Novel Lineages in Cercozoa and Their Feeding Strategies

Inaugural – Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

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Tag der mündlichen Prüfung: 24. 01. 2017

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Summary

The term protist describes an informal grouping of unicellular eukaryotic organisms that do not form tissues. With a tremendous diversity in morphology and ecology, they represent the vast majority of eukaryotic heterogeneity of which only a small fraction is yet known. Ubiquitously dispersed in marine, freshwater and terrestrial habitats they occupy various ecological niches as e.g. primary producers, osmotrophs, bacterivores, fungivores, algivores, omnivores, predators or parasites of e.g. animals and plants. However, methodological drawbacks in culturing impeded research of protists. Research focused on the easily culturable taxa, especially bacterivores and algae, leading to a skewed image of protist diversity and many 'unculturable' protist taxa are still unknown to science.

Therefore we focused on protists with unusual feeding types (in particular bacterivorous sit-and-wait predators and eukaryvorous predators) in the phylum Cercozoa. The Cercozoa CAVALIER-SMITH 1998, were discovered to be closely related based on molecular analyses although being highly divergent in morphology and ecology. Molecular surveys revealed a high genetic diversity in the Cercozoa of which only a small fraction can yet be linked to morphological data. Based on culture material, cell morphology, feeding processes and life history stages of several cercozoan amoebae have been studied by us. This was achieved by using mainly light microscopy and time-lapse photography but also ultra structure data was obtained. Genetic markers, e.g. SSU rDNA and LSU rDNA were subjected to phylogenetic analyses to draw conclusions on cercozoan evolution.

Based on six isolates from German and Spanish soils a novel lineage of bacterivorous amoebae was described. *Kraken carinae* gen. nov. sp. nov. is an amoeba distinguished by a scale bearing cell body (usually <10 µm in diameter) and a network of filopodia (up to 0.5 mm in diameter). *K. carinae* is one of the few known sit-and-wait predators in the Cercozoa, preying on bacteria that get in contact with its large filopodia network. Unlike other cercozoan amoebae that usually use the filopodia to drag prey to their cell bodies for ingestion, *K. carinae* ingests bacteria directly at the point of contact and transports them through the filopodia to the cell body for digestion. SSU rDNA phylogeny showed an affinity to the order Cercomonadida in the class Sarcomonadea with only weak support, but a concatenated approach, by combining SSU rDNA and LSU rDNA sequences, confirmed the results with higher (though still moderate) support, in particular with the family Paracercomonadidae. However, *Kraken carinae* still remains *incertae sedis* as ultrastructure revealed the presence of scales, a morphological character predominantly known from the class Imbricatea, contradicting the phylogenetic results.

By combining literature research with phylogenetic examination focusing on *Lecythium* (HERTWIG et LESSER, 1874) and its family the Chlamydophryidae (DE SAEDELEER 1934) we were able to clarify the confusing taxonomy of genera like *Plagiophrys, Lecythium, Rhizaspis* and others. All of these amoebae bear a flexible organic theca, branching and anastomosing filopodia. However, they differ in cell shape and show species (or strain) specific feeding preferences. SSU rDNA phylogenies reflected the phenotypic differences between those genera but also revealed surprising results: The genera *Lecythium* (Novel Clade 4) and *Rhizaspis* (Tectofilosida) were polyphyletic and had to be separated, resulting in a secession of *Fisculla* gen. nov. (Tectofilosida) from *Lecythium* and *Sacciforma* gen. nov. (Cryomonadida) from *Rhizaspis*.

As these thecofilosean amoebae, similar to the predominantly known eukaryvores in the Cercozoa, the Vampyrellida, have been shown to be eukaryvorous, we further focused on eukaryvorous protists in terrestrial habitats, by investigating (a) their feeding preferences (b) their physiological requirements to consume eukaryotic prey and (c) their dispersal in terrestrial and freshwater systems. This was achieved by conducting thorough sampling, observing individuals in their unaltered sample (if possible) and performing experiments on feeding preference, chemical sensing, and enzyme production with several omnivorous or eukaryvorous Cercozoa. In laboratory experiments, we could show that the eukaryvorous protist *Fisculla terrestris* is able to sense and select its preferred prey and produce a battery of enzymes needed to digest cell wall compounds of eukaryotes, such as chitin. *F. terrestris* preferred fungal prey (in particular *Saccharomyces cerevisiae*) and only fed to a small extend on algae. To get more insight into the dispersal of eukaryvorous protists in terrestrial systems, we screened metatranscriptomes of different terrestrial habitats for the eukaryvorous Vampyrellida and Grossglockneriidae, showing high dispersal, since they were present in all screened habitats, with up to 3% of total reads.

