogostoma* OTU2 represented by far the most abundant species in England, while it was less dominant in other sites, especially in Sweden. *Euglypha* OTU11 and *Trinema* OTU17 were more dominant in Italian compared with English soils, while Glissomonad_U OTU15 showed an inverse association with these two sites. *Neoheteromita* OTU5 was most dominant in French and Dutch soils especially in comparison to Italian soils (Figure 3; Supplementary Table 3). Other site difference patterns of the most abundant species are shown in Supplementary Table 3.

Discussion

This study reveals clearly distinct CCCs in different geographic locations, with Swedish coniferous forests most strongly differing, but even grasslands soils harbouring different cercozoan communities. Grassland sites were replicated across Europe with treatments in England, France, Italy and the Netherlands, but CCCs differed between them. Similarly, no common OTU was found that was indicative for grasslands, suggesting that a complex combination of geographic distance, land management and other abiotic factors shape cercozoan- and protist communities. In line, climatic factors such as moisture have been shown to strongly influence protist communities (Bischoff 2002, Bates et al. 2013, Heger et al. 2013).

LUI had much less effect than geographic distance, but slight changes between low and high LUI were observed. These were most pronounced in France, the site with highest differences in LUI between treatments. CCCs in the intensively managed permanent agricultural fields in France more closely resembled those from recently abandoned fields in the Netherlands, suggesting a dominant and long lasting legacy effect of agriculture (Foissner 1997). We could, however, not detect a reduction in diversity or absence of major OTUs, indicating that synchronous shifts in nearly all OTUs might be underlying reasons rather than changes in only few groups. Testate amoebae have been suggested to be strongly reduced by

agriculture, while species richness of ciliates sometimes even being higher in agricultural soils (Foissner 1997). Therefore, the entire CCCs might more closely resemble those of ciliates. We have not detected significant changes in testate amoebae, but as the resolution to species level was not possible, we might have missed species-specific changes and losses in some larger, k-strategist testate amoebae might have been compensated for by closely-related fast-growing r-strategists (Wanner et al. 2008).

We found that differences in CCCs become much more pronounced with increasing taxonomic resolution, resulting in largest differences on the OTU level. This likely explains why cultivation-based studies that are often limited to low taxonomic identification levels fail to detect substantial differences in community compositions of Cercozoa and protist in general (Finlay et al. 2000, Domonell et al. 2013). Interesting, however, is that differences in CCCs were even observed at the highest taxonomic levels, i.e. class level. Therefore, cercozoan and protist communities in general seem to be much more divergent in soils as suggested before (Finlay et al. 2000, Esteban et al. 2006).

The total of 957 distinct cercozoan specific OTUs placing at distant positions in phylogenetic analyses (Supplementary Figures 2 and 3), provide strong support for the enormous diversity of soil cercozoans (Bass and Cavalier-Smith 2004, Bass et al. 2009b, Howe et al. 2009, Howe et al. 2011a). Such an analysis depth within a single study can only be accomplished by HTS methods and confirms that HTS will eventually become the gold-standard in studying (soil) protist diversity (Bates et al. 2013, Stoeck et al. 2014).

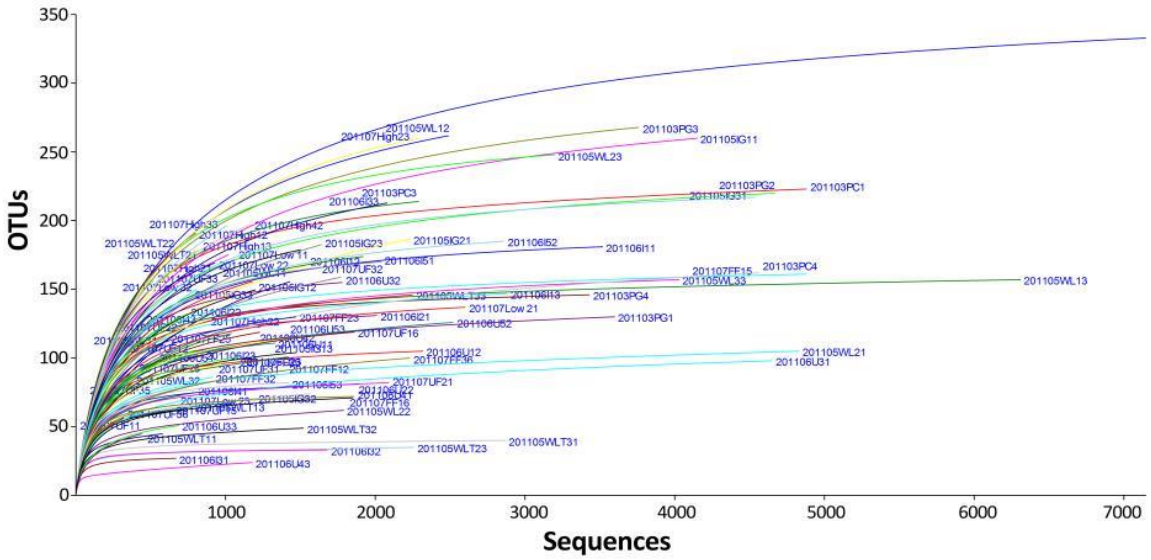
The results of different OTU clustering levels with an appropriate similarity threshold of 98 % is in line with a recent 454 study that targeted the V4 region in ciliates (Stoeck et al. 2014). Therefore it seems that this OTU clustering level when targeting the hypervariable region V4, as the suggested barcode region for protists (Pawlowski et al. 2012), is appropriate when analysing high-throughput data obtained with 454 pyrosequencing platforms. The need of OTU clustering to counteract sequencing errors and the non-existence of truly general, unbiased primers (Epstein and López-García 2008, Adl et al. 2014) currently lead to an underestimation of taxon richness in HTS. The use of recently developed PR2 database strongly reduced false OTU assignments that commonly introduce biases in HTS (Medinger et al. 2010, De Jonckheere et al. 2012, Stoeck et al. 2014). However, significant OTU numbers could not be assigned as protist sequence information is still highly underreported in

databases (Pawlowski et al. 2012, Stoeck et al. 2014). Intensive efforts using cultivation-based approaches are needed to fill the immense gaps in public databases with reliable sequence information.

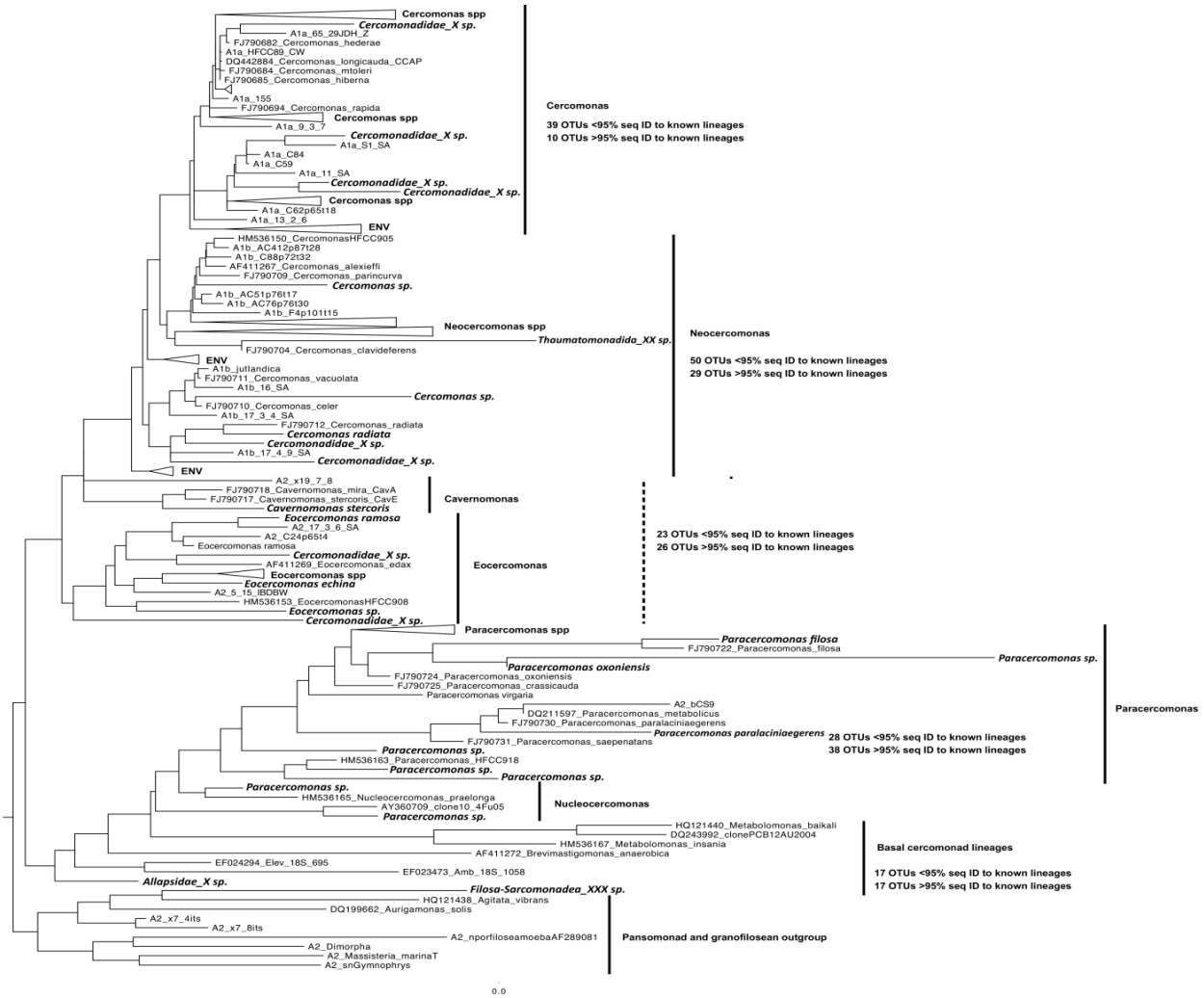
Little is known about soil Cercozoa, their biogeography and environmental factors shaping cercozoan soil communities, especially on the species level. Therefore, classical indicator or flagship species (Foissner 2006, Foissner 2009) could not reliably be assigned. However, we detected individual clades, especially at the OTU level, preferentially being associated with certain sites and LUI. Cercozoan spp. associated with moss were dominant in Swedish coniferous forest soils, confirming strikingly different communities of other protist groups present in coniferous forest soils (Bamforth 1980, Wanner 1991, Foissner 1998, Bobrov 2005). The testate amoebae *Corythion* spp. were among the dominant OTUs in Swedish forest, which have been shown to be dominant in moss-covered, grassland and forest soils (Heal 1965, Wanner 1991, Bamforth 2010, Carlson et al. 2010). The specious and widespread testate amoeba genus *Euglypha* is commonly found in mosses and litter layers (Wanner 1991, Bamforth 2010). However, *Euglypha laevis* was reported in high abundances in a range of orchards (Wanner 1991), which is in line with the high fraction of *Euglypha* among Cercozoa in wooden sites in Italy. In contrast, *Neoheteromita* OTU5 comprised only a small fraction among Italian and Swedish cercozoans. The genus *Neoheteromita* formerly belonged within the abundant and geographically widespread species complex "*Heteromita" globosa* (Howe et al. 2009), geographic location seems not to be the driving factor impacting this genus. Glissomonad_Z OTU 36 was another OTU dominating in Swedish forest soil. The sequences of as yet uncultivated is known from forest soils in the USA (Lesaulnier et al. 2008) and might be associated with forest soils. *Rhogostoma* is suggested to be abundant in freshwater and soil habitats (Howe et al. 2011b), rendering explanations of the dominance in English grassland sites difficult.

Taken together, we reveal that the community structure of Cercozoa shows biogeographic patterns with soil management also affecting the CCC, by the broadest study targeting soil protists to date. The enormous diversity sequences assigned to non-described taxa further suggests that a plethora of cercozoan species still is undiscovered, encouraging future studies to combine cultivation-based and molecular tools to broaden the understanding of taxon-specific functions within communities of Cercozoa and protist in general.

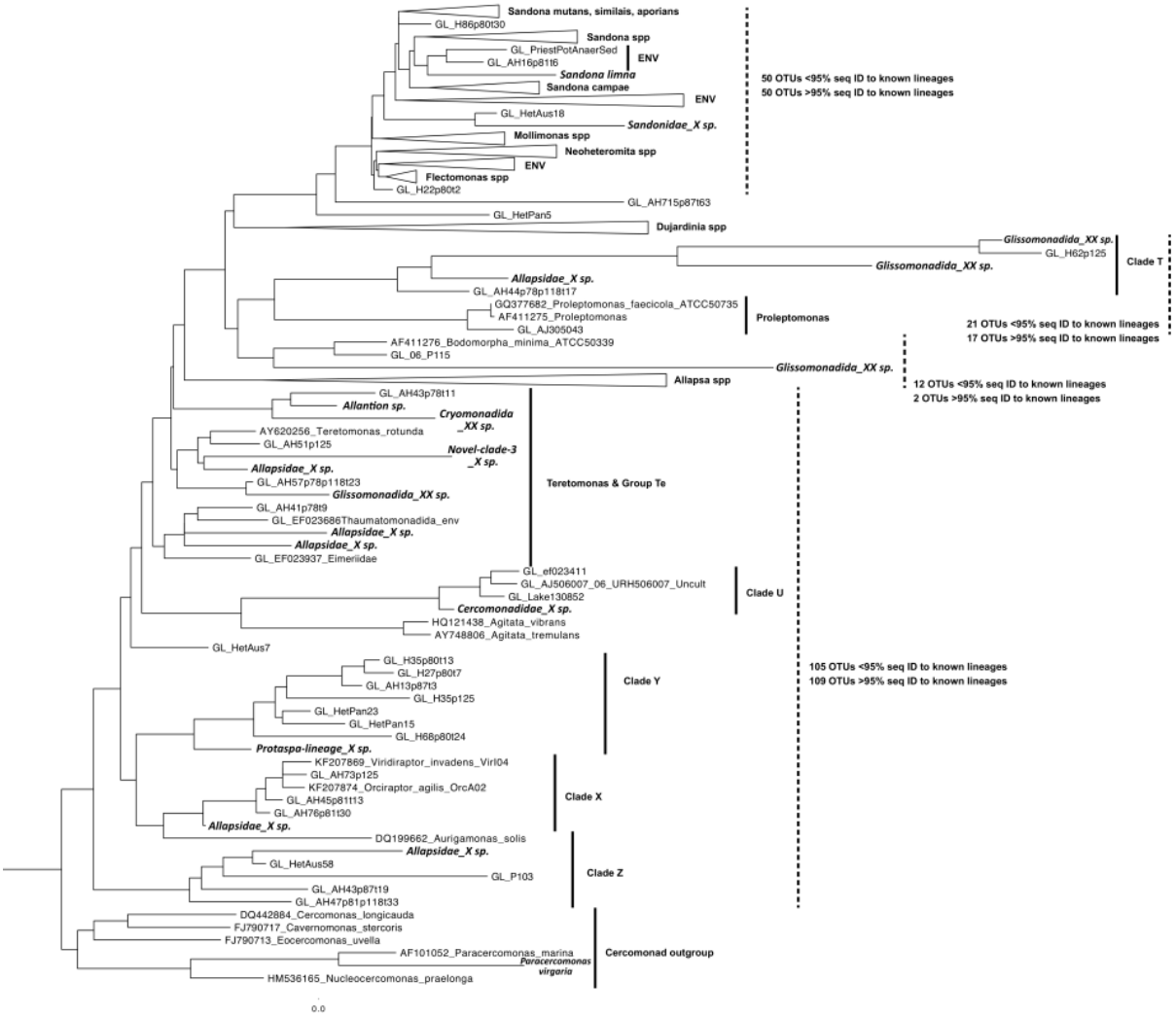
Supplementary



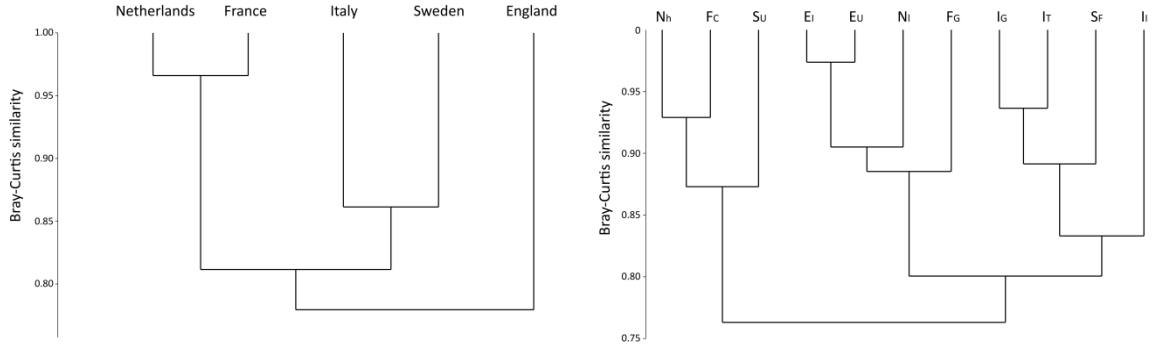
Supplementary Figure 1. Rarefaction curves of amplicon data sets from all individual soil samples.



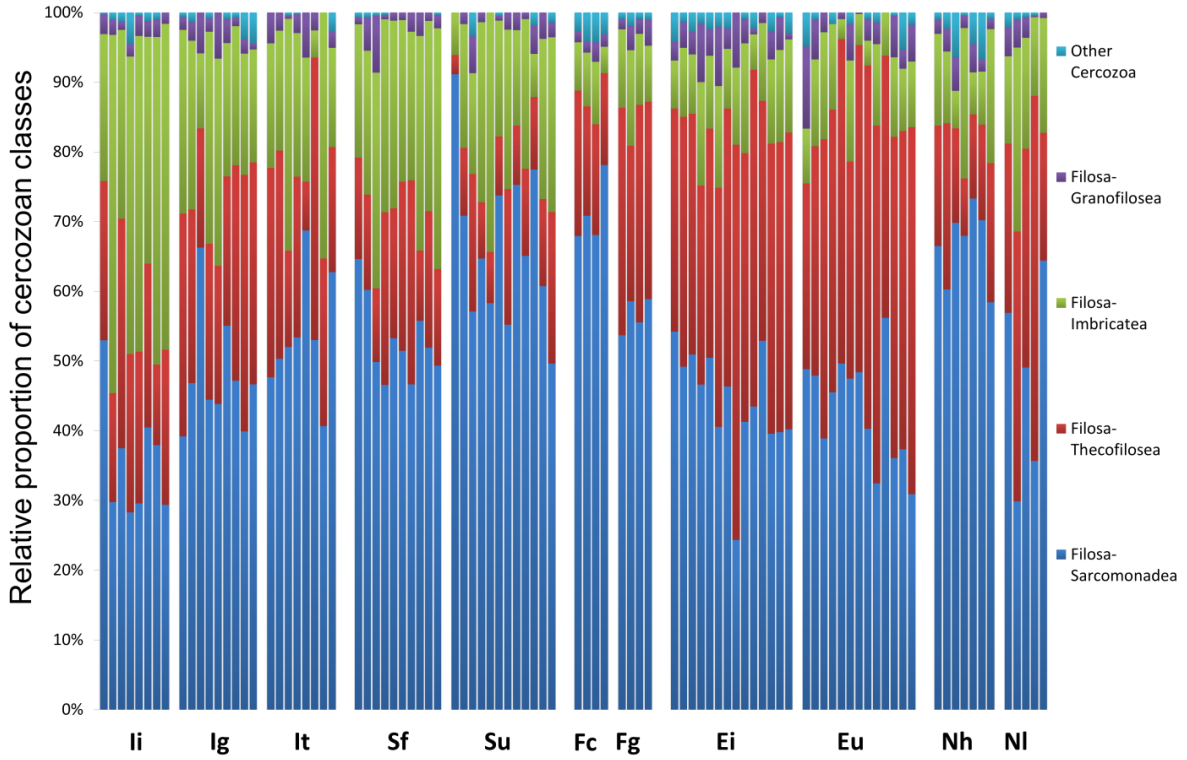
Supplementary Figure 2. Maximum likelihood phylogenetic analyses placing 28 cercomonad OTUs (top). Bold italic: OTUs obtained in this study.



Supplementary Figure 3. Maximum likelihood phylogenetic analyses placing 19 glissomonad OTUs. Bold italic: OTUs obtained in this study.



Supplementary Figure 4. Similarity of cercozoan communities between sites (left) and treatments (right) based on class level using the paired group (UPGMA) analyses with Bray Curtis similarity; see Table 1 for details on soil characteristics and treatment abbreviations.



Supplementary Figure 5. Rarified relative abundances of cercozoan classes in all samples at respective sites and treatments (x-axis); (see Table 1 for details on soil characteristics and treatment abbreviations).

Supplementary Table 1. Between-site comparisons of differences on cercozoan class level; E = England, F = France, I = Italy, N = Netherlands, S = Sweden; Stars indicate significant differences (* = q < 0.05, ** = q < 0.01)

Class	E-F	E-I	E-N	E-S	F-I	F-S	I-S
Imbricatea		**		**	*	*	
Sarcomonadea	*				*		
Thecofilosea	*	**	*	**			**

Supplementary Table3. Between-site comparisons of differences on cercozoan OTU level; E = England, F = France, I = Italy, N = Netherlands, S = Sweden; Stars indicate significant differences (* = q < 0.05, ** = q < 0.01)

OTU	E-F	E-I	E-N	E-S	F-I	F-N	F-S	I-N	I-S	N-S
Glissomonad_Z OTU 36				**			**		**	*
Cercomonas OTU216				**			*		**	
Cercomonas OTU12									**	
Glissomonad_Y OTU103				**						
Corythion OTU3				**			**		**	*
Teretomonas_Te OTU45				**						
Eocercomonas OTU7									**	
Euglypha OTU11		**							**	
Euglypha OTU14		*							**	
Euglyphida OTU21		**						*	**	
Nudifila-relative OTU 293				**			**		**	
Cercomonad_undet OTU29				**					**	
Glissomonad_U OTU15 sp.		**		**						
Limnofila OTU93		*								
Neoheteromita OTU5					*		**	*		
Neoheteromita OTU34								**		
Paracercomonas OTU6				**				**		
Peregrinia OTU63		**				*	*			
Rhogostoma OTU2	*	**	*	**			*		**	
Glissomonad_NF-D OTU167		**								
Thaumatomonas OTU32						*	**		**	
Trinema OTU17				**			**			
Trinema OTU13	*	**			*	*	**		**	

Supplementary Table 2. Overview of 30 most abundant cercozoan OTUs obtained in this study; blast from original

OTU	Class	Genus	Blast	Re-blast including own database	%IDENT	Genus
2	Filosa-Thecofilosea	*Rhogostoma*	HM628668	HQ121430	97,9	*Rhogostoma*
1	Filosa-Thecofilosea	*Rhogostoma*	AY620264	HQ121430	91,0	*Rhogostoma*
3	Filosa-Imbricatea	*Corythion*	EF456751	EF456751	96,2	*Corythion*
8	Filosa-Sarcomonadea	*Flectomonas*	AY965866	SCCAP_H251	100	*Flectomonas*
5	Filosa-Sarcomonadea	*Sandona*	U42447	ATCC50780p145at4____4173_bp	100	*Sandona*
9	Filosa-Sarcomonadea	*Eocercomonas*	AF372741	A2_244_25 2309_bpDA_2	100	*Eocercomonas*
15	Filosa-Sarcomonadea	*Glissomonad_U*	EF024447	AJ506007_06_URH506007_Ucultur	99,7	Glissomonad_U
12	Filosa-Sarcomonadea	*Neocercomonas*	FJ790711	Wyth3_8p68p138at6LS_2	100	*Neocercomonas*
167	Filosa-Sarcomonadea	*Glissomonad_NF-D*	JN207873	H63p80t19____2871_bp_____DA	97,9	Glissomonad_NF-D
6	Filosa-Sarcomonadea	*Paracercomonas*	AY884342	WA42p142at4	100	*Paracercomonas*
1155	Filosa-Thecofilosea	*Rhogostoma*	AB534325	HQ121430	97,5	*Rhogostoma*
45	Filosa-Thecofilosea	*Teretomonas_Te*	EF024805	DQ303924	87,1	Teretomonas_Te
18	Filosa-Thecofilosea	*Teretomonas_Te*	EF024805	AH51p125____3002_bp_____DA	86,2	Teretomonas_Te
13	Filosa-Imbricatea	*Trinema*	EF023588	EF456752	96,1	*Trinema*
41	Filosa-Thecofilosea	*Rhogostoma*	EU798722	HQ121430	92,3	*Rhogostoma*
4	Filosa-Thecofilosea	*Rhogostoma*	EF023854	HQ121430	92,6	*Rhogostoma*
17	Filosa-Imbricatea	*Trinema*	AJ418792	AJ418792	97,5	*Trinema*
36	Filosa-Sarcomonadea	*Glissomonad_Z*	EU709266	HetAus17____2820_bp_____DA	97,2	Glissomonad_Z
27	Filosa-Thecofilosea	*Rhogostoma*	AY620303	HQ121430	94,4	*Rhogostoma*
28	Filosa-Sarcomonadea	*Sandona*	EU709147	AH15p125	100	*Sandona*
7	Filosa-Sarcomonadea	*Eocercomonas*	EF023536	C24p65t4	99,7	*Eocercomonas*
11	Filosa-Imbricatea	*Euglypha*	GQ330594	EF456753	95,4	k
44	Filosa-Sarcomonadea	*Glissomonad_NF-E*	EU709178	AH11p125	99,3	Glissomonad_NF-E
25	Filosa-Thecofilosea	*Rhogostoma*	EF023854	DQ303924	93,0	*Rhogostoma*
29	Filosa-Sarcomonadea	*Cercomonad_undet*	AB695519	x7_4its	97,3	Cercomonad_undet
16	Filosa-Sarcomonadea	*Paracercomonas*	FJ790723	B1a_198_25f____2211_bp_____DA	100	*Paracercomonas*
95	Filosa-Imbricatea	*Trinema*	EF024634	AJ418792	97,2	*Trinema*
463	Filosa-Sarcomonadea	*Glissomonad_Z*	EU709266	HetAus17____2820_bp_____DA	98,9	Glissomonad_Z
23	Filosa-Sarcomonadea	*Eocercomonas*	AB534320	Z70.14_AH	88,1	*Eocercomonas*
26	Filosa-Thecofilosea	*Rhogostoma*	EU798722	HQ121430	92,3	*Rhogostoma*

Part 2 – Chapter 8

Surprisingly aquatic: Soil protist community structure revealed by metatranscriptomics

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Abstract

The vast number, enormous diversity and importance of protists in soil system has long been assumed, but the true diversity and community composition of these eukaryotes remains largely concealed. Traditional cultivation based methods miss a majority of taxa, while recent breakthroughs in sequencing technologies, promise to greatly add to the knowledge on soil protists. However, high-throughput sequencing (HTS) approaches have so far rarely been adopted to study protists and mainly targeted DNA, therefore introducing significant biases in obtained communities. Here we applied a metatranscriptomic approach specifically aimed at deciphering the protist community in high taxonomic resolution to compare 12 terrestrial samples of five different treatments (temperate litter from forests and soils from temperate grassland, forest and soils from two distinct artic peatlands. We found that protist communities strongly differed between treatments, while biological replicates strongly resembled another. More importantly, we detected a strikingly different community composition of soil protists than previously shown in cultivation-based and DNA-based studies and reveal an enormous diversity of soil protists. Amoebozoan-specific sequences were highly diverse and represented a large part of all protist sequences, contrasting previous DNA-based studies. However and despite using a manually curated database, automatically assigned sequences were often falsely assigned especially on lower taxonomic levels, strongly indicating that taxonomic expertise and manual inspection is still indispensable in working with protists even when using sequence-based information only. Typically aquatic Foraminifera and Choanoflaggellida were detected in nearly all samples further emphasizing the knowledge gap on soil protists. Therefore, this study strongly

encourages focused investigations of the soil protist community and highlights the need of careful investigations. Taking these steps will undoubtedly reveal additional interesting insights on soil protists, which are essential to understand entire soil systems.

Introduction

Soils harbour a spectacular microbial diversity. Among the least studied soil microorganisms are single-celled protists. Despite being microscopic, protist biomass in soils largely exceeds that of most animal taxa (Schaefer and Schauermann 1990, Zwart et al. 1994, Schröter et al. 2003), and protists have been shown to play major roles in controlling bacterial turnover and community composition, recycling of nutrients, and plant growth promotion (Clarholm 1985, De Ruiter et al. 1993, Bonkowski 2004). Protists display an enormous diversity of fundamentally different taxa, based on both, morphological features and phylogenetic relatedness (Cavalier-Smith 1993, Cavalier-Smith 2003, Adl et al. 2012), but a comprehensive understanding of the relative contributions of the dominant taxa and their activity in different soil systems has not yet been elucidated.

The pervasive lack of knowledge on soil protist communities is mainly caused by the need to establish enrichment cultures, as protists cannot be extracted from soils, and the opaqueness of soil particles prevents direct microscopic observation of the majority of taxa (Foissner 1987, Clarholm et al. 2007). Expert knowledge is needed for the time-consuming microscopic identification (Foissner 1987, Smirnov et al. 2008, Fenchel 2010, De Jonckheere et al. 2012), and therefore traditional attempts to describe the full diversity of protist taxa in natural soils are rare (Finlay et al. 2000, Bamforth 2007, Domonell et al. 2013). As mainly dormant stages are captured with cultivation-based approaches, these methods only reflect a sum-estimate of past protist production or ‘growth potential’ in soil (Finlay et al. 2000, Domonell et al. 2013). A further bias in the estimates of species richness is introduced as different growth media select for different species, and only a subset of taxa is estimated to grow in cultures (Ekelund and Rønn 1994, Foissner 1999b, Smirnov and Brown 2004). The advent of molecular techniques along with a revised species concept that is largely based on the small subunit ribosomal RNA (SSU rRNA) gene has fundamentally altered the view on the “protist world”; relatedness of species are being fundamentally revised and a huge number of formerly undistinguishable species have since been described (Cavalier-Smith 1993,

Cavalier-Smith 2003, Adl et al. 2012). Further, environmental sequencing studies based on the SSU rRNA gene have revealed a huge diversity of previously unknown and often most likely uncultivable protists (Bass and Cavalier-Smith 2004, Berney et al. 2004, Lara et al. 2007a, Lejzerowicz et al. 2010, Bates et al. 2013).

Despite molecular tools have diminished some problems to decipher the community structure of soil protists, new biases are being introduced and still obscure the true protist diversity. Fundamental problems are created by (i) the lack of reference sequences for major protist clades, (ii) vast mislabelled sequences in published databases, and (iii) the enormous phylogenetic diversity of protist taxa, often leading to wrong assignment of protist taxa (Epstein and López-García 2008, Smirnov et al. 2008, Adl et al. 2012, De Jonckheere et al. 2012). Further biases are introduced by the PCR step that usually precedes high-throughput sequencing (HTS) studies. “General” eukaryotic primers are often applied to decipher the community structure of protists, but these primers are in fact far from being truly universal (Adl et al. 2014). A strongly biased view of the true protist community in soils is being depicted by primer-based HTS studies as only a subset of the protist diversity is being recovered (Jeon et al. 2008, Hong et al. 2009) while others are being preferentially amplified by PCR-based methods (Berney et al. 2004, Medinger et al. 2010, Stoeck et al. 2014). Amoebae for instance, are notoriously underrepresented in molecular surveys due to long SSU sequences, common mismatches in primer regions and common presence of introns (Berney et al. 2004, Fiore-Donno et al. 2010, Pawlowski et al. 2012). Ciliates on the other hand are highly overrepresented due to their shorter SSU sequences that ease amplification, and the presence of extremely high SSU copy numbers (Gong et al. 2013). Similar to culture-based approaches, PCR-based estimates on protist diversity in soils are further biased by the fact that cysts of some protist taxa accumulate in soil and may survive for decades (Goodey 1915, Moon-van der Staay et al. 2006), and even DNA from dead organisms might be amplified, making a reliable estimation of active organisms impossible (Pawlowski et al. 2011).

Most of these obstacles are avoided when directly targeting SSU rRNA instead of genes encoding SSU rDNA combined with randomly-primed reverse transcription in metatranscriptomic approaches. For example, Urich et al. (2008) generated cDNA from the total extracted RNA of soil communities by a non-targeted approach applying reverse

transcription with random-hexamers. The cDNA was subjected directly to 454 HTS without any prior PCR or amplification steps. Several of the above-mentioned biasing steps could therefore be circumvented. Although the fraction of SSU rRNA stemming from protists is comparably small, recent studies show that the sequencing depth of current HTS platforms allows sizable datasets of protist SSU rRNA sequences (Urich et al. 2008, Tveit et al. 2012, Turner et al. 2013).

We used this PCR-free metatranscriptomic approach to characterise the active soil protist communities within six highly diverse natural soil systems in Europe, including forest soil and litter, grassland soil, and arctic peat substrates. We annotated all sequences to a database consisting of manually curated published protist sequences and compared the protist diversity and community composition between sites. Our non-targeted sequencing approach enabled for the first time an in-depth analysis of the community structure of the supergroup Amoebozoa, which, as mentioned above, is rarely detected in PCR-based approaches. Finally, our non-targeted approach led to the unexpected discovery of protist clades that are typically associated with freshwater and marine environments, but not considered as typical soil inhabitants.

Materials and Methods

Soil sampling and processing

Arctic peat soils were sampled as described in Tveit et al. (2012). The grassland site (Park grass, Rothamstead) was sampled by coring, with intact cores being brought to the laboratory, top soil (5 - 10 cm) sieved (5 mm mesh size) and subsequently flash-frozen in liquid nitrogen. Beech forest soils were sampled as described in Kaiser et al. (2010), the top soil (5 - 10 cm) sieved (5 mm mesh size) and subsequently flash-frozen in liquid nitrogen. Litter from the same sites was homogenized with a coffee grinder and subsequently flash-frozen in liquid nitrogen.

Nucleic acid extraction, cDNA synthesis and sequencing

Nucleic acids were extracted and processed as previously described (Urich et al. 2008). cDNA synthesis was performed as described in (Radax et al. 2012, Tveit et al. 2012).

454 - pyrosequencing was done either with FLX (forest soil) or FLX Titanium (grassland, peat soil) chemistry.

Sequence processing and analysis

Raw reads were processed as described in Tveit et al. (2012). Sequences were first filtered using LUCY (Chou and Holmes 2001), removing short (< 150 bp) and low-quality sequences (> 0.2 % error probability). Small subunit (SSU) ribosomal RNA sequences of eukaryotes were identified by MEGAN analysis of BLASTn files against a SSU rRNA reference database (Lanzen et al. 2011; parameters: min. bit score 150, min. support 1, top percent 10; 50 best blast hits). All eukaryotic SSU rRNAs were reanalysed with CREST (Lanzén et al. 2012) using the Silvamod database with LCA parameters min bit score 250, top percent 2 for classification of protist sequences. Correct taxonomic assignment was verified by manual BLASTn searches against the NCBI Genbank nt database. For the high-resolution taxonomic annotation of amoebozoa sequences, a custom-made database was constructed consisting of 1,164 sequences from Silva (www.arb-silva.de) and currently unpublished sequences (Stefan Geisen lab). The taxonomy was set according to the most recent taxonomy of Amoebozoa (Smirnov et al. 2011b, Adl et al. 2012, Lahr et al. 2013) to enable high-resolution taxonomic placement of sequences. An overview of the taxonomic levels within Amoebozoa distinguished in this study is shown in Figure 1. Reference database and taxonomy were then generated with CREST, and Amoebozoa sequences were classified using the same parameters in Megan as described above.

Supergroup	Subphylum	Class	Subclass	Order	Suborder	Family	Genus
Amoebozoa	Lobosa	Tubulinea		Euamoebida		Several unresolved	
				Arcellinida	Arcellina	Several unresolved	
					Difflugina	Several unresolved	
					Phryganellina	Several unresolved	
				Nolandida		Nolandellidae	Nolandella
				Echinamoebida		Vermamoebidae	Vermamoeba
						Echinamoebidae	Echinamoeba
				Leptomyxida		Leptomyxidae	Leptomyxa
							Rhizamoeba
				Vannellida		Vannellidae	Vannella
			Flabellinia	Himatismenida		Cochliopodiidae	Cochliopodium
				Dactylopodida		Vexilliferidae	Vexillifera
		Discosea				Paramoebidae	Korotnevella
				Thecamoebida		Thecamoebidae	Stenamoeba
							Thecamoeba
			Longamoebia	Dermamoebida		Mayorellidae	Mayorella
						Dermamoebidae	Paradermamoeba
				Centramoebida		Acanthamoebidae	Acanthamoeba
						Balamuthiidae	Balamuthia
	Conosa	Variosea		Varipodida		Filamoebidae	Filamoeba
							Flamella
						Acramoebidae	Unresolved
				Schizoplasmodiida		Unresolved	Unresolved
		Mycetozoa				Unresolved	

Figure 1. Overview of taxa and taxonomic classification of sequences assigned as Amoebozoa.

Unweighted Pair Group Method (UPGMA) as a cluster analysis was applied to evaluate differences between the protist community composition in all samples (Sokal 1961).

Results

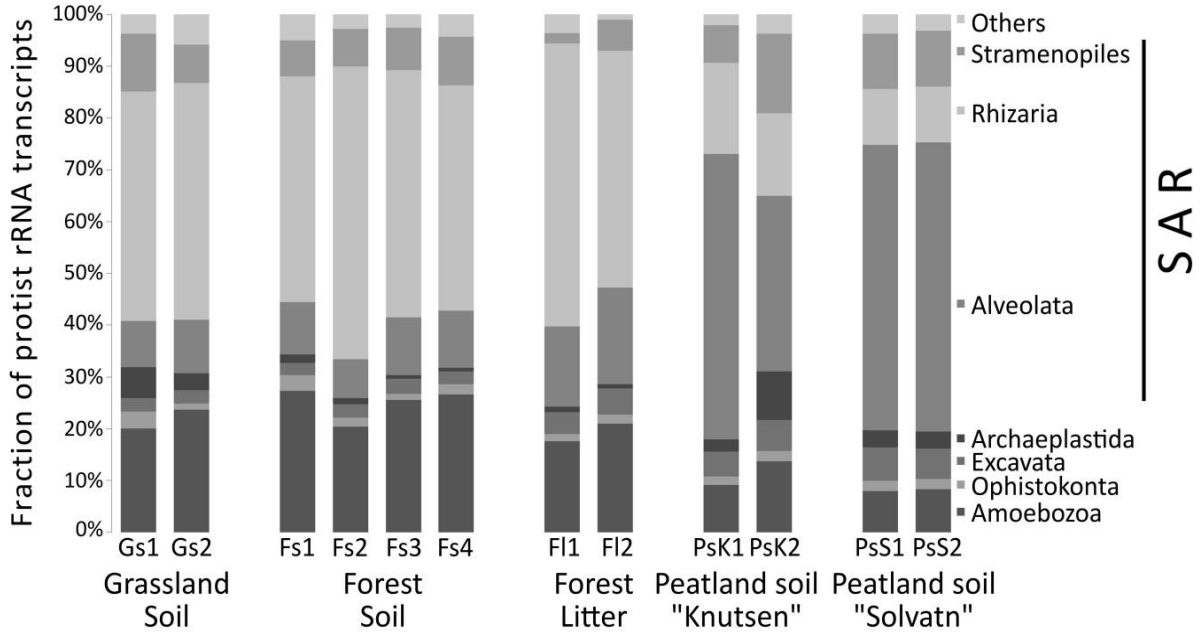

Figure 2. Community composition of protist supergroups in the investigated soils. For detailed information see Table 1.

In total, 32,808 SSU rRNA sequences of protists were obtained from the 12 soils (average of 2,734 sequences per site). The numbers of recovered sequences strongly increased with the organic matter content of soils and in litter samples (Table 1). In all cases, both biological replicates of each site yielded a very similar community composition (Figure 2) and grouped together in cluster analysis (Figure 3).

Table 1. Site description and sequence numbers (± SD) obtained from each site; NA = Data not available; in italics: eukaryotic supergroups

Sample name	Grassland soil (Gs)	Forest soil (Fs)	Forest litter (Fl)	Peatland soil Knutsen (PsK)	Peatland soil Solvatn (PsS)
# of replicates	2	4	2	2	2
pH	NA	4.8	NA	7.3	7.6
Moisture (%)	25	20	15	91	90
Horizon / soil type	Mineral / A	Mineral / A	Organic / Litter horizon	Organic peat / Top layer	Organic peat / Top layer
Vegetation	Grassland	Beech forest	Beech forest	Fen wet land, moss dominated	Fen wet land, moss dominated
Climatic zone	Temperate	Temperate	Temperate	Arctic	Arctic
Location	Rothamstead, UK	Vienna woods, Austria	Vienna woods, Austria	Svalbard, Norway	Svalbard, Norway
Protist sequences	253±21	961±470	4862±2579	2722±773	6631±210
SAR	163±15	662±287	3494±1735	2058±844	5265±187
·Stramenopiles	24±8	77±26	234±238	294±71	731±25
·Alveolata	24±1	96±34	870±542	1295±756	3797±144
·Rhizaria	115±7	489±236	2391±954	470±160	737±18
Amoebozoa	65±4	250±87	1009±633	322±6	586±29
Excavata	7±1	25±11	233±141	148±20	420±23
Archaeplastida	12±6	11±7	47±19	144±93	222±10
Ophistokonta	6±4	19±10	82±52	50±7	139±6

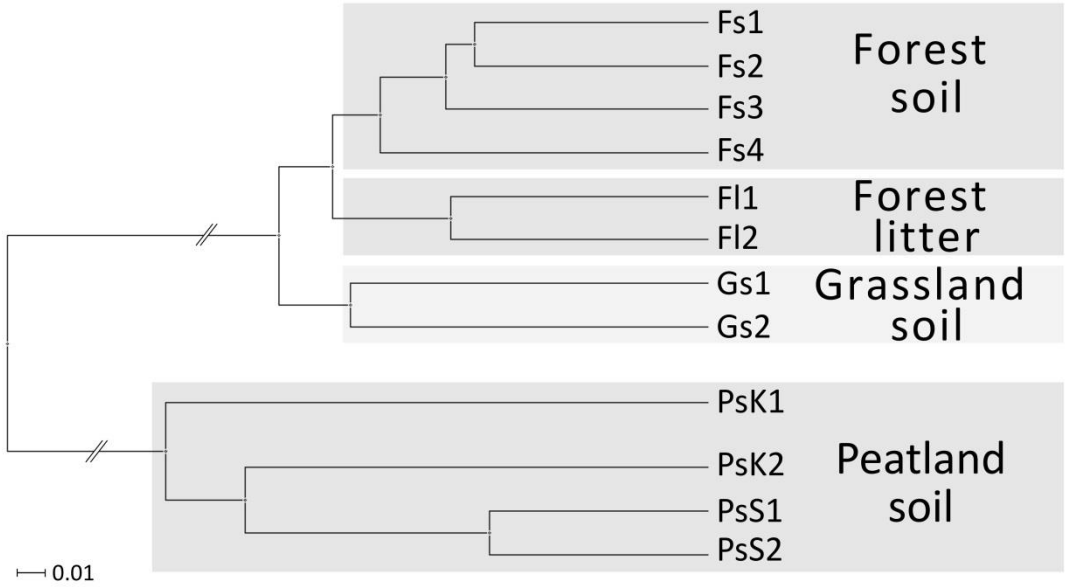

Figure 3. UPGMA clustering analysis to evaluate differences shown by the soil protist communities between samples. For detailed information on soil characteristics and abbreviations see Table 1.