The phenotypic, phylogenetic and ecologic data on the investigated cercozoan amoebae resulted in a comprehensive characterization of the Thecofilosea and the novel lineage Krakenidae. Based on intensive literature research and a critical evaluation of it, first steps for a phylogeny-based taxonomy of these cercozoan lineages were made. Finally, this thesis provides an evaluation of the hidden diversity of eukaryvorous Cercozoa in terrestrial and freshwater habitats.

Zusammenfassung

Der Begriff Protist beschreibt einzellige, eukaryotische Organismen die kein Gewebe ausbilden. Mit einer enormen Mannigfaltigkeit in Morphologie und Ökologie repräsentieren sie die Mehrheit der eukaryotischen Diversität, von der bisher nur ein kleiner Teil bekannt ist. Weltweit verbreitet, in marinen, limnischen und terrestrischen Habitaten, besetzen sie unzählige ökologische Nischen, z. B. als Primärproduzenten, Osmotrophen, Bakterivoren, Fungivoren, Algivoren, Omnivoren, Prädatoren oder Parasiten z. B. von Tieren und Pflanzen. Probleme im Kultivieren von Protisten sorgten allerdings für eine bevorzugte Erforschung von Bakterivoren und Algen, was zu einem verzerrten Abbild der Protistendiversität führte. Viele „nicht kultivierbare" Protisten Taxa sind der Forschung noch immer unbekannt.

Deshalb befassten wir uns mit Protisten mit unüblichen Ernährungsweisen (insbesondere bakterivore Lauerjäger und eukaryvore Räuber) in den Cercozoa. Die Cercozoa CAVALIER-SMITH 1998, wurden aufgrund von molekularen Untersuchungen als phylogenetische Entität entdeckt obwohl sie starke Unterschiede in Morphologie und Ökologie aufweisen. Molekulare Untersuchungen deckten eine hohe Diversität in den Cercozoa auf, von der bisher nur ein geringer Bruchteil mit morphologischen Daten verknüpft werden kann. Auf Basis von angelegten Kulturen wurden Zellmorphologie, Ernährungsweise und Zellzyklen von verschiedenen cercozoen Amöben untersucht. Dafür wurden hauptsächlich Lichtmikroskopie und Zeitraffer-Mikrofotographie und auch durch Ultrastrukturaufnahmen Daten erhoben. Genetische Marker, d. h. SSU rDNA und LSU rDNA, wurden sequenziert und für phylogenetische Untersuchungen genutzt, um Rückschlüsse über die Evolution der Cercozoa zu ziehen.