Each of the five protist supergroups according to Adl et al. (2012) was represented at each site by a high number of different protist sequences (Table 1; Figure 2). The SAR group, consisting of the formerly independent supergroups Stramenopiles, Alveolata and Rhizaria, always dominated the active communities (62.7 - 81.2 %, average 71.0 ± 6.4 of all sequences [oas]) with sequences of Rhizaria being most numerous (11.1 - 57.8 %, average 37.2 ± 17.7 % oas) and those again being almost exclusively comprised of Cercozoa (95.5 %)

with dominance of sequences specific for the small flagellates in the order Cercomonadida (42 %) and both, flagellated and amoeboid protists in the order Silicofilosea (31 %). Surprisingly, Foraminifera occurred in relatively constant, albeit low numbers in all samples (1.5 ± 1.7 % oas). Alveolata sequences were quite variable among samples (7.8 - 57.8 %, average 25.0 ± 20.5 % oas) with the dominant phylum Ciliophora (91.2 %) and its main orders Spirotrichea (26.9 %), Colpodea (19.7 %) and Oligohymenophorea (16.0 %). The third group in SAR, Stramenopiles, was less abundant (2.1 - 15.8 %, average 8.8 ± 3.4 % oas). The supergroup Amoebozoa represented overall 20.0 ± 7.8 % oas (8.7 – 29.9 %). The other supergroups, i.e. Excavata (2.3 - 6.7 %; average 4.1 ± 1.6 % oas), Archaeplastida (0.7 - 9.6 %; average: 2.9 ± 2.6 % oas) and Ophistokonta (1.3 - 3.4 %; average: 2.0 ± 0.7 % oas) were generally less abundant. The high variability in the occurrence of individual supergroups reflected marked differences in the distribution of major protist taxa depending on organic matter content and soil type. Sequences specific for Rhizaria in SAR were dominant in mineral forest and grassland soils, while alveolate sequences composed the majority of sequences in substrates of high organic matter content, such as litter and peat soil samples. Generally, the community composition of protists in artic peat soils differed most strongly to all other samples, partly due to a reduction of Amoebozoa and Rhizaria and an increased dominance of Alveolata (Figure 2).

A more detailed taxonomic analysis of all detected protist taxa would exceed the scope of our manuscript, but the supergroup Amoebozoa is particularly suited to demonstrate the advantages of our approach, as it is usually markedly underestimated in PCR-based HTS studies (Baldwin et al. 2013, Bates et al. 2013), revealing further caveats in molecular protist community analyses. Our data enabled reliable deep phylogenetic analyses to the order level, often even to the genus level. The sequences affiliated to Amoebozoa were highly diverse at each sampling site, and could be assigned to at least four major classes, i.e. Tubulinea, Discosea, Variosea and Mycetozoa (Smirnov et al. 2011b). Only sequences of the anaerobic and parasitic Archamoebae were not represented. Tubulinea contributed the majority of sequences followed by Discosea, Mycetozoa and Variosea (53.2 ± 17.7, 23.7 ± 12.3, 11.4 ± 7.1 and 8.1 ± 3.8 %, respectively). Major orders with decreasing dominance were Euamoebida, Leptomyxida, Arcellinida and Centramoebida (21.0 ± 6.4, 15.4 ± 8.7, 14.8 ± 4.6 and 14.7 ± 8.6 % of all amoebozoan sequences).

The community composition within Amoebozoa differed largely between sites (Figure 4). The dominant class Tubulinea made up 53.2 ± 17.7 % of amoebozoan sequences on average (27.4 ± 0.0 % in Solvatn peat soil to 69.2 ± 7.3 % in forest soils). Discosea, being generally the second most abundant class represented 23.7 ± 12.3 % of amoebozoan sequences and reached dominance in Solvatn peat soil (41.6 ± 5.2 % oas). The classes Mycetozoa and Variosea were always less abundant (11.4 ± 7.1 % and 8.1 ± 3.8 % oas, respectively; Figure 4). Euamoebida dominated mineral soils of grasslands and forests on the order level in the class Tubulinea, while Echinamoebida and Nolandida were generally rare. The testate order Arcellinida became more abundant in rich organic layers of litter and peat soils. Leptomyxida were highly abundant in forest habitats. Among the class Discosea, the subclass Longamoebia represented 15.2 % oas, and was almost entirely (96.1 %) composed of the order Centramoebida. Sequences of the Longamoebian subclass Flabellinia was generally lower (7.0 % oas), mostly composed of the order Vannellida (50.1 % of Flabellinia). Sequences assigned to the subphylum Conosa could only reliably be assigned on the class level, as taxonomy and phylogeny affiliations especially of protists in the class Variosea are still largely unresolved (Adl et al. 2012). Variosea were most abundant in grassland, and forest soils and the Solvatn peat soil (7.7, 8.3, 14.5 % oas, respectively), while Mycetozoa were more abundant in forest litter and Knutsen peat soil (16.5 and 23.4 % oas, respectively). An overview of relative abundances of all amoebozoan clades up to the order level is shown in Figure 4.

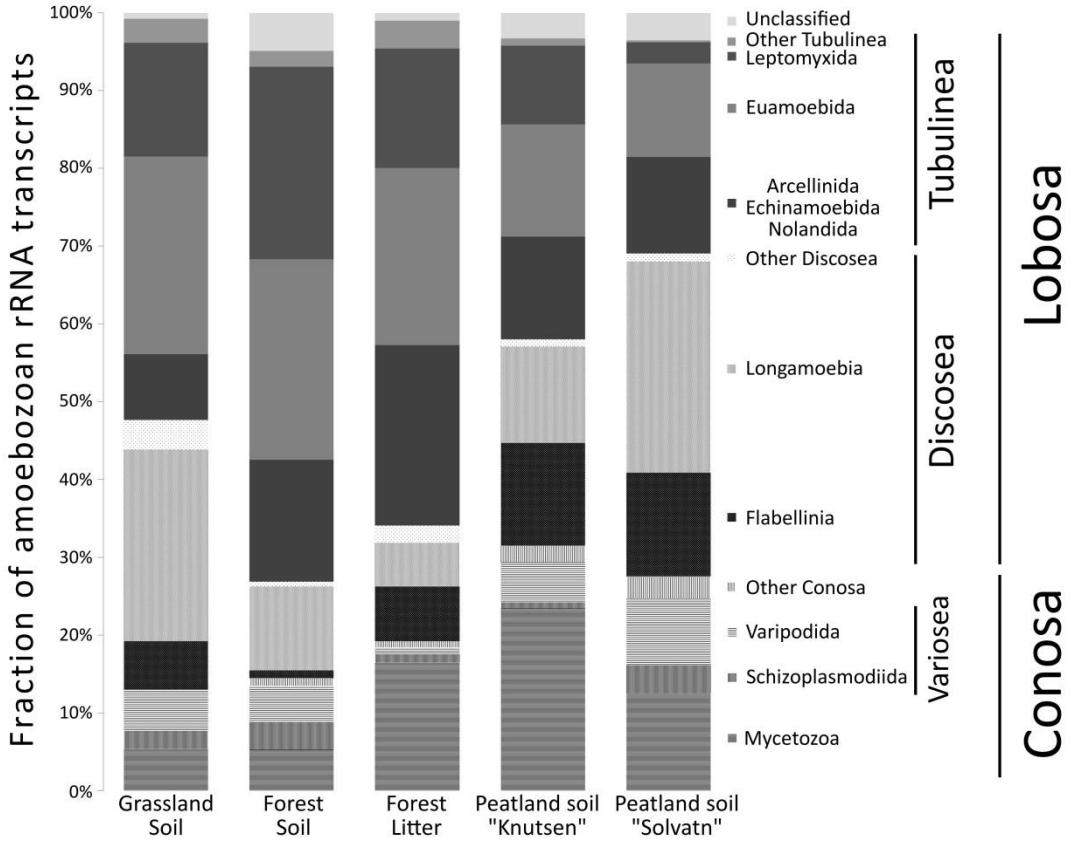

Figure 4. Diversity and community composition on the order level within the supergroup Amoebozoa in investigated soils. Labels and sites as described in Table 1 and Figures 1 and 2.

Widespread were protist sequences closely resembling potential human pathogens. Among them and occurring at most sites were diverse sequences affiliated with the genus *Acanthamoeba* and the species *Balamuthia mandrillaris*. In addition, sequences assigned as *Naegleria fowleri* (Heterolobosea in the supergroup Excavata) were found at all four arctic peatland samples. One sequence at Solvatn closely resembled the malaria causing agent *Plasmodium vivax*. It must be noted, however, that only few of the sequences assigned to these human pathogens showed perfect matches to described species.

Finally, our approach revealed the occurrence of protist groups that have been only rarely reported from soil. Sequences of the typically marine groups Foraminifera and Choanoflagellida confirm their presence and activity in all investigated soils (Figure 6). They comprised between 0.1 and 3.5 % of all protist SSU rRNAs.

Among Foraminifera three unknown SSU contigs ranging from 742 to 890 bp showed substantial sequence dissimilarity (maximum identity of 91 to 93 % to known sequences). Closest similarities were shared with sequences obtained in a focused molecular survey on

soil Foraminifera (Lejzerowicz et al. 2010). Several other sequences, however, closely matched sequences typically obtained from freshwater and marine environments, such as the genera *Astrammina*, *Bathysiphon*, *Allogromia* and diverse uncultured species among Foraminifera. Several sequences specific for Choanoflagellida matched with a maximum identity of ≥ 99 % with published sequences of uncultivated choanoflagellates (e.g. HQ219439, EF024012, JF706236, EF024012 and GQ330606) while the other sequences reached similarities of > 95 % with uncultivated choanoflagellate genera among typical freshwater and marine taxa, such as *Monosiga*, *Codonosiga, Salpinoeca*, and more rarely with *Lagenoeca*, *Stephanoeca*, *Didymoeca*, *Diaphanoeca*, *Desmarella* and *Acanthoeca.* Five contig reads (763 to 1163 bp) showed sequence similarities of 96 to 99 % to uncultivated freshwater choanoflagellates, but the closest hit to a formerly described species was < 92 %. Most sequences branched together with uncultivated choanoflagellates in a phylogenetic analysis (Figure 5).

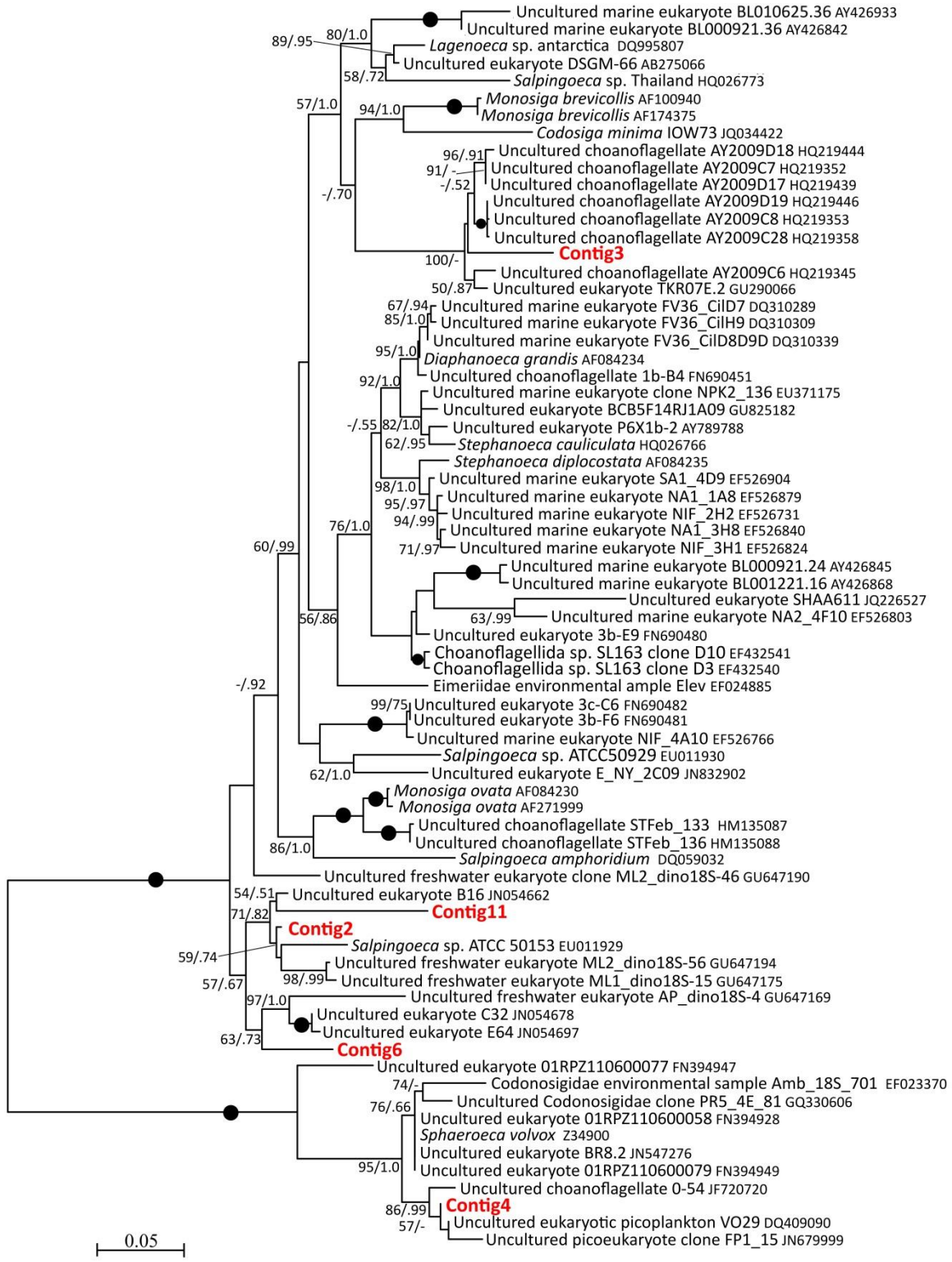

Figure 5. Maximum likelihood tree of Choanoflagellida: The placement of obtained long contigs (red) is specific for newly discovered choanoflagellate lineages in soil.

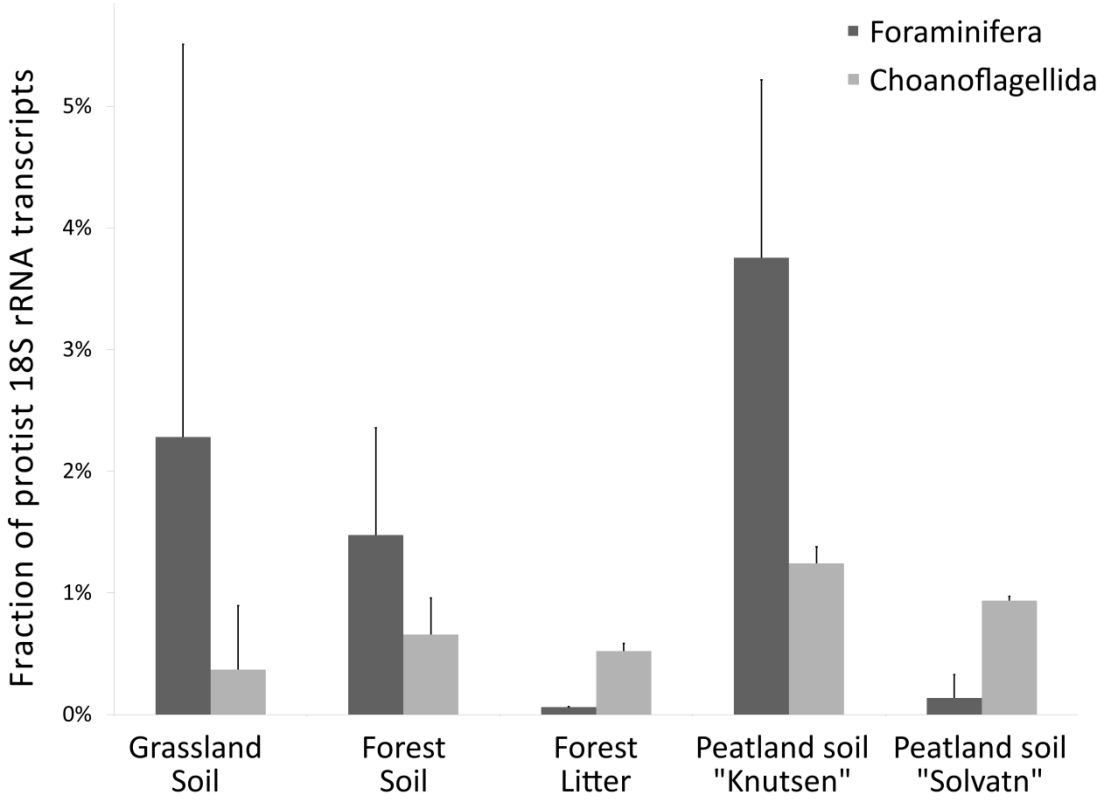

Figure 6. Sequence abundance ± SD of formerly neglected soil protist groups Foraminifera and Choanoflagellida. Labels and sites as described in Figure 2 and Table 1.

Discussion

Cultivation-based studies have shown that protists are diverse, abundant and important (Clarholm 1985, Ekelund and Rønn 1994, De Ruiter et al. 1995, Finlay et al. 2000, Bonkowski 2004, Epstein and López-García 2008, Adl et al. 2012, Pawlowski et al. 2012), but still miss a majority of taxa (Epstein and López-García 2008). In contrast, cultivation independent DNA-based sequencing studies have revealed a far much higher diversity of soil protists as previous suggested (Lara et al. 2007a, Lejzerowicz et al. 2010, Bates et al. 2013), but fail to amplify a wide range of protists and cannot estimate abundance (Epstein and López-García 2008, Weber and Pawlowski 2013, Stoeck et al. 2014). We here applied a primer-free metatranscriptomic approach to avoid the above-mentioned issues to obtain a more accurate picture of the true protist diversity in a wide range of terrestrial samples.

We obtained on average 2,700 protist-specific SSU rRNA gene sequences per site, corresponding to a two- to fourfold higher coverage than earlier primer-based DNA- (Baldwin et al. 2013, Bates et al. 2013) and metatranscriptome studies (Urich et al. 2008, Tveit et al. 2012, Turner et al. 2013). Generally, HTS results impose major caveats, especially

when DNA serves as a template: (i) amplification of dormant cysts and extracellular DNA (Stoeck et al. 2007, Not et al. 2009, Pawlowski et al. 2011) and large differences in SSU copy numbers between protist taxa make DNA as a target molecule unreliable to obtain relative quantitative estimates (Zhu et al. 2005, Parfrey et al. 2012, Gong et al. 2013, Weber and Pawlowski 2013); (ii) Even the best 'universal primers' (Adl et al. 2014) are in fact unable to detect many common and even dominant taxa, showing a highly biased view of the protist community composition (Jeon et al. 2008, Hong et al. 2009, Lee et al. 2012, Pawlowski et al. 2012), and (iii) PCR amplification rates strongly differ between taxa due to e.g. length variation in the amplification region, presence of introns, and variation of GC contents (Huber et al. 2009, Amend et al. 2010, Engelbrektson et al. 2010, Xie et al. 2011, Lee et al. 2012, Shakya et al. 2013, Strien et al. 2013). For example, the negative selection for amoebozoan sequences when applying a primer based approach on DNA is well-known (Berney et al. 2004, Amaral-Zettler et al. 2009) and explains the absence of nearly the entire supergroup Amoebozoa in DNA-based HTS surveys (Baldwin et al. 2013, Bates et al. 2013), while on the other hand high rDNA copy numbers in ciliates (Gong et al. 2013) lead to their over-proportional representation in DNA-based HTS studies. Therefore, primer-based methods are inherently prone to severe biases and appear largely inappropriate to reveal the true community composition of soil protists.

Not surprisingly, primer-free metatranscriptomic studies reveal a fundamentally different soil protist community structure than shown by those applying primers (and targeting DNA) (Urich et al. 2008, Tveit et al. 2012, Baldwin et al. 2013). Similarities are merely the dominance of the supergroup SAR with high sequence abundances of especially Rhizaria (and Alveolata in Arctic peatland soils) (Baldwin et al. 2013, Bates et al. 2013). However, sequence numbers assigned as Amoebozoa replaced those of Alveolata in all non-arctic soils. This is in line with cultivation-based studies that clearly show that small flagellates, especially Cercomonadida (Rhizaria) (Finlay et al. 2000, Ekelund et al. 2001, Howe et al. 2009) and amoebae appear to be the numerically dominant protists in mineral soils (Schaefer and Schauermann 1990, Ekelund and Rønn 1994, Finlay et al. 2000, Robinson et al. 2002, Domonell et al. 2013). Taking into account the high sequence coverage in our study, we are confident that our study provides the most detailed and potentially closest picture of the true composition of active protist communities in soils shown so far.

Still, also data from metatranscriptomic studies are not immune to misinterpretations, and a thorough taxonomic expert knowledge on protists is still indispensable, especially if sequences are analyzed to lower taxonomic levels. Public databases provide major obstacles that are filled with a great number of misidentified species and wrongly assigned sequences (Smirnov et al. 2008, Lahr et al. 2012), and even for many distinct and common taxa sequence information is still entirely lacking. Therefore we used our own manually curated database to assign our sequences, which fundamentally improved assignments, especially in the supergroup Amoebozoa.

Therefore, we are confident to provide the first thorough analysis of the eukaryotic supergroup Amoebozoa in diverse European terrestrial samples. While recent metatranscriptomic approaches confirmed cultivation based studies showing high relative abundances of Amoebozoa (Urich et al. 2008, Tveit et al. 2012, Turner et al. 2013), within amoebozoan analyses have not yet been conducted. The diversity of Amoebozoa in terrestrial samples is largely unknown, because of the above-mentioned issues in cultivability of amoebae and difficulties in taxon identifications (Smirnov et al. 2005, Smirnov et al. 2008). Morphotype assignments that are mostly used in cultivation based approaches suffer from artificial establishment of paraphyletic morphogroups (Anderson and Rogerson 1995, Finlay et al. 2000, Bass and Bischoff 2001, Domonell et al. 2013), and thus fail to distinguish and resolve major taxa (Brown and De Jonckheere 1999, Smirnov et al. 2007). Using our approach we detected enormous sequence diversity in all classes of Amoebozoa. The general dominance of the two classes Tubulinea and Discosea also confirms cultivation based studies (e.g. Finlay et al. 2000, Bass and Bischoff 2001, Domonell et al. 2013) but only metatranscriptome studies are able to reveal the high representations of Mycetozoa among Amoebozoa (and protists in general) (Urich et al. 2008). Mycetozoa are extemely species-rich and most species pass complicated life cycles that switch between single-celled amoebae, flagellate and cyst stages and large mulitcellular plasmodia (Stephenson et al. 2011). Their general absence in primer-based surveys can be easily explained by their long, intron-rich and strongly diverging SSU sequences (Stephenson et al. 2011). The high abundance of Varipodida is entirely novel. The order Varipodida was only erected in 2004 and hosts mostly large plasmodial, branching or reticulate amoebae (Cavalier-Smith et al. 2004, Smirnov et al. 2008, Smirnov et al. 2011b). Only few species of Varipodida are formally described resulting in sparse molecular information (Berney et al. 2013). In order to verify these

metatranscriptomic data, we conducted targeted cultivation studies, and this indeed revealed an unprecedented diversity of these extremely large amoebae in mineral soils (Chapters 3 + 5). The addition of several of these new sequences to our reference database resulted in strongly increased sequence assignments of Varipodida-specific sequences and shows that this highly diverse and active group of amoebae has entirely been overlooked in earlier studies.

Some other amoebozoan clades also remain unresolved. Especially taxonomic affinities within the order Euamoebida could not be resolved to deeper taxonomic levels. *Hartmannella* and *Saccamoeba* branch paraphyletically within Euamoebida with the entire taxonomy of Euamoebida strongly being debated (Dyková et al. 2008, Corsaro et al. 2010, Brown et al. 2011, Lahr et al. 2013). A recent metatranscriptomic study identified *Glaeseria* as one of the dominant soil protist genera in this group (Turner et al. 2013), which was confirmed by high relative abundance of automatic assigned sequences in our study. However, subsequent manual BLASTn searches of sequences assigned as *Glaeseria* revealed that none of the assigned sequences in fact matched *Glaeseria mira*, but more closely resembled other described and, to an even larger extend, undescribed taxa within Euamoebida. Therefore we omitted the lower level classification in the highly abundant order Euamoebida. Future re-classifications of this order are urgently needed to allow family and genus sequence assignments in future studies.

Amoebozoan sequences closely resembling potential human pathogenic protists indicate that soils are potential reservoirs of pathogens. *Acanthamoeba* spp. are causative agents of the eye infection amoebic keratitis and *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri* (Excavata, Heterolobosea) can cause Amoebic Encephalitis, a fatal human disease (Visvesvara et al. 1993, Schuster and Visvesvara 2004, Visvesvara et al. 2007, Siddiqui and Ahmed Khan 2012). We found sequences closely resembling all these protist taxa. The presence of *Acanthamoeba* in all samples was not surprising as this genus is ubiquitously present in soils and probably one of the most abundant amoebae (Page 1988), a number of which are pathogenic (Risler et al. 2013). The molecular identification for most heterolobosean amoebae such as *N. fowleri* is based on the ITS region and only few SSU sequences are available (De Jonckheere 1998, 2004). Therefore, sequences assigned to *N. fowleri* could have derived from non-pathogenic relatives and would need more reliably

reference database to be evaluated. *B. mandrillaris* is the only described species in the genus *Balamuthia* (Visvesvara et al. 1993), but as none of our sequences perfectly matched, but most closely resembled *B. mandrillaris,* it is likely that this genus is more diverse than previously suggested. Due to the high number and diversity of yet undescribed species, and our poor knowledge of protist pathogens, it remains elusive how many species in soil are true human pathogens.

As many sequences could not reliably be assigned beyond orders or sometimes even class, significant taxonomic information gaps prevail in all soil protist supergroups, especially in Amoebozoa. In line, new species and genera are continuously being described (Epstein and López-García 2008, Atlan et al. 2012, Geisen et al. 2014). Therefore, traditional taxonomy and the desciption of new species in cultivation based methods are an essential prerequesite to increase the protist reference database and joint efforts are needed to curate and improve existing databases.The surprising finding of several typically marine protist groups corroborates the notion that a plethora of soil protist taxa have formerly been missed in both, cultivation- and primer-based studies. For example, we detected Choanoflagellata in all samples. These protists are typically marine and only few taxa are known from freshwater systems (Tong et al. 1997, Arndt et al. 2000, Stoupin et al. 2012). Very few cultivation-based studies have reported the choanoflagellate genera *Monosiga*, *Codosiga* and *Salpingoeca* in soils, but they did not provide molecular data (Ekelund and Patterson 1997, Ekelund et al. 2001, Tikhonenkov et al. 2012). Only few molecular soil surveys found choanoflagellate specific sequences (Lesaulnier et al. 2008, Lara et al. 2011), several of those closely being resembled by sequences obtained in our study. However, other sequences, among them the longer contigs, more closely resembled sequences obtained in freshwater surveys (Chen et al. 2008, Monchy et al. 2011, Stoupin et al. 2012). As most sequences showed highest similarity with uncultivated species, the need to connect sequence information with morphological and functional information on the respective protist species in soil is essential. The substantial unknown diversity of choanoflagellates in soil might be significant for evolutionary studies, since choanoflagellates are based at the branching point to multicellular eukaryotes.

Foraminifera represent another group of typically marine protists, commonly not associated with soils. *Edaphoallogromia australica* is to date the only foraminiferan species known from

soil (Meisterfeld et al. 2001). We detected Foraminifera-specific sequences in all samples, supporting a recent targeted molecular survey that detected diverse Foraminifera-specific sequences in 17 out of 20 soils (Lejzerowicz et al. 2010), confirming their wide distribution in terrestrial soil enironments. A number of our contig sequences strongly resembled the sequences obtained by Lejzerowicz et al. (2010), but the majority of sequences most closely resembled the typically marine genera *Astrammina*, *Bathysiphon* and *Allogromia*. Although Foraminifera comprise a small fraction of the total soil protist community, they appear highly diverse and ubiquitously distributed in terrestrial habitats. The finding of sequences reliably assigned as choanoflagellates and foraminiferans indicates that, despite being relatively rare, these organisms are widespread members of active soil protist communities.

Taken together, our study clearly demonstrates the advantages of a metatranscriptomic sequencing approach, by circumventing major biases commonly associated with high-throughput studies. Still, taxonomic expert knowledge is needed to interpret metatranscriptomic data on protists and a reliable, manually curated database is indispensable for the correct assignment of taxa. Accordingly, the composition of soil protist communities in our investigation differed strongly from previous studies and revealed an unprecedented diversity of soil protists, many belonging to formerly unknown taxa and widespread protists groups commonly associated with marine environments. The possibility to achieve a high taxonomic resolution with our approach was exemplified by a detailed description of the abundant supergroup Amoebozoa, which is generally largely underrepresented in primer-based studies. We are highly confident that our primer-free metatranscriptomic approach along with a curated reference database provides the best estimate of the community composition of active protist communities in European soils to date.

Part 3

Ecological studies to broaden the knowledge on soil protist function other than feeding on bacteria

Part 3 – Chapter 9

The neglected link in soil food webs: Mycophagous protists

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Abstract

Soil biologists generally discriminate the nutrient flows in soil food webs into a bacterial and a fungal-based energy channel. Protists are considered to be the main consumers in the bacterial energy channel, while microarthropods and mycophagous nematodes are suggested to be the predominant consumers in the fungal energy channel. Taxonomic studies have, however, revealed that a range of protists are mycophagous. To get deeper insights on the distribution and functional role of mycophagous protists we tested facultative mycophagous feeding of several isolated soil protists. In addition, sequence data from high throughput sequencing studies were mined for known mycophagous protists, vampyrellid amoebae and grossglockneriid ciliates. The feeding studies revealed that a wide range of soil protists are able to feed and proliferate on yeast, some even on potential phytopathogens, such as *Fusarium*-fungi. The data-mining approach revealed that sequences specific for the two mycophagous protist groups were present in all terrestrial habitats representing a significant proportion of the entire protist community. These results strongly suggest that mycophagy among soil protists is common and the potential of this ecological function should be considered in soil food web analyses. Future studies need to investigate taxon-specific (facultative) mycophagy, decipher changes caused in the fungal community and quantitatively evaluate the functional importance of this trophic position in soil ecosystems.

Introduction

In soil biological studies, protists are mainly considered as major consumers of bacterial biomass (Hunt et al. 1987, De Ruiter et al. 1995, Bonkowski 2004). The notion that soil protists are merely bacteria grazers stands in sharp contrast to taxonomic studies. Taxonomists have long realized that obligate and facultative protists are widespread in soils (Old and Darbyshire 1978, Petz et al. 1985, Ekelund 1998), but knowledge on their functional importance is largely lacking. A major reason for the biased view on soil protists derives from the extraction and cultivation methods of soil protists. Traditional cultivation methods rely on enrichment cultivation using bacterial growth media (Berthold and Palzenberger 1995, Ekelund 1998), thereby missing the majority of mycophages and selecting for bacterivores.

Nevertheless, taxonomic studies have revealed a diverse range of mycophagous protists. All described ciliates of the family Grossglockneriidae are obligate mycophagous (Foissner and Didier 1983, Petz et al. 1985, Petz et al. 1986). Further facultative mycophagous protists (= omnivores) are soil vampyrellid amoebae (Old and Darbyshire 1978, Hess et al. 2012), *Thecamoeba* spp. (Bamforth 2004) and diverse testate amoebae (Mitchell et al. 2008, Wilkinson 2008, Wilkinson and Mitchell 2010), all being large often exceeding 100 µm. Even small flagellates have been shown to feed on fungi (Hekman et al. 1992, Flavin et al. 2000) and high abundances of undetermined mycophagous flagellates with biomasses in the range of those from bacterivores have been reported from soils (Ekelund 1998).

Molecular tools nowadays provide an alternative to get insights on formerly uncultivable protists and revealed that the cultivable fraction of soil protists represents only a minor part of the actual protist diversity in soils (Foissner 1999b, Kamono et al. 2013, Risse-Buhl et al. 2013). Among the molecular techniques of studying soil protists are cloning and sequencing (Lara et al. 2007a) and high-throughput sequencing approaches. It is, however, difficult to link molecular phylogenetic information with ecological functioning. Molecular information needs to be supplemented by functional information on ecological traits, which relies mainly on in-*vitro* studies on cultivated organisms. Directly targeting functional genes or using metatranscriptomic approaches promise a cultivation independent method to evaluate functional traits (Urich et al. 2008, Mitra et al. 2011, Tveit et al. 2012).

We hypothesized that also with traditional bacterial growth media a significant fraction of facultative fungal feeders can be isolated and that mycophagous protists are widespread and common in soils.

Materials and Methods

Evaluating facultative mycophagy among soil protists

Soil samples were taken in Pulheim Stommeln (Germany; 51°01'N, 6°45'E); Müncheberg (52°30'N, 14°07'E), in Les Verrines (France; 46°25'N, 0°7'E) and Cologne (Germany; 50°55'N, 6°55' E). The organic soil horizon was sampled in two locations (upper 2 cm in Pulheim Stommeln, 10 cm in Müncheberg and 10 cm in Cologne), while soil from earthworm burrows (2 mm around burrows) was samples at Les Verrines (Table 1).

Enrichment cultures with bacterial growth medium were established to isolate facultative mycophagous protists. From each soil sample, 1 g dry wt of soil was suspended in 250 ml Neff's Modified Amoeba Salina (NMAS) according to Page (1988). After shaking on an orbital shaker (Köttermann, Germany) at 100 rpm for 10' and fourfold dilution with NMAS, 20 µl of the strongly diluted soil suspension was added to wells of a 24 multiwell-plate (Sarstedt, Germany). 80 µl of a 0.4 $g * l^{-1}$ NMAS solution of dried *Saccharomyces cerevisiae* (Ruf, Germany) was added to each well and 160 µl of a *Fusarium culmorum* spore solution with a concentration of four spores * μl^{-1}. Plates were sealed with Parafilm and stored at 15 °C in the dark. These enrichment cultures were examined microscopically for mycophagous protists, i.e. growth on fungi and ingestion of fungal material, 7 and 21 days after incubation using an inverted microscope (Nikon Eclipse TS100, Japan) at 100 x and 400 x magnification.

Subsequently, enrichment cultures with fungal growth medium, were initiated using malt extract agar (MEA; 1.5 %). MEA plates were prepared by adding malt extract (1.5 %; AppliChem, Darmstadt, Germany) and agarising it by adding non-nutrient agar followed by autoclaving (122^{o}C, 20'). MEA plates were inoculated with 100 µl suspension of *F. culmorum* spores and hyphae in H_2O_{dest} to establish active fungal cultures. Yeast cultures with *Cryptococcus laurentii* were incubated on potato glucose agar (1.5 %; Sigma-Aldrich, St. Louis, USA) supplemented with yeast extract (0.5 %; Oxoid Limited; Hampshire, England).

For feeding experiments, specific protists amoebae and amoeboflagellates that showed indications of mycophagy (Table 1) were cultivated monoxenically on bacteria, by transferring individual protists to 60 mm Petri dishes filled with NMAS using a tapered glass pipette under an inverted phase-contrast microscope (Nikon TS-100, Japan). According to the observed feeding behaviour *S. cerevisiae* or *F. culmorum* were added as food source. These monoclonal protist cultures were incubated at room temperature. Observations and microphotographs of protists were performed on a Nikon Eclipse 90i (Japan) equipped with phase contrast and Differential Interference Contrast optics at 100 – 400 x magnification.

Cultures of the potentially mycophagous protists were tested for their feeding preferences on *S. cerevisiae, F. culmorum and C. laurentii.* A low-density fungal suspension (100 µl fungal suspension) with a concentration of 300 cells * μl^{-1} (*S. cerevisiae* and *C. laurentii*) or 40 cells * μl^{-1} (*F. culmorum*) was directly added to protist cultures. The protist cultures were growing on accompanying bacteria in 60 mm petridish for one week. The cultures were microscopically investigated for uptake of fungal material 2 and 24 hours after inoculation, and microphotographs of protists ingesting fungal material were recorded.

A more detailed experiment was initiated to test the mycophagous potential of the bacterivorous model protist *Acanthamoeba castellanii* on four different fungi. Two strains of the single celled *S. cerevisiae* and two filamentous fungi, *Neurospora crassa* and *Coprinus cinerea*, were given as potential prey for *A. castellanii* axenically grown in proteose peptone-yeast extract-glucose medium (PYG in a 4:2:1 mixture). The experiment was run in 96 well-plates (flat-bottom; Sarstedt, Nümbrecht, Germany), filled with a 150 µl sterile mixture of NMAS enriched with nutrient broth (Merck, Darmstadt, Germany) at 1:9 v/v (NB-NMAS). The four fungi were grown either alone or with *A. castellanii*. The control treatment contained only NB-NMAS or *A. castellanii* in NB-NMAS. All treatments were replicated eight-fold. Before use, *A. castellanii* cultures were washed three times with sterile NMAS before approximately 100 amoebae were added to each well of the *A. castellanii* treatments. Plates were sealed with Parafilm and directly placed in an automated microplate reader (Varioscan, Thermo Scientific, Waltham, USA). Optical density (OD) as an estimate of fungal biomass was measured every hour for a total of four days. Plates were additionally examined microscopically for amoebae growth and contamination. For subsequent statistical analyses, 24 OD measurements per analysis time were combined and growth changes were

compared. Biomass (OD) changes for *N. crassa*, *C. cinerea* and strains of *S. cerevisiae* were analysed by repeated measures analyses of variance with factors time and amoebae. Data were log (x + 1) transformed when required to satisfy the assumption of ANOVA. SAS 8.0 (Statistical Analysis System, SAS Institute Inc., Cary, USA) software package was used for statistical analyses.

Table1. Overview on potential facultative mycophagous amoebae and amoebaflagellates Isolated in this study

Genus	Order	Supergroup	Morphotype	Length	Sample	Coordinates
Cercomonas	Cercomonadida	SAR	Amoeboflagellate	~12µm	Müncheberg	52°30'N;14°07'E
Cercomonas	Cercomonadida	SAR	Amoeboflagellate	~30µm	Müncheberg	52°30'N;14°07'E
Cryptodifflugia	Arcellinida	SAR	Testate amoeba	~18µm	Pulheim Stommeln	51°01'N;6°45'E
Acanthamoeba-like	Centramoebida	Amoebozoa	Naked amoeba	~40µm	Müncheberg	52°30'N;14°07'E
Mayorella-like	Dermamoebida	Amoebozoa	Naked amoeba	~40µm	Les Verrines	46°25'N;0°7'E
Mayorella	Dermamoebida	Amoebozoa	Naked amoeba	~100µm	Cologne	50°55'N;6°55'E
Leptomyxa	Leptomyxida	Amoebozoa	Naked amoeba	>100µm	Cologne	50°55'N;6°55'E
Thecamoeba	Thecamoebida	Amoebozoa	Naked amoeba	~50µm	Cologne	50°55'N;6°55'E
Acanthamoeba castellanii	Centramoebida	Amoebozoa	Naked amoeba	~30µm	Pacific Grove	36°60'N;121°93'W

The presence and activity of mycophagous protists in soils

Data obtained in a high-throughput amplicon sequencing (HTAS) study that investigated carbon flux from root exudates through bacterial, fungal and protist communities (Hünninghaus et al. in prep) were analysed for sequences specific to known fungal feeding vampyrellid amoebae, and for ciliates of the family Grossglockneriidae. In this experiment bulk soil and rhizosphere soil was sampled from rhizoboxes containing 135 g of fresh soil from an agricultural field side and young maize plants. RNA was extracted as described by Lueders et al. (2004) with minor modifications The primers 20f (5' - TGC CAG TAG TCA TAT GCT TGT - 3') and euk302r+3 (5' - ATT GGA GGR CAA GTC TGG T - 3') were used to amplify a > 500 bp long fragment from a wide range of soil eukaryotes using the conditions described by Euringer and Lueders (2008). HTAS data were obtained for two time points, 23 and 26 days after planting. Only sequences longer than 250 bp were taxonomically assigned by MEGAN (settings: min. bit score 330, min support 1, top percent 2) using BLASTn against a Silvamod database (Urich et al. 2008). All sequences assigned to the orders Vampyrellida or the family Grossglockneridididae were manually subjected to BLASTn searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using default parameters. Relative percentages of both vampyrellids and grossglockneridididae of the entire protist community were calculated for each sample.

Similarly, sequence data from diverse terrestrial samples (artic peatland soils, forest soils, forest litter, and grassland soils) obtained in a recent metatranscriptomic study (Chapter 8) were analysed for vampyrellid and grossglockneriid sequences. Parameters used were the same as described above, except that sequences longer than 155 bp were taxonomically assigned.

Results

Microscopic examination of facultative mycophagous protists

A wide range of morphologically different amoebae and amoeboflagellates showed clear mycophagy in enrichment cultures and clonal cultures (Table 2). Microscopic observations revealed that all eight protist taxa ingested *S. cerevisiae* and *C. laurentii*, while four cultures, *Cryptodifflugia* sp, *Cercomonas* sp, *Leptomyxa* sp, and *Mayorella* sp. fed on spores of *F. culmorum* (Table 2, Figure 1). All these protists also reproduced in presence of the respective fungal prey.

Table 2. Feeding experiment of isolated mycophagous protists on the three different fungi, *F. culmorum*, *C. laurentii* and *S. cerevisiae.* X = successful ingestion of fungal material; - = no ingestion observed

Protist genus	***F. culmorum***	***C. laurentii***	***S. cerevisiae***
Cercomonas	X	X	X
Cercomonas	-	X	X
Cryptodifflugia	X	X	X
Acanthamoeba-like	-	X	X
Mayorella-like	-	*X*	*X*
Mayorella	X	X	X
Leptomyxa	X	X	X
Thecamoeba	-	X	X

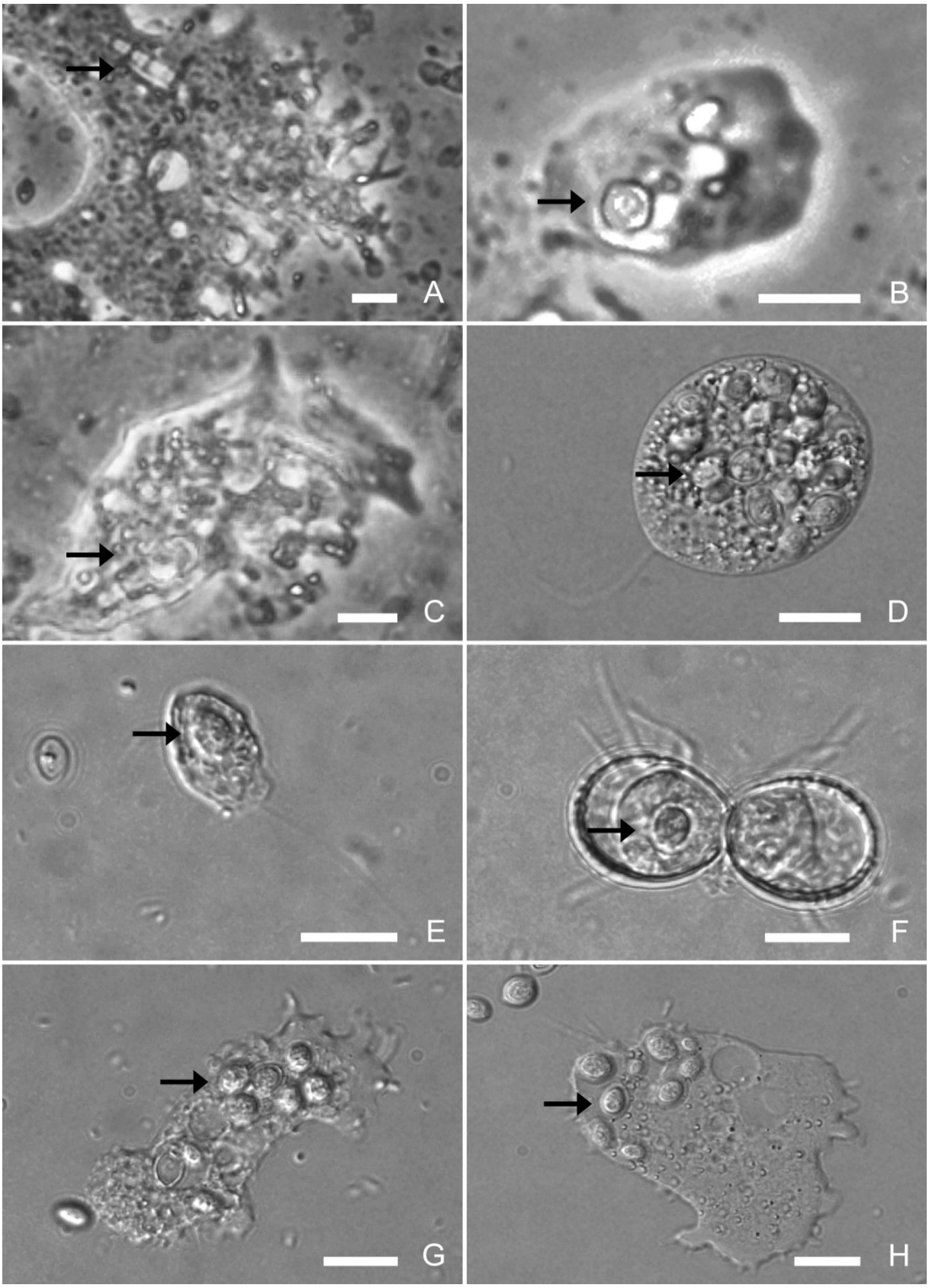

Figure 1. Isolated protists with ingested fungi; Pictures show: (a) *Leptomyxa* sp. with ingested *F. culmorum* (b) *Thecamoeba* sp. with ingested *S. cerevisiae* (c) *Mayorella* sp. feeding on *F. culmorum* (d) *Cercomonas* sp. with ingested *S. cerevisiae* (e) *Cercomonas* sp. with ingested *C. laurentii* (f) Cryptodifflugia sp. (g) *Mayorella*-like amoebae with engulfed *S. cerevisiae* (h) *Acanthamoeba*-like with ingested *S. cerevisiae*. Arrows indicate ingested fungal material; Scale bar = 10 µm.

Mycophagy in *Acanthamoeba castellanii*

A. castellanii reduced optical density that we subsequently define as a measure of cellular biomass, and inhibited growth of *C. cinerea* and both *S. cerevisiae* strains (Figure 2). Trophozoits and cysts of *A. castellanii* were present throughout the experiment in controls, with *C. cinerea* and both yeast strains, but a reliable quantification of amoebae was impossible within the dense layer of fungal material. *C. cinerea* and both *S. cerevisiae* strains were strongly reduced in comparison to non-amoebae controls (Figure 2). However, biomass of *N. crassa* was not reduced and totally overgrew the amoebae (Figure 2), indicating that feeding differences strongly depended on the fungal prey. Both yeast strains immediately started to grow, while growth of *N. crassa* was delayed by > 40 hours until an increase in biomass was observed (Figure 2). *A. castellanii* reduced the initial growth of *S. cerevisiae* and *C. cinerea* by 30 and 90 % during the initial growth phase of the fungi ($F = 17.5$, $p < 0.001$; $F = 545.1$, $p < 0.001$, respectively), but *S. cerevisiae* gradually compensated predation losses, while *C. cinerea* remained in low numbers, resulting in 15 and 82 % biomass reductions at the end of the experiment after 4 days ($F = 5.2$, $p < 0.05$; $F = 99.9$, $p < 0.001$; Figure 2).

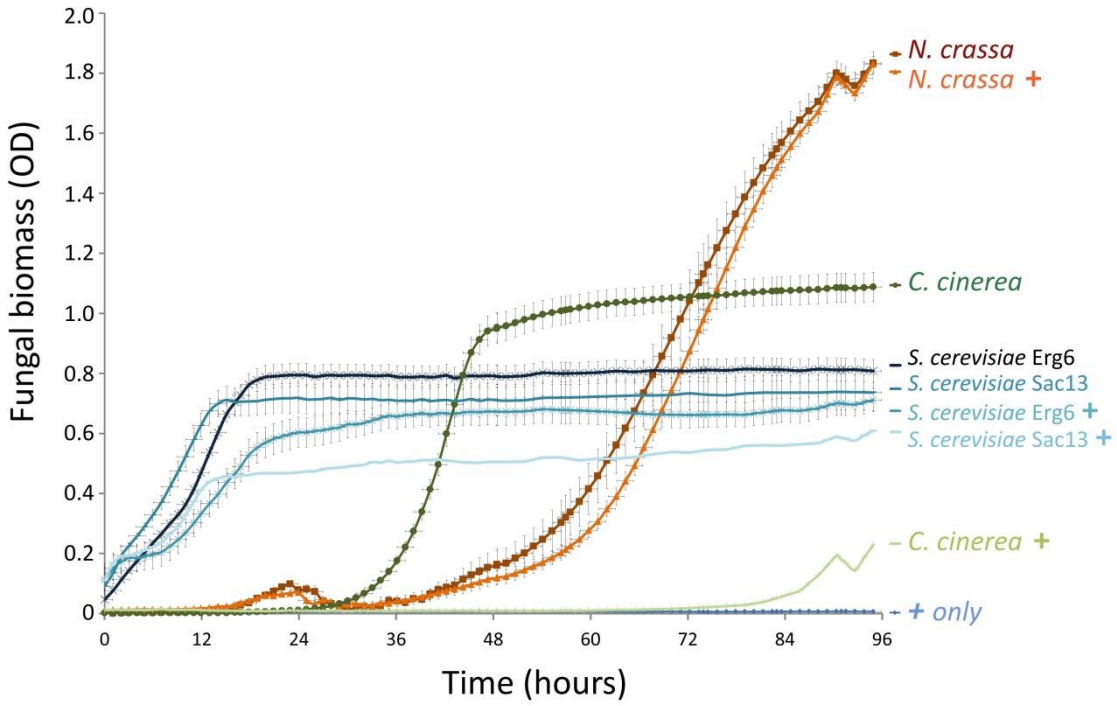

Figure 2. Changes in fungal biomass over a period of 95 hours determined by changes in optical density (OD). Different colours represent different fungal treatments (*Neurospora crassa*, *Coprinus cinerea* and *Saccharomyces cerevisiae*) (± SD); + = fungi with *A. castellanii.*

Presence and relative abundance of mycophagous protists in soils

In the plant rhizosphere we detected a substantial fraction of fungal feeding protists using a DNA based HTAS approach. Sequences of Grossglockneriidae and Vampyrellidae were obtained from all datasets. Four weeks after maize planting, sequences of Grossglockneriidae represented ~ 0.5 and 1 % of all protist sequences in bulk and rhizosphere soil, respectively. Sequences of Vampyrellidae represented ~ 6 % in bulk soil but only ~ 3.5 % of protist sequences in rhizosphere soil, respectively (Figure 3). BLASTn searches of sequences assigned as Grossglockneriidae yielded often perfectly matches to *Pseudoplatyophora nana* or *Mykophagopphrya terricola*. However, several sequences showed closer affinities to uncultured species with *P. nana* or *M. terricola* as the closest described species.

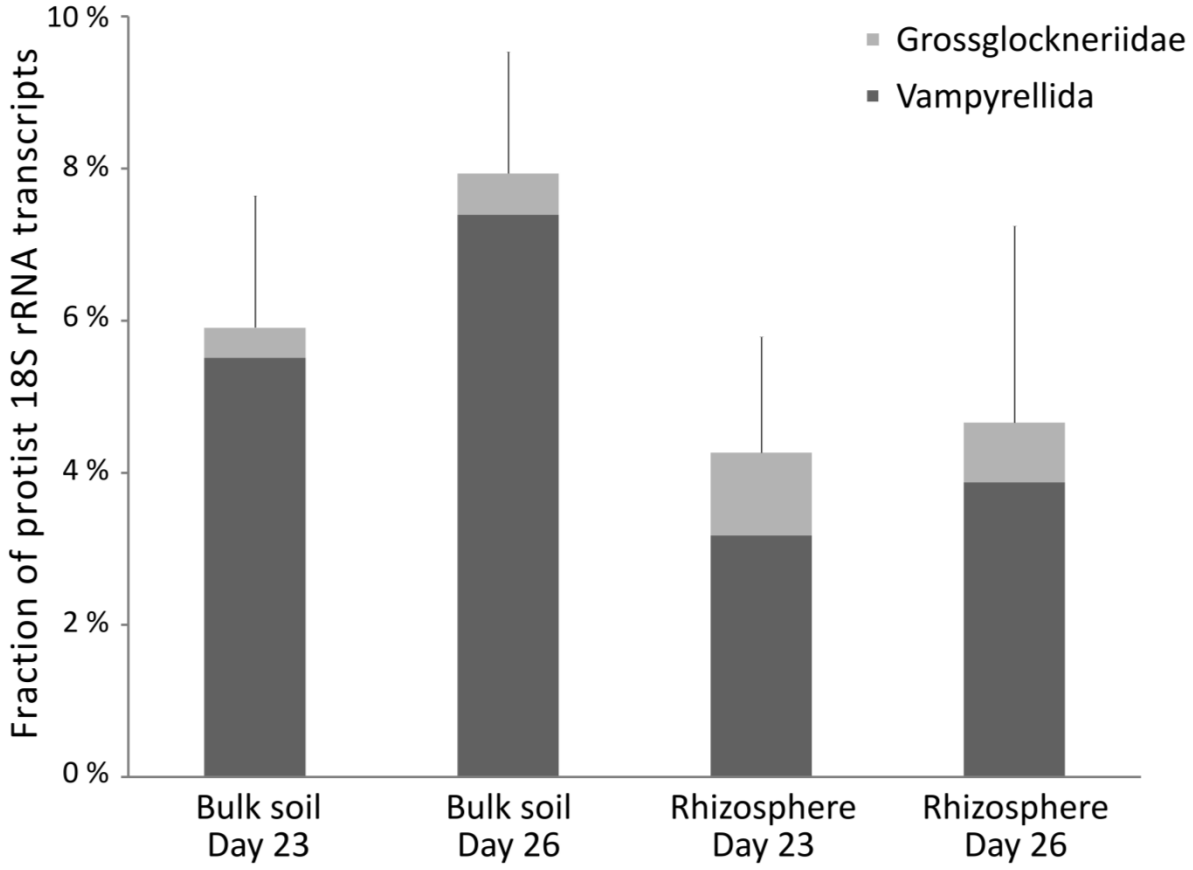

Figure 3. Sequences assigned as grossglockerniids and vampyrellids shown as relative abundance of all protist sequences obtained in an amplicon sequencing study of soils using rRNA as a substrate; ± SD of both mycophagous groups together.

The diversity of sequences assigned as species within Vampyrellidae was more diverse, closely resembling different members of Vampyrellida, such as *Theratromyxa weberi*, *Platyreta germanica* or *Vampyrella* sp. However, BLASTn searches of many sequences yielded highest identity to sequences of undescribed vampyrelliids. Most sequences were not perfectly matching known taxa with similarities sometimes more than 5 % different to described species.

Presence and relative abundance of metabolically active mycophagous protists in soils

Sequences of vampyrellids and grossglockneriids were found in all soils in a metatranscriptomic dataset, representing between 0.1 and 3.0 % of all protist-specific sequences. While Vampyrellida and Grossglockneridae contributed little to the diversity of protists in artic peatlands (< 0.5 % of all protists), higher fractions were found in both forest soil and litter (1.5 % of all protists) with highest relative abundance in grassland (3.0 %; Figure 4).

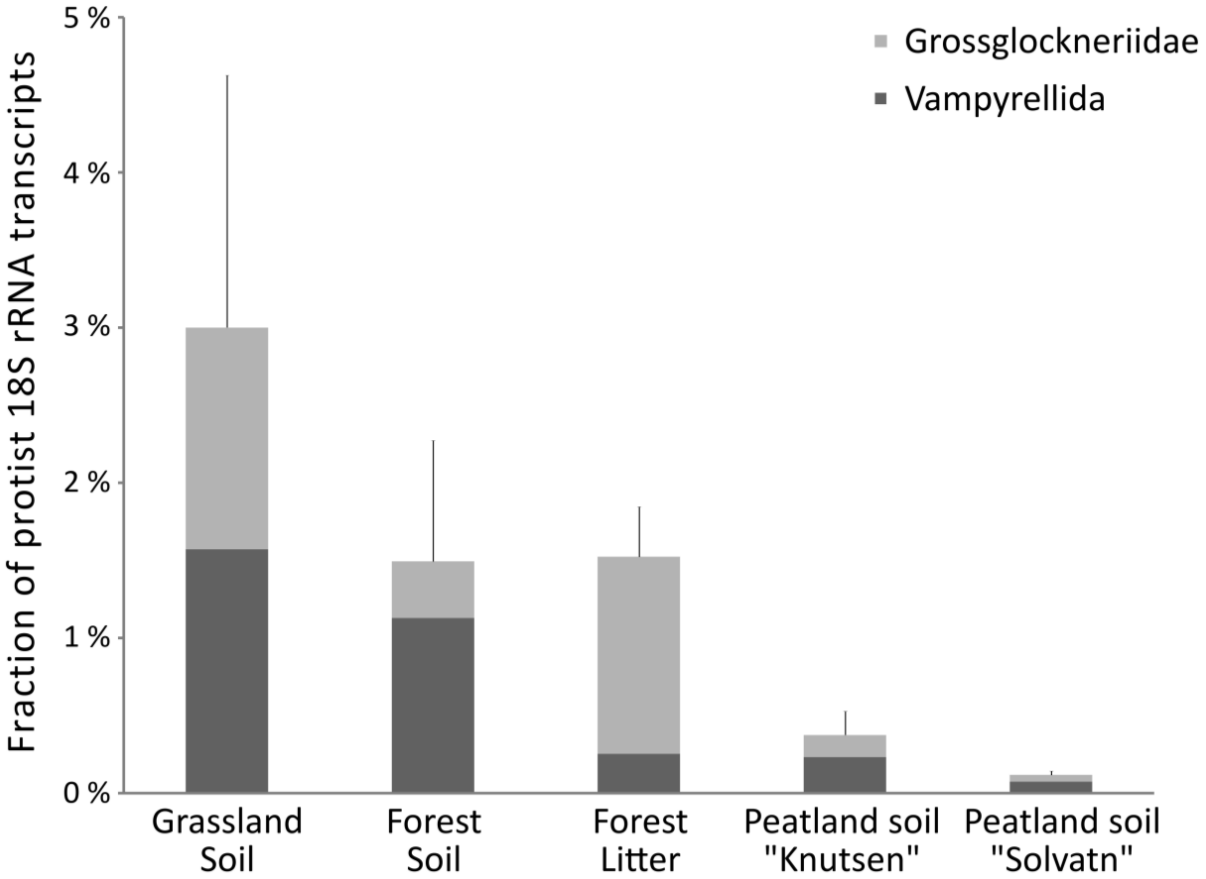

Figure 4. Sequences assigned as grossglockneriids and vampyrellids shown as relative abundance of all protist sequences obtained in a metatranscriptomic analyses of different soils.

P. nana represented the majority of sequences in Grossglockneridae and sequences specific to *M. terricola* were second most abundant. Especially short sequences showed identical similarities to both species, while others more closely resembled uncultivated species with *P. nana and M. terricola* being the closest hits.

The majority of sequences assigned to Vampyrellida resembled most closely *Theratromyxa* and *Arachnula*, but also sequences closely resembling uncultivated vampyrellids were common.

Discussion

A hitherto unconsidered functional role of soil protists as mycophages and omnivores in soil food webs is highlighted in this work. Several soil protists, previously classified as bacterivores, were shown to selectively feed on yeasts and on spores of soil fungi, among them *F. culmorum*. It appears that a wide range of protists taxa are in fact opportunistic omnivores.

Despite their enormous diversity, soil protists were generally considered as bacterivores in soil food webs (Hunt et al. 1987, De Ruiter et al. 1995). Taxonomists have shown that some protists feed on fungi, such as several amoebae, e.g. *Thecamoeba*, *Gephyramoeba*, *Mayorella*, *Saccamoeba* and *Leptomyxa* (Page 1977, Chakraborty and Old 1982, Chakraborty et al. 1983), ciliates of the family Grossglockneridae (Foissner 1980, Petz et al. 1986, Foissner 1999a) and even small flagellates (Hekman et al. 1992, Ekelund 1998, Flavin et al. 2000). The importance of mycophagy among protists and the diversity of mycophagous protists in terrestrial ecosystems remain, however, largely unknown.

Non-toxin producing yeast strains were preferentially being preyed upon by a variety of protists. Similarly, *A. castellanii* almost entirely prevented the growth of the non-toxic *C. cinerea*, and also reduced growth of baker's yeast (*S. cerevisiae)*, while *N. crassa* was entirely unaffected. The presence of putative antibiotics in the genome of *N. crassa* (Galagan et al. 2003) presumably acts anti-microbial, liberating *N. crassa* from being preyed upon by *A. castellanii*. If these results can be extrapolated to other fungi, fungal ability of toxin production seems to be an important factor determining potential mycophagy of soil protists. Future studies evaluating interactions of a wider range of fungi and mycophagous

protists are needed to prove this hypothesis. However, Tapilskaja (1967), Old and Oros (1980), Chakraborty and Old (1982) and Chakraborty et al. (1983) have shown that soil amoebae fed on spores of plant pathogenic fungi and even reduced the outbreak of pathogenic fungi in field experiments.

Size and structure pf prey fungi, however, also most likely influence the edibility, as former studies often found that bacterivorous / facultative mycophagous protists ingest single-celled yeasts (Heal 1963, Bunting et al. 1979, Allen and Dawidowicz 1990). Transport of yeasts inside amoebae without damage of the cells has been reported (Heal 1963, Chakraborty and Old 1982), but we could clearly oberve active digestion inside food vacuoles of protists leading to inhibition of fungal growth. All protists tested in this study consumed yeast cells, *C. operculata* and *Cercomonas* sp. ingested single yeast cells, whereas larger protists ingested several cells simultaneously.

A. castellanii has been used as a bacterivore protist in a number of experiments (Weekers et al. 1993, Bonkowski and Brandt 2002, Rønn et al. 2002b, Neidig et al. 2010, Koller et al. 2013) and was confirmed to strongly alter bacterial community composition in the plant rhizosphere (Kreuzer et al. 2006, Herdler et al. 2008, Rosenberg et al. 2009). Our results show that *A. castellanii* also exhibis selective feeding preference for fungal species. However, the formation of fungal hyphae was supressed by *A. castellanii* showing that also the growth of filamentous fungi can significantly be reduced by common opportunistic omnivorous protist species, such as *Acanthamoeba* spp. This genus is ubiquitous and highly abundant in aquatic and terrestrial environments (Page 1988, Rodríguez-Zaragoza 1994). Generally, amoebae in the genus, but especially those identified as *A. castellanii,* are treated as purely bacterivorous (Chakraborty et al. 1983, Bamforth 1988). *Acanthamoeba* spp. rely on phagocytosis to take up prey unlike larger mycophagous amoeba such as vampyrellids that have been shown to perforate hyphae (Old and Darbyshire 1978, Chakraborty and Old 1982), excluding long hyphae from their food spectrum. However, we show that single-cell *S. cerevisiae* and fungal spores were ingested and reduced, but also hyphae of *C. cinerea* could not be produced under feeding pressure exhibited by amoebae. *Acanthamoeba* is very diverse (Gast et al. 1996, Stothard et al. 1998, Gast 2001, Corsaro and Venditti 2010, Qvarnstrom et al. 2013) with niche differentiation likely existing between taxa. The immense enzymatic repertoire detected in the sequenced *A. castellanii* Neff strain contained

chitinases (Anderson et al. 2005, Clarke et al. 2013), suggesting that *A. castellanii* might thrive on fungi. Also other *Acanthamoeba* spp. are likely to be opportunistic mycophages, indicated by presence of chitinases in *A. culbertsoni* (Krishna Murti and Shukla 1984) and consumption of *Cryptococcus neoformans* by *A. polyphaga* (Bunting et al. 1979). The obvious omnivory of *Acanthamoeba* spp. combined with their broad enzymatic repertoire might explain the ubiquity and high abundance of Acanthamoebae in basically all environments (Sawyer and Griffin 1975, Page 1988, Rodríguez-Zaragoza 1994). Further, it is shown for the first time that even hyphae-forming fungi can be impacted by *A. castellanii* probably by feeding on fungal spores.

Distribution of mycophagous protists

As the knowledge on mycophagous protists is sparse, a focused molecular sequence analyses was performed targeting only vampyrellids and grossglockneriids as known representatives of mycophagous protists (Old and Darbyshire 1978, Foissner 1980, Petz et al. 1986, Hess et al. 2012). Grossglockneriidae have only been described in 1980 with currently nine species in six genera being described, all being obligate mycophages (Foissner 1980, Petz et al. 1985, Petz et al. 1986, Foissner 1999a). Cultivation based studies indicate their presence in almost all soils (Foissner 1999a), but still little is known on the distribution and ecological importance of these ciliates. Our molecular analyses supported these studies as we found sequences of grossglockneriids in all samples, representing up to 3 % of all protist sequences. The diversity in this family seems to be high in most samples, as several sequences showed highest similarity to undescribed grossglockneriid species, indicating that the entire diversity of this family is still not known. The only two available sequence data of *Mykophagophrys terricola* and *Pseudoplatyophrya nana* (Lynn et al. 1999, Dunthorn et al. 2008) thus urgently need to be supplemented by sequences of the remaining described species to investigate how many species are likely to exist within this family of mycophagous ciliates.

Vampyrellid amoebae have been the focus of a recent detailed investigation (Berney et al. 2013). The authors found sequences of new vampyrellids in HTS datasets especially in marine environments, but also retrieved diverse vampyrellid sequences from soils. Our HTAS and metatranscriptomic data clearly support the findings of Berney et al. (2013), as vampyrellid sequences were highly diverse and ubiquitously found. Sequences of described

species, such as *Theratromyxa weberi* and *Platyreta germanica* were detected, but also a wide array of undescribed and uncultivated species in Vampyrellida. These data suggest that facultative mycophagous vampyrellids could play important ecological roles in soils (Figure 5). More cultivation based efforts are needed to clarify their ecological functions in terrestrial soil samples.

We identified sequences specific for mycophagous protists using ribosomal rRNA mirrors the living and active community of protists (Urich et al. 2008, Tveit et al. 2012, Turner et al. 2013). Vampyrelliids and grossglockneriids included in the molecular analyses only represent two clades of mycophagous protists and most likely represent a fraction of all (facultative) mycophagous soil protists shown in this work and before (Pussard et al. 1979, Chakraborty et al. 1983, Old et al. 1985, Ekelund 1998, Flavin et al. 2000). Therefore, and due to the ubiquituously detectable representation of grossglockeriids and vampyrellids in soils, mycophagy evidently should be considered as an important ecological function of soil protists supplementing nematodes and microarthropods as major consumers of fungi (Figure 5).

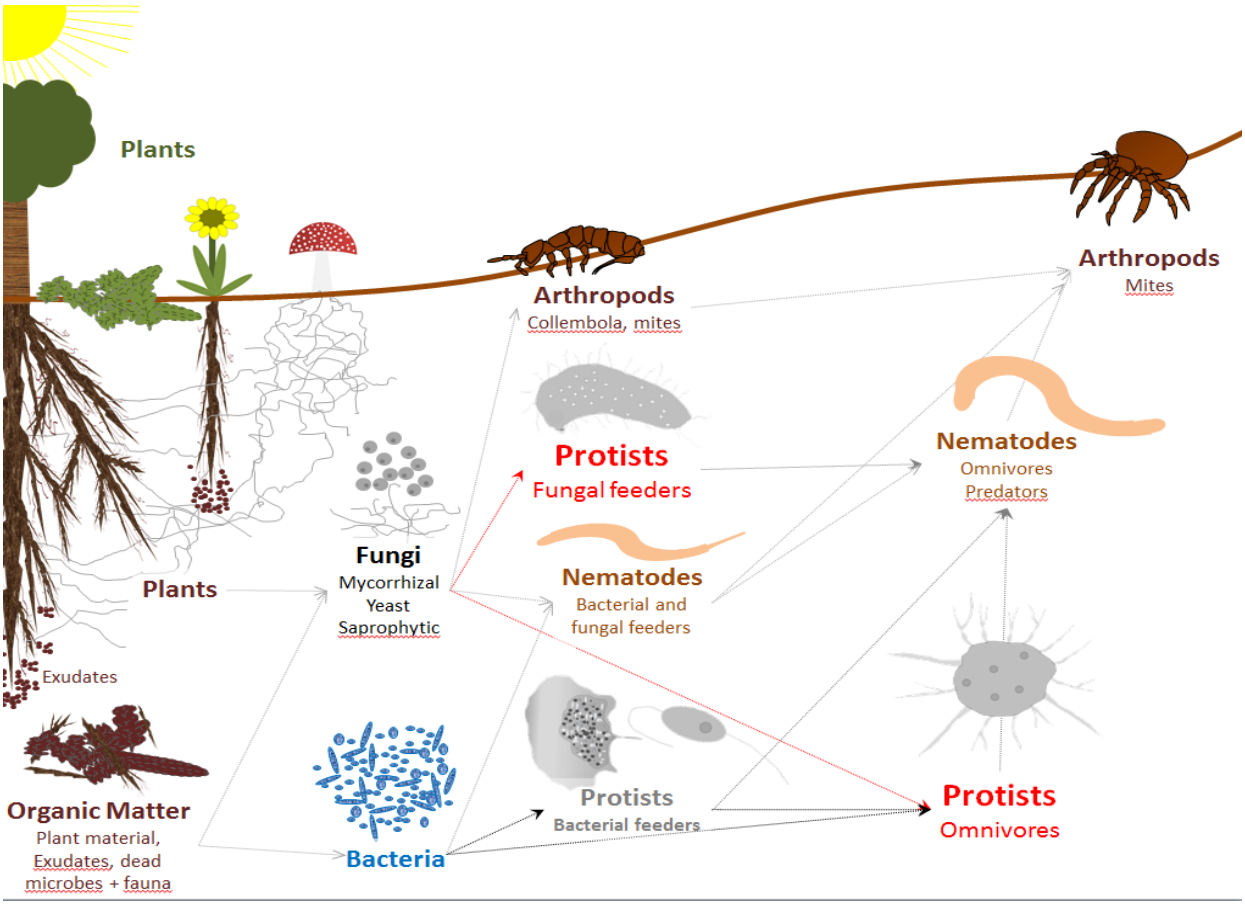

Figure 5. The soil food web revisited showing the diverse role of free-living heterotrophic protists; red: formerly largely ignored positions of protists.

Part 3 – Chapter 10

St. George belowground: Armored amoebae attack dragon-like soil nematodes

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Abstract

In soil biology, heterotrophic protists are assumed to occupy a key position in soil food webs by controlling bacterial biomass. Their enormous phylogenetic diversity, however, suggests that bacterivory alone strongly underestimates the true functional importance of protists in soil. We investigated feeding interactions of the testate amoeba *Cryptodifflugia operculata* with other soil organisms and investigated the relative contributions of active *C. operculata* among soil protists in soil and litter samples using a metatranscriptomic approach. We show that tiny *C. operculata* actively preyed on a range of much larger bacterivorous nematodes. In turn, protist abundances strongly increased by preying on nematodes, while protist turnover with bacteria as sole nutrient source was much lower. This indicates that facultative nematophagous feeding of *C. operculata* is of essential importance for population fitness especially in view of the ubiquity of nematodes in soil. We detected sequences assigned to *C. operculata* in all samples investigated, representing up to 4 % of the entire protist community. This is the first study to show that even tiny protists feed and proliferate on nematodes and that these taxa are very common members of the active protist community in soils. Therefore, feeding on nematodes by distinct soil protist groups might be an important component in soil food webs.

Introduction

Heterotrophic protists and bacterivorous nematodes are commonly assumed to be the major controllers of bacterial biomass in soils (Hunt et al. 1987, De Ruiter et al. 1995, Bjørnlund and Rønn 2008). Based on coarse morphological features heterotrophic protists, hereafter simplified as protists, have traditionally been classified as flagellates, ciliates and amoebae (Darbyshire 1994). Recent phylogenetic information, however, revealed that flagellates and amoebae are composed of a vast number of paraphyletic species, spreading all across the whole eukaryotic tree of life (Cavalier-Smith 1993, Cavalier-Smith 1998, Adl et al. 2012). In view of this enormous diversity, the concept used by soil biologists of treating protists as bacterivores is rather unlikely.

Taxonomic studies have already provided evidence other trophic feeding groups, such as algivores (Smirnov et al. 2011a, Hess and Melkonian 2013) and mycophages (Old and Darbyshire 1978, Chakraborty and Old 1982, Petz et al. 1986, Ekelund 1998). Even nematodophagous protists are known, which generally are large taxa that directly consume nematodes; among them are the vampyrellid amoebae *Arachnula impatiens*, *Platyreta germanica* and *Theratromyxa weberi* (Sayre 1973, Hess et al. 2012), the testate amoebae *Nebela vas* and *Difflugia lanceolata* (Yeates and Foissner 1995) and the ciliates *Stylonychia pustulata* and *Urostyla* sp. (Doncaster and Hooper 1961). Direct feeding of smaller protists (< 30 µm) on nematodes (> 250 µm) is not known, despite small protists affect nematode behaviour (Bjørnlund and Rønn 2008, Neidig et al. 2010). Yeates and Foissner (1995) isolated nematodes with damaged tails and suggested that it might have been caused by predacious testate amoebae.

Testate lobose amoebae are intermingeled with naked amoebae in Amoebozoa, placing monophyletic in the order Arcellinida (class: Tubulinea, supergroup: Amoebozoa) (Nikolaev et al. 2005, Smirnov et al. 2011b, Adl et al. 2012, Lahr et al. 2013). Amoebae of this order are well characterized and high numbers of species are described due to distinctions based mainly on scale morphology. Nearly 20 *Cryptodifflugia* spp. are described based on morphological differences mostly attributed to size and shape of the shell in the genus, which comprise among the smallest testate amoebae (10 - 55 µm in length) and show a wide distribution (Page 1966, Hedley 1977, Nicholls 2006).

We investigated intra-guilt predation interactions of *Cryptodifflugia operculata* on nematodes. Further we investigate the ecological distribution and activity of these amoebae in a wide range of soil habitats analysing a dataset obtained in a metatranscriptomic approach, to revealing presence, relative abundance and therefore potential significance of *C. operculata* in soil food webs.

Materials and Methods

Isolation and description of *Cryptodifflugia operculata*

C. operculata was cultivated from the mineral layer of a pasture soil in the Netherlands (N52°01', E5°99'). Cultures on accompanying bacteria were established in Petri dishes with Prescott-James medium (Page 1991), enriched with 0.15 % wheat grass (Weizengras, Sanatur, Singen, Germany), i.e. WG medium. *C. operculata* grew well in cultures with a diverse soil bacterial community. Cell sizes were determined microscopically using a Nikon Eclipse TE2000-E inverse microscope at 400 x magnification by measuring the average of 20 cells. Shell dimensions were on average 16.9 ± 2.1 µm in length and 14.9 ± 1.8 µm in width. Genomic DNA was isolated using a guanidine isothiocyanate method (Maniatis et al. 1982) followed by amplification of the small subunit (SSU) ribosomal DNA with primers, cycling conditions and enzymatic purification as described in Geisen et al. (2014). A partial sequence obtained revealed a perfect match to the *C. operculata* strain deposited under GenBank accession number JF694280 (Lahr et al. 2011)

Intra-guilt predation of *C. operculata* on bacterial feeding nematodes

The bacterial feeding nematodes *Acrobeloides buetschlii*, a bisexual *A*. sp.*, Rhabditis belari, R. dolichura* and *R. terricola* were grown on *Escherichia coli* OP-50 on 2 % agar plates enriched with 0.1 % of 0.4 g cholesterol in 80 ml 95 % ethanol. Nematodes were suspended in WG medium, washed twice by centrifugation in fresh WG medium and numbers were subsequently determined microscopically using a Neubauer improved counting chamber (Roth, Karlsruhe, Germany).

Interactions between *C. operculata* and all nematode species were studied in 24 well-plates (flat bottom, Sarstedt, Germany). Treatments containing *C. operculata* were prepared one week before start of the experiment as enumeration in suspensions proved to be unreliably

due to extremely low numbers. Therefore, *C. operculata* was pre-inoculated in 500 µl WG-medium and enumerated at the beginning of the experiment. 500 µl sterile WG-medium was added to the remaining wells. The experiments were started by inoculating 33 ± 9 nematodes in 200 µl WG-medium to each well of the respective treatments while 200 µl sterile WG-medium was added to non-nematode treatments. In total, four replicates of each treatment (*C. operculata* alone or in combination with each of the nematodes) were setup resulting in 24 replicates in total. The 24 well-plates were sealed with Parafilm and incubated in the dark at room temperature for 18 days. The abundance of *C. operculata* in each well was determined microscopically at 100 x magnification 2, 4, 6, 8, 10, 14 and 18 days past inoculation, while living numbers of nematodes were determined at days 8 and 18. For convenience analyses were restricted to differences between initial and final abundances.

To investigate potential feeding of *C. operculata* also on dead nematodes, *A. buetschlii* in WG medium were killed by addition of boiling H_2O_{dest} (1:1 v/v). 1 ml of the heat-killed nematode suspension was then added to wells of a 24 well-plate that had been pre-inoculated with *C. operculata*. Attachment and feeding of *C. operculata* on the dead nematodes was investigated with an inverse microscope (Nikon Eclipse TE2000-E) at 100 - 400 x magnification.

Presence and activity of *C. operculata* in soils

Sequence data retrieved in a metatranscriptomic analyses analysing forest litter and soils from grasslands, forests and arctic peatlands (Chapter 8) were analysed for *Cryptodifflugia* sequences. Only sequences longer than 150 bp, with high quality (< 0.2 % error rate) were taxonomically assigned by MEGAN using BLASTn against the Silva SSU database (Pruesse et al. 2007, Urich et al. 2008). All sequences assigned as *C. operculata* were manually controlled by performing BLASTn searches against GenBank using default parameters.

Statistical analyses

Differences in the abundances of *C. operculata* and nematode spp. were calculated as relative values and square root arc-sin transformed to homogenize variances and analysed by a generalized linear model (GLM) using SAS 9.1 (Cary, FL, USA). Comparison of the means for the individual treatments was done at the 5 % probability level with a Tukey-test (Tukey's

honestly significant difference, HSD). Percentage data were square root arcsin-transformed prior statistical analyses.

Results

C. operculata consumed both, active and dead nematodes, and all nematode species were attacked and consumed. The feeding procedure was the following: nematodes were attached at their posterior end by one amoeba. Then other amoebae were attracted and fixed the anterior end. Attached nematodes were subsequently disintegrated from both ends by an increasing number of amoebae. The process of joint amoebae feeding dissolved a nematode within 12 hours. A sequential array of time-lapse photographs of the feeding procedure is shown in Figure 1.

The different nematode species had strongly different effects on the reproduction of *C. operculata*. Populations of *C. operculata* decreased in control treatments by 41 %, but increased by factors of 2.4, 2.4, 2.2, *A. buetschlii, R. dolichura, R. terricola, A. bisex* and *R. belari*, respectively ($F = 6.37$, $p < 0.01$; Figure 2). However, the growth increase in *C. operculata* did not reflect the declines in nematode numbers. After 18 days, *C. operculata* had reduced the numbers of all nematode species. *R. terricola* by 89 % ($F = 864$, $p < 0.001$), *A. bisex* by 77 % ($F = 99.4$, $p < 0.001$), *R. belari* by 32 % ($F = 10.7$, $p < 0.05$), *A. buetschlii* by 31 % ($F = 5.12$, $p = 0.06$) and *R. dolichura* by 25 % ($F = 9.3$, $p < 0.05$; Figure 3). Interestingly, the decline in *A. buetschlii* the nematode species that entailed the highest abundance increase of *C. operculata* was only marginally significant.

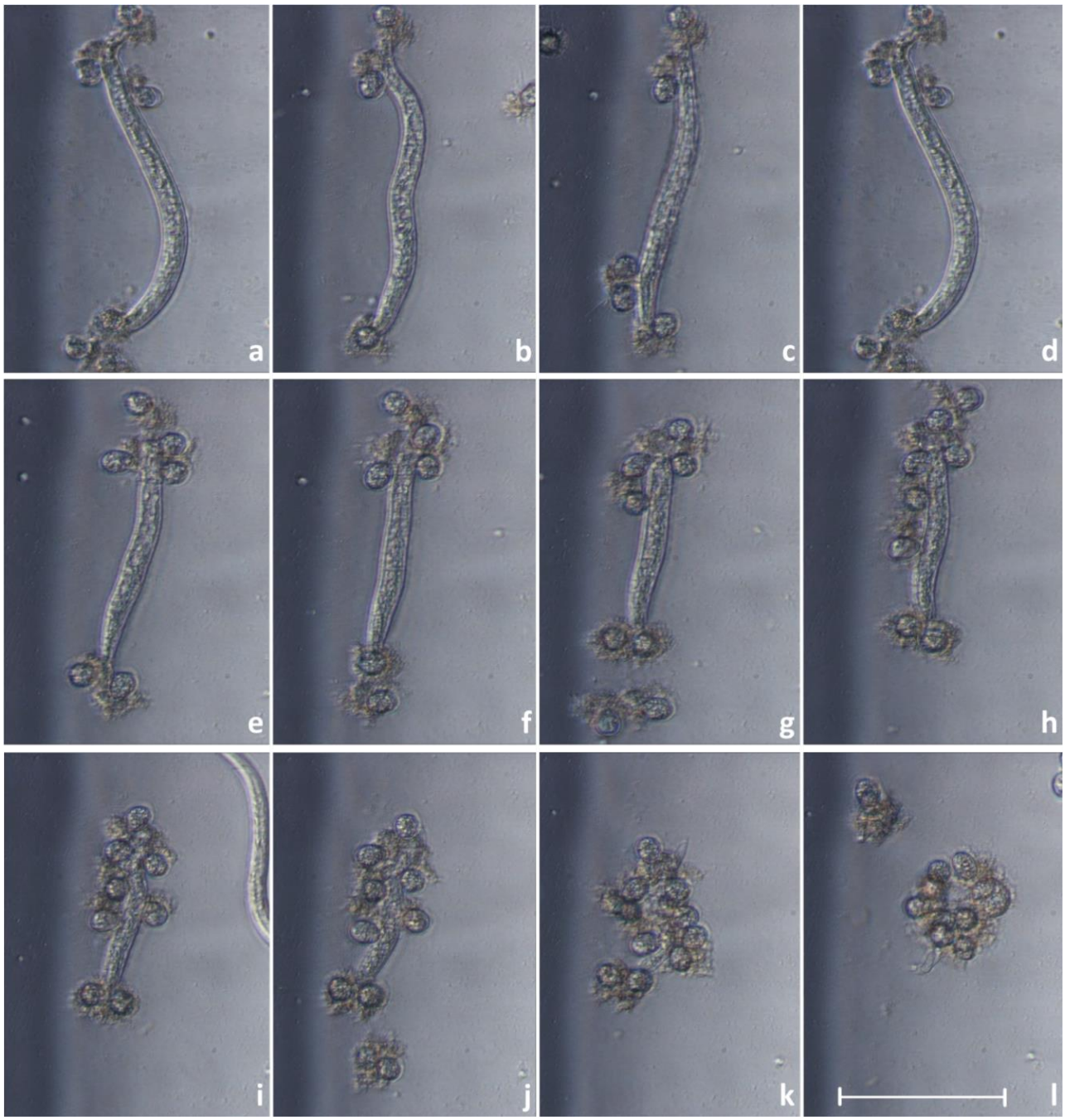

Figure 1. Feeding procedure of *C. operculata* on the nematode *A. buetschlii* over a period of 12 hours in intervals of one hour; scale bar = 100 µm.

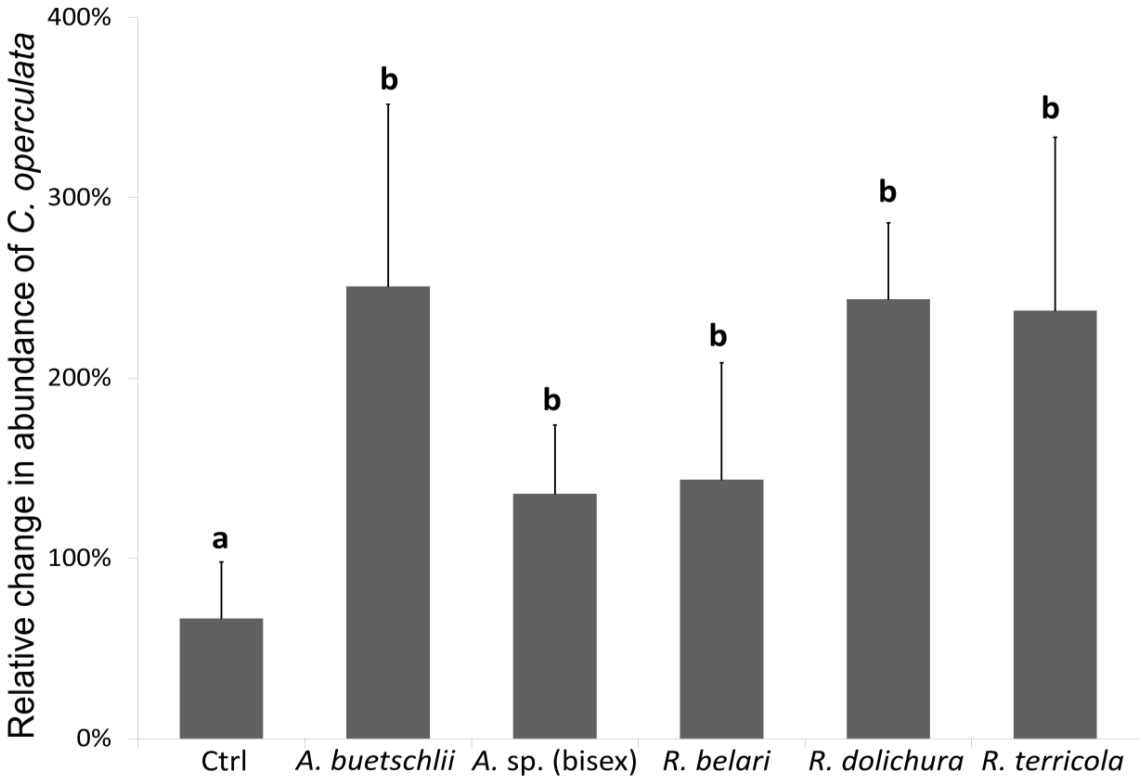

Figure 2. Relative change in abundance of *Cryptodifflugia operculata* over the course of the experiment. Treatments are without nematodes (Ctrl) or with *Acrobeloides buetschlii, A.* sp. (bisex), *Rhabditis belari, R. dolichura* or *R. terricola* as nematode prey species. Different letters indicate significant differences (p = 0.05).

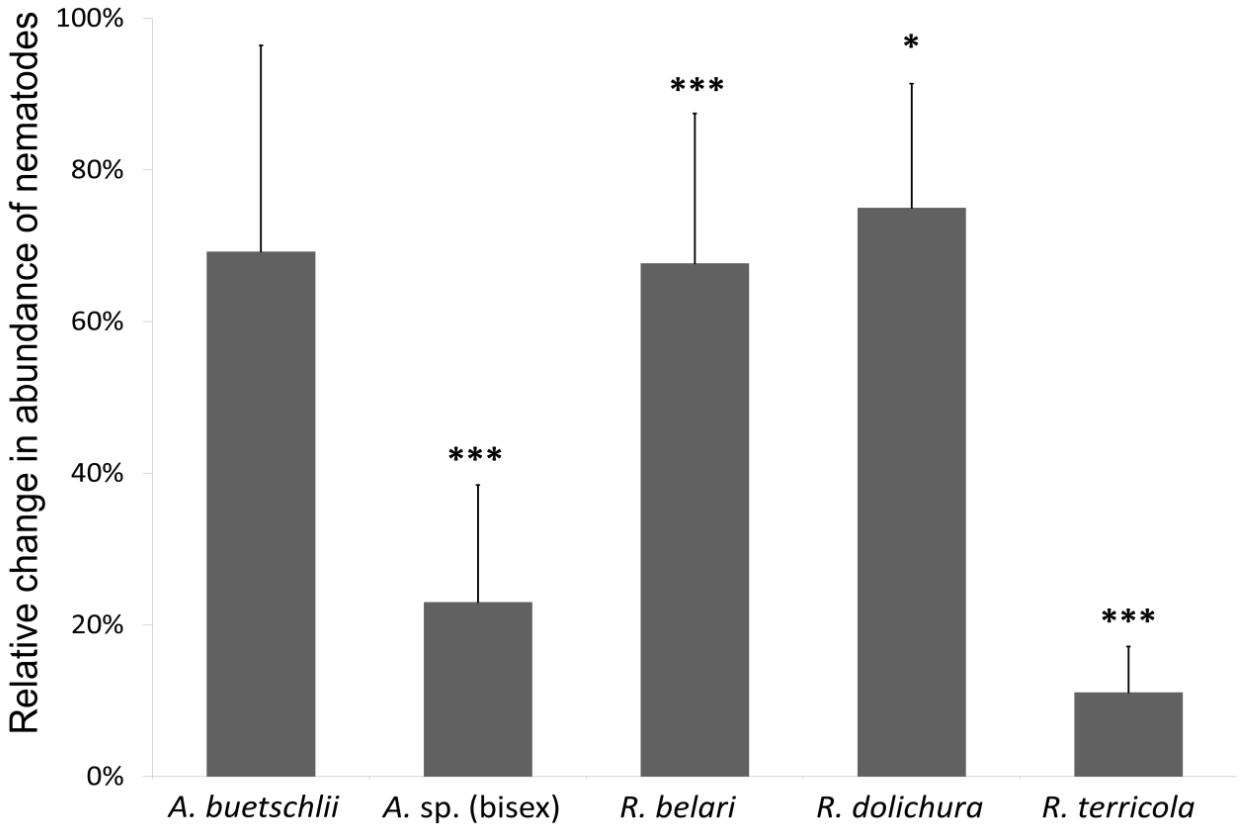

Figure 3. Relative change in abundance of nematode species *Acrobeloides buetschlii, A.* sp. (bisex), *Rhabditis belari, R. dolichura* or *R. terricola* in presence of *Cryptodifflugia operculata*. Stars indicate significant differences at * $p < 0.05$; and *** $p < 0.001$.

The general abundance of active *Cryptodifflugia* spp. in different soils

All samples from diverse terrestrial habitats in Europe contained sequences resembling *C. operculata* (Figure 5). Sequences assigned as *C. operculata* generally showed > 98 % sequence identity in BLASTn searches to the type species. A similar sequence identity was found for *C. oviformis*. However, especially sequences from forest sites more closely resembled uncultivated species with *C. operculata* as the first described species. Less than 1 % of sequences were wrongly assigned to *C. operculata* and were manually removed as they most closely matched uncultivated *Arcella* spp.

C. operculata sequences were particularly abundant in forest litter and soil samples, comprising up to 18 % of total Amoebozoa and 4 % of all protists sequences (Figure 5). *C. operculata* still represented 4.8 % of the amoebozoan sequences (1.1 % of all protists) in grasslands, while both Arctic peatland sites only hosted a relatively low fraction of *C. operculata* (< 5 % of Amoebozoa and < 0.7 % of all protist sequences; Figure 5).

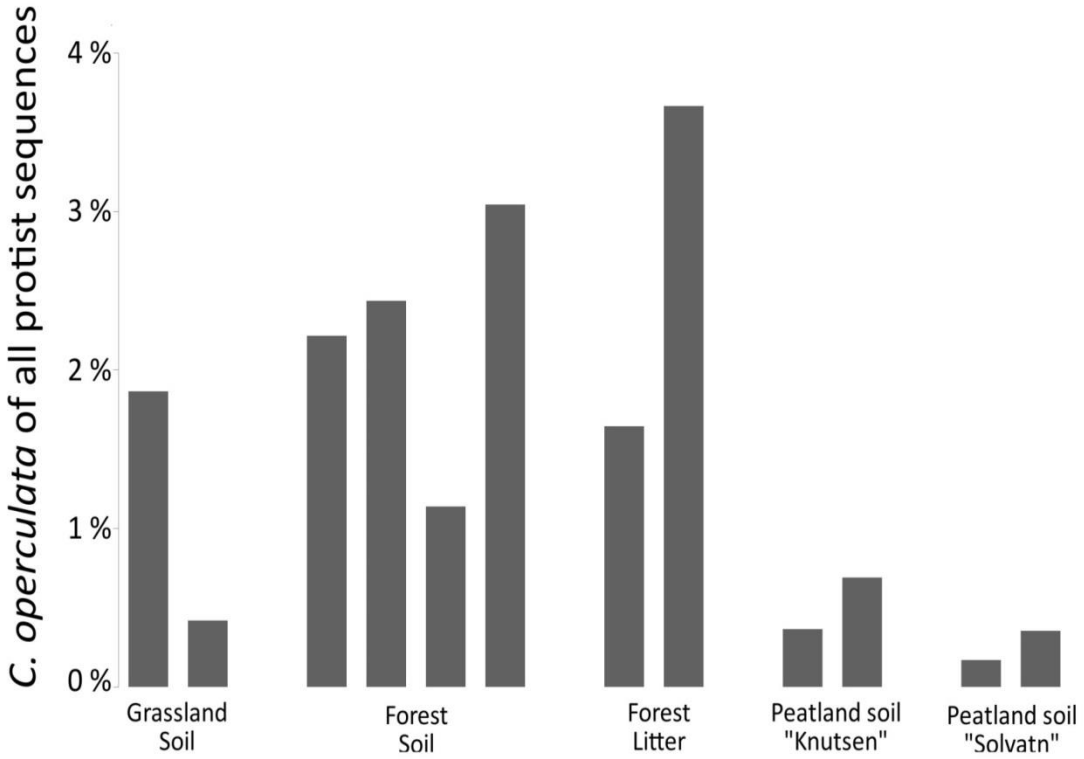

Figure 5. Sequences assigned as *C. operculata* shown as relative abundance of all sequences assigned to protists obtained in a metatranscriptomic study (Chapter 8).

Discussion

Predation of testate amoebae on nematodes has been shown before (Yeates and Foissner 1995), but predation of protist on nematodes was seen more as the exception rather than the rule. Our data unambiguously demonstrate that predation of protists on nematodes must be regarded as an important trophic link in the soil food web.

Sequences assigned as *C. operculata* are shown to represent high relative abundances of the entire active community of soil protists shown in metatranscriptomic datasets. Therefore, an inverse link of nematophagous protists in soil food webs might be of major importance. So far*, C. operculata* represents the smallest nematophagous soil protist, as the few taxa reported to feed on nematodes are 5- to 10-fold larger (Sayre 1973, Yeates and Foissner 1995). Large vampyrellid amoebae *Theratromyxa weberi* directly fed, digested and reduced nematodes, but densities of amoebae appeared largely unaffected by nematophagy (Sayre 1973).

More recently, more sophisticated negative intra-guild interactions were reported between protists and nematodes. Exoproducts of amoebae repelled nematodes and exhibited marked nematostatic activity, while exoproducts of nematodes increased encystation and reduced growth of their bacterivore competitors (Neidig et al. 2010). Likewise Bjørnlund and Rønn (2008) found flagellates that killed nematodes without proliferating at high flagellate abundance, whereas nematodes consumed flagellates at lower density, both not benefiting from this interaction.

In recent years it was shown that interactions of “bacterivorous” protists and nematodes are much more complex than depicted in current food-web models. Evidence from a number of experiments has clearly shown that “bacterivorous” nematodes can be important predators of naked amoebae (Anderson et al. 1977, Alphei et al. 1996, Rønn et al. 1996, Bonkowski et al. 2000b). Here, we demonstrate that intra-guild predation can be also an important strategy of amoebae to acquire a huge amount of food resources from nematodes.

Interestingly, *C. operculata* can grow entirely on bacteria, while the other nematophagous protists are broader omnivores or predators, all needing nematodes, algae or fungi as supplementary food (Yeates and Foissner 1995, Hess et al. 2012, Berney et al. 2013). Also, the predation strategy of *C. operculata* strongly differs from other known nematophagous

protists. The larger amoebae *T. weberi*, *N. vas* and *Difflugia* sp. attack nematodes individually usually from the posterior end or at mid-body and ingest them entirely (Sayre 1973, Yeates and Foissner 1995). Strongly different was the mode of pack hunting of *C. operculata*, where more and more conspecifics were attracted to an attacked nematode, most likely by chemical signals. However, reproduction of *A. buetschlii*, the nematode species that favoured most strongly reproduction of *C. operculata*, was only marginally affected, while other nematodes were strongly reduced. *A. buetschlii* might be less prone to predation losses as this species is an r-strategist characterized by asexual reproduction and short generation times (Frey 1971), while *A*. sp. was a sexual species that was more prone to predation. This suggests that indirect effects of *C. operculata* affecting the community composition of nematodes might be much more important than direct predation and the overall reduction in nematode numbers per se.

The metatranscriptomic data revealed that sequences assigned as *C. operculata* represented a significant proportion of the entire protist community in a broad variety of soil and litter samples across Europe. Since the biomass of protists in soil is at least an order of magnitude greater than the biomass of nematodes (Paustian et al. 1990, Schaefer and Schauermann 1990), relative abundances of *C. operculata* of up to 4 % of all protist sequences indicate a significant potential for widespread intraguild predation of protists on nematodes. It must be noted, however, that several sequences showed higher similarities to unknown uncultivated species, which might represent other unknown or unsequenced species within the genus *Cryptodifflugia*. Based on morphology, *Cryptodifflugia* is a species-rich genus with more than 20 described species (Page 1966), while sequence data is only available for *C. operculata* (Lahr et al. 2011) and *C. oviformis* (Gomaa et al. 2012).

Taken together, this work shows that intraguild predation of a small testate amoebae *C. operculata* on nematodes might be of fundamental importance in soil food webs by shaping the nematode community composition. Sequences assigned as *C. operculata* represented a significant proportion of the entire fraction of active protists indicating an important ecological function of *C. operculata* in soils by controlling both bacterial biomass and nematode abundances.

General Discussion

The work described in this thesis made significant contributions to fill the gaps of knowledge on the extremely abundant, diverse, and functionally important group of soil protists (Clarholm 1985, Foissner 1987, Ekelund and Rønn 1994, Finlay et al. 2000, Bonkowski 2004, Bonkowski and Clarholm 2012). It has been shown that specific protist taxa, and in particular amoebae, can exhibit important functions, but knowledge on the diversity and community composition of protists in soils was scarce. Those studies investigating and showing important ecological functions exhibited by soil protists largely focused on one or few taxa (Bonkowski and Brandt 2002, Krome et al. 2009, Pedersen et al. 2009, Koller et al. 2013, Saleem et al. 2013). Taken into account the enormous diversity of (soil) protists (Foissner 1987, Cavalier-Smith 1998, Adl et al. 2012, Bates et al. 2013), generalizations on protists functioning derived from these simplistic studies more than likely provide an artificially oversimplified picture. Further, methodological drawbacks prevent assessing the real soil protist community composition with many species remaining undiscovered (Foissner 1999b, Moreira and López-García 2002, Epstein and López-García 2008).

Within this thesis, several hundred amoebae were cultivated, classified into morphogroups and more specifically described using sequence information in order to increase the knowledge of soil protists. Using this information a total of 16 new species and 7 new genera have been formally described (Part 1, Chapters 1 - 4). These basal cultivation efforts targeting amoebae emphasized the enormous diversity of soil protist. To get a more exhaustive knowledge on the protists community, Part 2 of this thesis aimed to describe the community composition of soil protists by applying and optimizing a range of cultivation-based, and cultivation-independent molecular methods (Part 2, Chapters 5 - 8). These studies further emphasise the enormous diversity of the soil protist community revealing that protist communities differ between soils and that the results strongly depend on the method being used. The last part aimed at increasing the knowledge of species-specific ecological functions performed by soil protists (Part 3, Chapter 9 - 10) and reveals that protists are far more than bacterial feeders. Distinct taxa were shown to feed on fungi and nematodes and environmental sequencing revealed that protists with these ecological functions are widely distributed and abundant in soils.

Cultivation efforts remain an essential component in the study of soil protists (Part 1)

As the first major part of this thesis, the cultivation and description of 16 new species and even 7 genera of soil amoebae reveals that soil protists and especially amoebae are largely undersampled and reinforces the notion that a plethora of currently unknown protists inhabit soils (Moreira and López-García 2002, Epstein and López-García 2008).

The description of two new *Stenamoeba* spp. shows that this genus is species-rich, despite the short history of the genus *Stenamoeba* (Smirnov et al. 2007) with currently only three described species (Dyková et al. 2010b). The presence of MTOCs in *Stenamoeba* further demonstrates the scarce knowledge about taxonomic and morphological characteristics specific for or shared between amoebae within the supergroup Amoebozoa. Therefore, morphological features still add pivotal information to taxonomic affinities of unresolved groups based on phylogenetic information, such as between members of the class Discosea (Cavalier-Smith et al. 2004, Smirnov et al. 2005, Kudryavtsev and Pawlowski 2013), here shown by the ultrastructural feature of MTOCs (Chapter 1).

The description of another discosean amoeba, *Cochliopodium plurinucleolum* reveals that species-specific morphological characters and phylogenetic affinities even within a well-investigated amoebozoan genus such as *Cochliopodium* are far from being deciphered. Several *Cochliopodium* spp. with nearly identical sequences are further shown to exhibit inconsistent morphological characters, indicating that only a combination of molecular and morphological tools enables reliable identification of *Cochliopodium* spp. These discrepancies need to be considered when identifying amoebae based on morphological characters. Even the differentiation between higher taxonomic ranks often are impossible, such as between vahlkampfiids (Brown and De Jonckheere 1999, De Jonckheere and Brown 2005), tubulinids (Page 1985, Smirnov et al. 2011b), or the focus group of Chapter 3, i.e. “Variosea-like” amoebae (Smirnov et al. 2008, Lahr et al. 2012, Berney et al. 2013). Therefore, it is hardly surprising that amoebae described without providing molecular information have commonly been misidentified and later transferred to other positions in the eukaryotic tree (Brown and De Jonckheere 1999, Smirnov et al. 2007, Smirnov et al. 2008, Smirnov et al. 2011b, Lahr et al. 2012). But as indicated by the results obtained within

this chapter, species-level identification based on purely molecular information need careful interpretation. Intra-specific differences are often higher than inter-specific differences (Smirnov et al. 2007, Qvarnstrom et al. 2013) and morphologically clearly distinguishable species or genera sometimes share identical sequences, especially when partial sequences are being used (De Jonckheere and Brown 2005, Smirnov et al. 2009, Anderson and Tekle 2013). Further, annotated sequences are commonly mislabeled and some taxonomic affinities of and between groups remain uncertain, preventing reliable sequence assignments without knowledge on morphology (Berney et al. 2004, Smirnov et al. 2008, De Jonckheere et al. 2012). Therefore, identification up to species level should only be made by combining morphological with sequence information.

The description of six new genera of amoebae in the class Variosea helped at strongly increasing the knowledge on the diversity of and phylogenetic affinities within Variosea. Morphologically species-differentiation within Variosea remains hardly impossible, due to profound intra-clonal morphological plasticity that often surpasses differences even between genera. The variosean morphology, i.e. cells more or less branching and extended with filose pseudopodia, generally seems widespread among amoebae and is adopted by amoebae in the orders Leptomyxida, Centramoebida and Varipodida (Amoebozoa) and Vampyrellida (Cercozoa) (Adl et al. 2005, Bass et al. 2009a, Smirnov et al. 2011b, Hess et al. 2012). Therefore it is hardly surprising that many taxa have taken a long way until finding their current taxonomic affinity. One striking example is Leptomyxida that long combined all variosean-like amoebae but was later divided based on molecular information; *Leptomyxa* and *Gephyramoeba* are now placed in Leptomyxida*, Balamuthia* in Centramoebida, while *Acramoeba dendroida* (initially mislabelled as "*Gephyramoeba*") (Smirnov et al. 2008), *Grellamoeba robusta* (Dyková et al. 2010a) and *Telaepolella tubasferens* (initially mislabelled "*Arachnula impatiens*") (Lahr et al. 2012) found their home in Varipodida (Amaral-Zettler et al. 2000, Smirnov et al. 2011b). Morphological and molecular information on the new cultivated genera provided and by reliable placing many sequences from uncultivated species inside Variosea an enormous diversity of the class Variosea is shown.

Similar to those species descriptions of amoebae in the supergroup Amoebozoa, seven new vahlkampfiid species including the new genus *Pagea* are described, revealing a high unknown diversity of heterolobosean amoebae (supergroup Excavata). Six new species

placed in the recently erected genus *Allovahlkampfia* (Walochnik and Mulec 2009) were isolated from all geographically distant soils indicating a wide distribution of *Allovahlkampfia* spp. in soils. "*Solumitrus" palustris* is included as *A. palustris* as it reliably placed inside this genus, confirming previous studies (Brown et al. 2012, Harding et al. 2013) and shows that taxonomic affinities of other protists and amoebae might still change in the future due to increased taxon-sampling and multi-gene approaches. *Pagea alta* as the type species of a new genus branched with only uncultivated taxa in phylogenetic analyses pointing out that cultivation efforts are necessary in assigning sequences obtained in cultivation-independent soil surveys such as by using high-throughput sequencing (HTS).

The formal description of all these new species and genera of amoebae is in line with other recent descriptions of soil amoebae, which, however, remain rare (De Jonckheere et al. 2011b, Atlan et al. 2012). Cultures of several other new species and genera were obtained as part of this PhD work. An overview of sequenced amoebae of the supergroup Amoebozoa is shown in Figure 1, demonstrating that several species only known from environmental sequencing approaches have been successfully cultivated, such as Vannellidae (e.g. strains Nl174, Sar32 and Tib97), Dermamoebidae (strains Sar17, Nl179 and Tib196), *Angulamoeba* sp. F2 and Variosea sp. (strains G5, Tib48, Tib90). Most other clones branch inside known genera but often sharing low identity to described species, so that they are likely to represent new species, such as *Hartmannella* sp. (strains Nl117, Sar7 and Tib2), *Cochliopodium* sp. (strains Tib64 and Tib174), *Vannella* sp. (strains Nl7, Nl176 and Sar88) and *Filamoeba* sp. (strain Tib69). Among the cultivated amoebae are also many known species, such as *Saccamoeba limax* (strain Nl46), *Vermamoeba vermiformis* (strains Sar34 and Tib103), *Vannella simplex* (Sar36) and *Vexillifera bacilipedes* (strain Nl6). Several more examples for each of those three categories are illustrated in Figure 1. Despite less diverse and lower abundant in cultures, several heterolobosean amoebae were also cultivated that can be placed in these three categories and also an amoeba showing unique morphology and molecular patterns that could not reliably be assigned to any eukaryotic supergroup (data not shown). All these examples prove that many species remain unknown or only known from environmental sequencing approaches (Lara et al. 2007a, Lejzerowicz et al. 2010, Berney et al. 2013). However, the cultivation of entirely unknown amoebae or known only from environmental sequencing approaches reveals that at least a subset of the so-called uncultivable amoebae are in fact cultivable. Molecular techniques such as HTS are

now rapidly being improved, replacing traditional cultivation-based studies (Lara et al. 2007a, Urich et al. 2008, Medinger et al. 2010, Bates et al. 2013). Much, if not major information is currently lost as many sequences cannot reliably be assigned to known species, reinforcing the notion that traditional cultivation-based methods can- and should not entirely be replaced by molecular tools. In addition to the benefit of being able to assign molecular sequences to morphological information, cultivation-based efforts allow detailed ecological investigations on distinct species to eventually allow functional assignment to respective sequences.

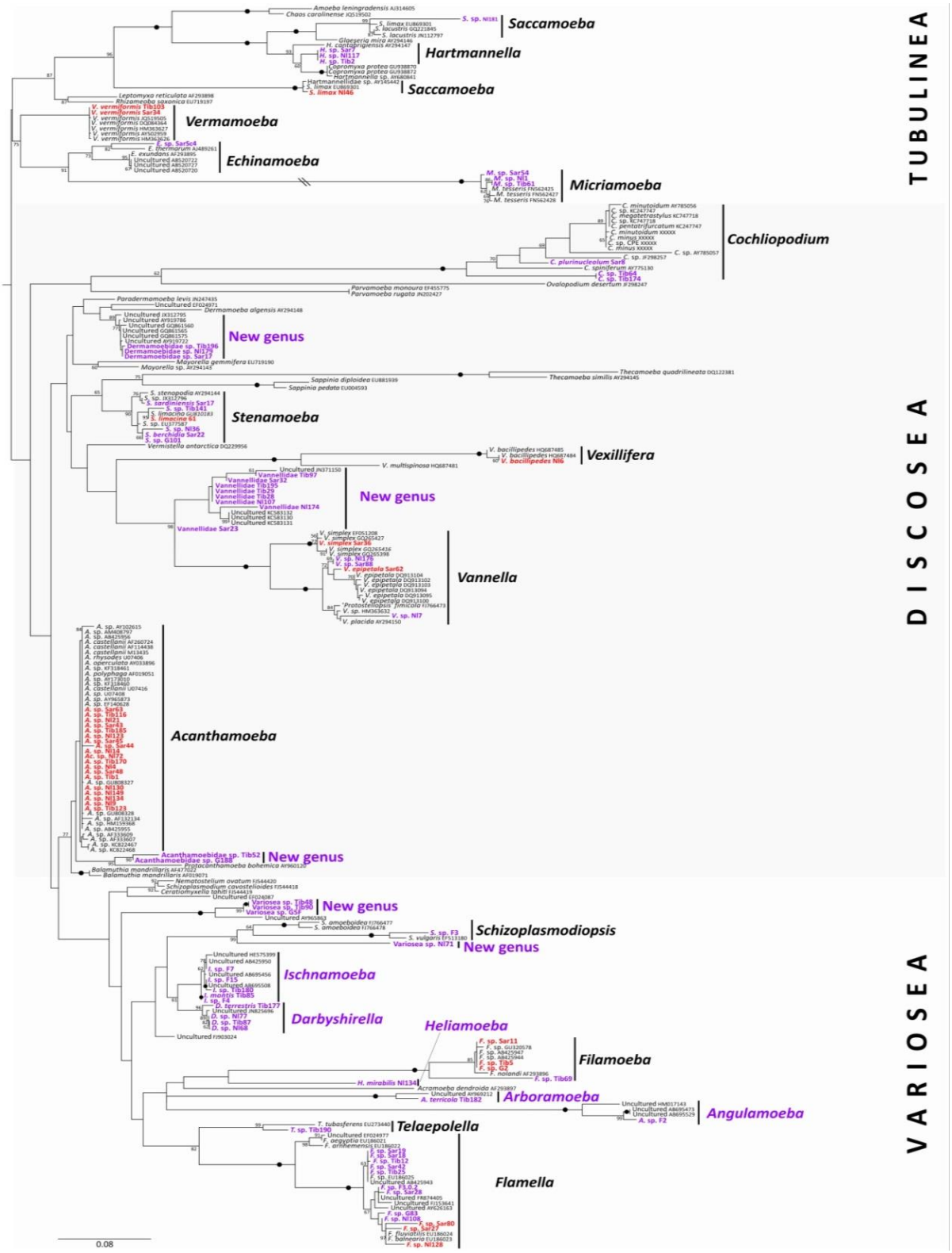

Figure 1. Maximum likelihood phylogenetic tree of amoebozoan amoebae (classes Tubulinea, Discosea and Variosea) cultivated in this thesis with closest BLASTn hits (default parameters) obtained of each respective sequence. Amoebae most likely resembling known species are shown in red, new species and genera in pink; Genera of amoebae cultivated in this study highlighted in bold.
RaxML analysis (version 7.3.2, Stamatakis 2006), 1,113 nucleotide positions of the SSU-rDNA gene, GTR+γ+I model of nucleotide substitution, rooted with Tubulinea; bootstrap values shown > 60, solid circles = 100. Branches with a break cut in half; scale bar = 0.08 substitutions / site.

Diversity, distribution and community structure (Part 2)

The second major part of this thesis aimed at increasing the knowledge on the diversity and community structure of soil protists by applying a battery of different methods. Among those were a traditional cultivation based enumeration study combined with morphological protist identification (Chapter 5), a combination of cultivation and subsequent molecular identification of *Acanthamoeba* clones (Chapter 6), a HTAS study (Chapter 7) and a metatranscriptomic approach (Chapter 8).

Traditional cultivation based methods have the longest history and used to provide the only possibility of studying the entity of soil protists (Darbyshire et al. 1974, Foissner 1987, Smirnov 2003). As a first method to study soil protist communities a modified liquid aliquot method (LAM) (Butler and Rogerson 1995), decreasing workload, allowing deeper taxonomic identification of protist clades and enabling to obtain information on abundances of respective taxa. The high taxonomic resolution according to the most recent taxonomic classification (Smirnov et al. 2011b, Jeuck and Arndt 2013) is the first in this depth and provides evidence, that global climate changes might impact distinct protist clades differentially and that soil moisture has a profound impact on the abundance of soil protists. Therefore, cultivation based studies allow answering specific questions such as in ecological studies and remain unique in providing biomass estimations of a range of soil protists. A major advantage of the LAM is that protist taxa are usually growing in monoclonal cultures enabling downstream deep taxonomic classifications using molecular sequencing (e.g. Chapter 6) and functional investigations (Chapters 9 and 10).

An example for an approach using morphologically classified cultivated taxa from geographically distant locations with subsequent high-resolution molecular identification was applied in Chapter 6 as a second example of studying soil protist communities. Interestingly, all sequences obtained from morphologically often indistinguishable *Acanthamoeba* spp. are different to previously published sequences. Further, none of the sequences obtained at one location is identical to sequences recovered at another location and also the community composition was found to differ between sites. The fairly low number of sequenced clones might have missed known and strains of *Acanthamoeba* identical between sites. Nevertheless, the observed difference in the *Acanthamoeba*

community structure with only previously unknown strains sequenced strongly suggests that a huge diversity of *Acanthamoeba* strains remain to be discovered. Analysing a wider range of soils by deep-sequencing are needed to decipher the dispersal of sequence-identical *Acanthamoeba* strains and evaluate the factors that determine the community composition of *Acanthamoeba* spp. The observed patterns are even more profound taken into account that *Acanthamoeba* is among the best studied protists due to the presence of several facultative human pathogenicity strains (Schuster 2002, Schuster and Visvesvara 2004). One of the strains isolated in Dutch soils showed dramatic cytopathogenic characteristics, indicating that soils serve as a reservoir for pathogenic protists. Further functional investigations are highly desirable to investigate whether sequence differences between strains can be used to derive ecological functions and / or pathogenicity.

A high-throughput amplicon sequencing (HTAS) approach using cercozoan specific primers is used in Chapter 7 as a third technique to investigate the diversity and community composition of soil protists. Sample throughput and depth of community analyses are highly increased and the need to cultivate protists is circumvented by directly targeting DNA (Dawson and Hagen 2009, Creer et al. 2010, Medinger et al. 2010). Fundamental differences in the cercozoan community are detected that differ depending on geographic location and soil treatment. Interestingly, cercozoan communities differ strongly even between comparable soil environments strongly opposing the famous hypothesis that “everything is everywhere, the environment selects” (Baas-Becking 1934, Finlay 2002) and support recent findings that challenged this concept (Foissner 2006, Bass et al. 2007, Fontaneto and Hortal 2013, Heger et al. 2013). Further, this study shows that differences between soils become much more evident when increasing the level of taxonomic resolution suggesting that HTS approaches allow deciphering even minor differences in soil protist community structures.

Using metatranscriptomics as a forth method to study the entire diversity and community structure of soil protists allows deep analyses and comparisons of soil and litter samples from distinct locations in Europe. The deepest resolution of the soil protist community obtained to date reveals that amoebae represent a high proportion of the protist community contrasting previous HTAS approaches (Baldwin et al. 2013, Bates et al. 2013), that protist communities differ between locations on taxonomic ranks from supergroup to genus level and that protist groups basically unknown from soils such as choanoflagellates and

foraminifera comprise a significant part of the protist community. Therefore it seems that research on soil protist diversity and community composition up to now only scratched the tip of the iceberg, with presumably only a small fraction of the entire protist community being known and a virtual absence of reliable information on protist community compositions.

Biases for all of the applied methods remain and need to be considered when studying protists; despite applying a modified LAM (Chapter 5), cultivation based techniques suffer from low sample-throughput, the prerequisite of protist cultivability (Berthold and Palzenberger 1995, Foissner 1999b) and the need of expert skills to morphologically identify many protist groups even to shallow taxonomic levels, e.g. family or genus level (Foissner 1999b, De Jonckheere and Brown 2005, Smirnov et al. 2008, Howe et al. 2009). Downstream sequencing of cultivated protists increases the taxonomic resolution and this combination of morphological and molecular tools remains the only reliable method to identify the majority of protists up to species level (De Jonckheere 1998, Brown and De Jonckheere 1999, Pawlowski et al. 1999, De Jonckheere and Brown 2005, Pawlowski and Burki 2009, Brabender et al. 2012). However, the work- and time-load synchronously increases dramatically, lowering the sample throughput to a minimum (Chapter 6). As the name suggests, “high-throughput” sequencing methods in form of HTAS or metatranscriptomics solve this problem. Additionally, HTS methods enable identification of uncultivable protists and avoid expert knowledge in protist identification. On the downside, high start-up costs, advanced bioinformatic capabilities and skills, taxonomic expertise in sequence interpretation by dealing with wrongly annotated sequences need to be considered (Berney et al. 2004, Epstein and López-García 2008, Medinger et al. 2010, Pawlowski et al. 2011). Not only do all those methods for studying protist diversity necessitate different prerequisites and skills, while all being affected by distinct sources of error, each method reveals a different picture on protist communities. This suggests that no single method allows deciphering the entire soil protist community in all aspects (Figure 2) and that the method of choice depends on the question being addressed.

It has to be noted, however, that the methods applied here targeted different questions and were used to study dissimilar soils. Differences in observed protist communities are affected at least in part by abiotic factors such as differences in soil texture, organic matter content,

plant communities, land-use and moisture (Foissner 1997, 1999b, Anderson 2002, Bates et al. 2013). These differences render direct method comparisons based on our results impossible, but it remains unquestionable that each method reveals a different part of the protist community. Future direct comparisons of the entire battery of methods to study the soil protist community are essential to decipher inter-methodological differences that will ultimately allow to reliable identify information that can be extrapolated between methods. Only this knowledge will finally allow a reliable estimation of protist abundances, dispersal, diversity and community composition in soils, which until then remains highly speculative.

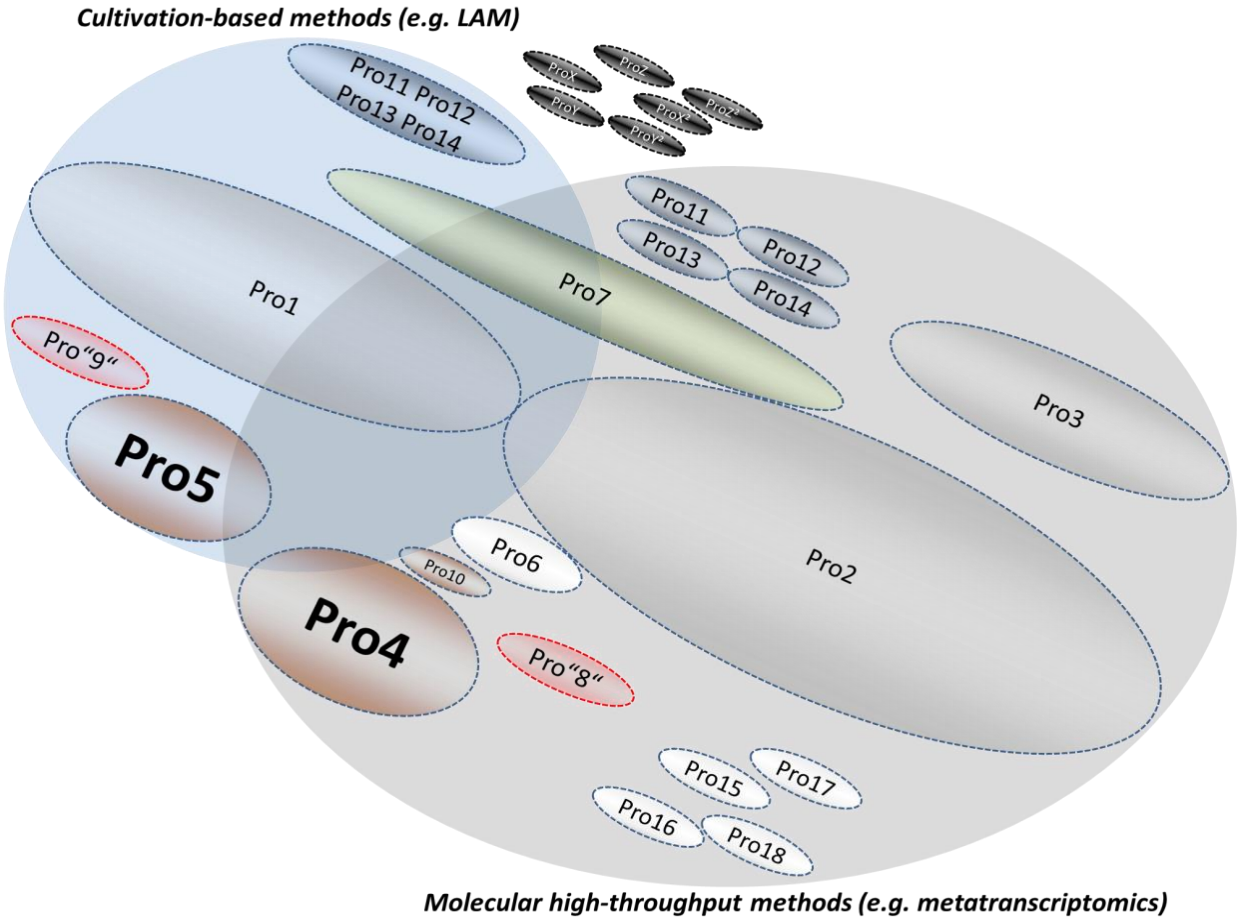

Figure 2. Simplified schematics comparing two major methodological approaches for studying soil protists, i.e. cultivation based (smaller blue circle; top left hand corner) and cultivation-independent (larger grey circle; bottom right hand corner). Differences between approaches are illustrating showing methodological artefacts that need to be considered when studying protist communities. Sphere sizes indicate (relative) abundances of respective protist clades; Equal colored spheres: comparable errors between methods
Pro1 - 18: protist taxa. **Pro1**: abundant, well cultivable; **Pro2**: abundant, not-well cultivable; **Pro3**: abundant, uncultivable; **Pro4**: overrepresented when applying HTS technologies (e.g. multiple copies of target molecules, amplification of extracellular DNA as well as PCR-artefacts in HTAS or high numbers of rRNA transcripts in metatranscriptomics); **Pro5**: overrepresented in cultivation-based studies, e.g. mainly present inactively; **Pro6**: underrepresented due to low abundances close to the detection limit in cultivation efforts; **Pro7**: equally represented between methods; **Pro„8"**: wrongly assigned (e.g. sequencing errors or mislabeled published sequence); **Pro„9"**: morphologically misidentified; **Pro10**: adversely affected in sequencing (e.g. extraction of target molecules prevented by incomplete cell lyses or mismatches of primers in HTAS); **Pro11 - Pro14**: morphologically (nearly) identical, distinguishable only using cultivation-independent techniques; **Pro15 - 18**: low abundant taxa recovered only by HTS; ProX-ProZ²: uncultivable protists, sequences obtained but removed in quality filtering or very low abundant taxa, i.e. „missing diversity".

Ecological importance (Part 3)

The third major part of this thesis reveals that protists are more than bacterivores as is usually been suggested in soil food web models (Hunt et al. 1987, De Ruiter et al. 1995, Crotty et al. 2011). Mycophagous (Chapter 9) and nematophagous protists (Chapter 10) are described, both functional groups showing high relative abundances in diverse soil and litter samples.

In the first study (Chapter 9) several protists previously suggested as bacterivorous are shown to feed on distinct fungi. True obligate mycophagous protists have rarely been studied, but few are known, such as ciliates in the family Grossglockneriidae (Foissner 1980, Petz et al. 1986, Foissner 1999a) and some facultative mycophagous groups, such as vampyrellid amoebae (Old and Darbyshire 1980, Old and Oros 1980, Hess et al. 2012). Sequences specific for these two protist groups were discovered in targeted sequence-mining of HTAS and metatranscriptomic (Chapter 8) datasets, being present in all samples investigated and represent a substantial fraction of the entire protist community. Therefore, soil protists are likely more than bacterivores and mycophagous protists should be considered as an important trophic node in soil systems (Figure 3).

A small and common testate amoeba, *Cryptodifflugia operculata*, is shown in a second study to interact with a range of other soil organisms, most profoundly feeding and proliferating on a range of nematodes. *C. operculata* grows in monoclonal cultures on bacteria only, but in presence of nematodes, abundances strongly increased suggesting that this facultative nematophagy suits as an important feeding strategy in soils. The high representation of *Cryptodifflugia*-like sequences among all protist sequences in the metatranscriptomic analyses of several soils (Chapter 8) provides further evidence that the trophic level of protists is not identical for all of these highly diverse taxa. Nematophagous or omnivorous protists are likely to deserve an own node in soil food webs and potentially constituting an important alternative link to other trophic levels (Figure 3). Also conceivable is that the microbial loop in soil and eventually plant growth (Clarholm 1985, Bonkowski 2004) is further stimulated by omnivorous protists as they supplement plants and bacteria with nutrients released from presumably higher trophic levels.

Due to their enormous diversity, vastly outreaching that of all multicellular eukaryotes (Cavalier-Smith 1993, Cavalier-Smith 1998, Adl et al. 2012) it is little surprising that protists occupy distinct environmental niches and perform alternate ecological functions, despite the contradictory view of treating protists in a single functional unit as bacterivores (Hunt et al. 1987, De Ruiter et al. 1995). The studies in this chapter provide further evidence for feeding differences between protist taxa, confirming studies revealing strongly diverging feeding strategies, such as by feeding on other protists (Page 1977, Smirnov et al. 2007, Berney et al. 2013), fungi (Old and Oros 1980, Petz et al. 1985, Ekelund 1998) and nematodes (Doncaster and Hooper 1961, Sayre 1973, Yeates and Foissner 1995). Peculiar, however, is the small size and high abundance of the newly found nematophagous amoeba *C. operculata* suggesting that food web anomalies are common and that higher turnover and abundances of these smaller organisms are of major ecological importance in controlling nematode numbers. The sequence information obtained from both mycophagous and nematophagous protists further shows their high representation in soils and litter indicating that traditional soil food webs need to be complemented by several nodes of functionally distinct protist clades, where e.g. nematophagous and mycophagous protists should find their home (Figure 3). Taken together, soil protists are a diverse assembly of organisms that host diverse functionally different groups most likely functioning as key nodes in soil food webs.

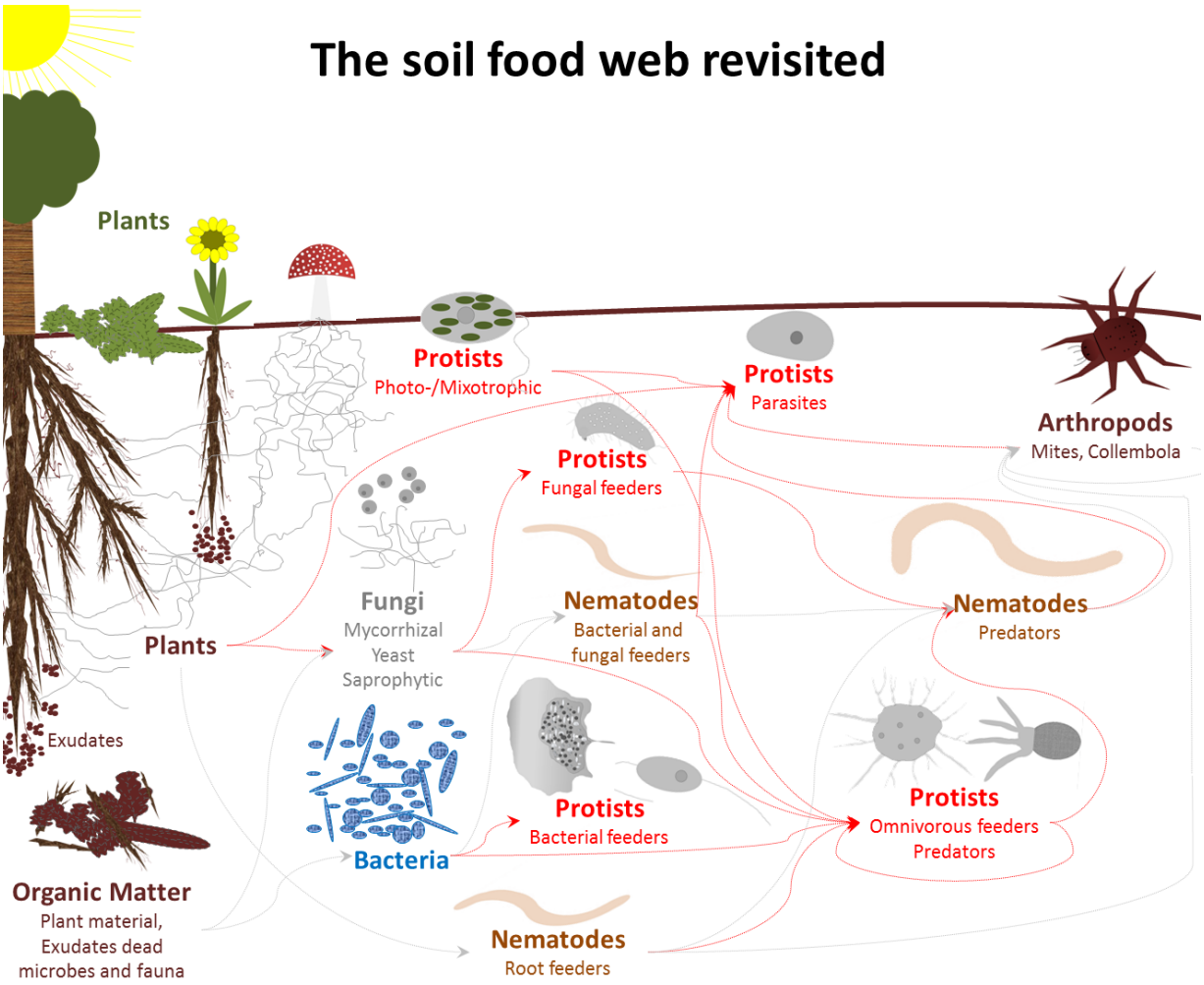

Figure 3. The soil food web focusing on protists; direct interactions indicated with arrows (red: interactions of protists with other soil organisms, grey: interactions between non-protists); different organisms encoded by different colors.

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Erklärung

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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