Von einer unbeschriebenen bakterivoren Amöbe wurden sechs Stämme aus deutschen und spanischen Böden isoliert. *Kraken carinae* gen. nov. sp. nov. ist eine Amöbe, unterteilt in einen schuppentragenden Zellkörper (normalerweise <10 µm im Durchmesser) und ein Netzwerk aus Filopodien (bis zu 0,5 mm im Durchmesser). *K. carinae* ist einer der seltenen Lauerjäger der Cercozoa, sie bewegt sich nur selten und erbeutet Bakterien, die mit ihrem Netzwerk aus Filopodien in Kontakt kommen. Anders als andere amöboide Vertreter der Cercozoa, die üblicherweise ihre Filopodien nutzen, um ihre Beute zum Zellkörper zu ziehen und dann zu phagozytieren, ingestiert *K. carinae* Bakterien direkt am Kontaktpunkt der Beute mit den Filopodien und transportiert diese dann intrazellulär zum Zellkörper um sie dort zu verdauen. SSU rDNA Phylogenie zeigte eine nähere Verwandtschaft mit den Cercomonadida auf, wenn auch nur mit mäßiger statistischer Unterstützung. Ein weiterer Anlauf, diesmal mit verketteten SSU rDNA und LSU rDNA Sequenzen, bestätigte die Verwandtschaft zu den Cercomonadida, insbesondere mit den Paracercomonadidae, mit erhöhter (aber dennoch nur moderater) statistischer Unterstützung. Allerdings bleiben die genaue Verwandtschaftsverhältnisse von *Kraken carinae* immernoch unklar, da die Ultrastrukturdaten konträr zu den phylogenetischen Ergebnissen, auf eine nähere Verwandtschaft mit den Imbricatea hinweisen, da *Kraken carinae* genau wie sie Schuppen auf dem Zellkörper trägt. Durch die Kombination von intensiver Literaturrecherche mit phylogenetischen Untersuchungen von *Lecythium* (HERTWIG et LESSER, 1874) und seiner Familie, der Chlamydophryidae (DE SAEDELEER 1934) konnten wir die unklare Taxonomie von Gattungen wie (*Plagiophrys, Lecythium, Rhizaspis,* …) enträtseln. All diese Amöben haben gemeinsam, dass sie eine hyaline flexible Schale tragen und verästelnde und anastomisierende Filopodien aufweisen, aber haben auch klare Unterschiede zueinander. SSU rDNA Phylogenien haben die phänotypischen Unterschiede zwischen den Gattungen

widergespiegelt, bargen aber auch Überraschungen: Die Gattungen *Lecythium* (Novel Clade 4) und *Rhizaspis* (Tectofilosida) waren beide polyphyletisch und mussten daher geteilt werden. Daher wurden *Fisculla* gen. nov. (Tectofilosida) von *Lecythium* und *Sacciforma* gen. nov. (Cryomonadida) von *Rhizaspis* abgetrennt. Weiterhin zeigen diese Schalenamöben, ähnlich wie die meistbekannten Eukaryvoren in den Cercozoa, die Vampyrellida, eukaryvores Verhalten. Deshalb führten wir weitere Studien über die Ökologie von eukaryvoren Protisten in terrestrischen Habitaten durch. Vor allem geben wir Einsicht auf (a) ihre Nahrungspräferenzen, (b) den physiologischen Voraussetzungen, um eukaryotische Beute zu konsumieren und (c) ihrer Verbreitung in terrestrischen und limnischen Systemen. Dazu wurden umfassende Probenahmen, gefolgt von Experimenten durchgeführt, welche Fütterungsversuche und Experimente über die chemische Wahrnehmung und Enzymproduktion mit diversen omnivoren oder eukaryvoren Cercozoa umfassten. Wir konnten zeigen, dass die eukaryvore Amöbe *Fisculla terrestris* fähig ist, die Anwesenheit ihrer bevorzugten Beute (*Saccharomyces cerevisiae*) wahrzunehmen und darauf zu selektieren. Außerdem produzieren sie eine Auswahl an Enzymen zum Abbau der Zellwand der Beute, wie z. B. Chitin. Um mehr Einblick auf die Verbreitung von terrestrischen Eukaryvoren zu erhalten, durchsuchten wir Metatranskriptom-Datenbanken von verschiedenen terrestrischen Habitaten nach den eukaryvoren Vampyrellida und Grossglockneriidae, die auf eine weite Verbreitung von Vampyrellida und Grossglockneriidae hinwiesen, da diese nicht nur in allen überprüften Habitaten nachgewiesen werden konnten, sondern auch bis zu 3% der absoluten Sequenzen ausmachten.

Die phänotypischen und phylogenetischen Daten der untersuchten Amöben der Cercozoa resultieren in einer umfassenden Charakterisierung der Thecofilosea und der neuen Familie Krakenidae. Auf Grundlage einer intensiven Literaturrecherche und einer kritischen Auswertung dieser im Kontext der selbstständig durchgeführten Arbeiten wurden erste Schritte in Richtung einer phylogeniebasierten Taxonomie gemacht. Diese Doktorarbeit umfasst weiterhin eine Diskussion über die noch unerforschte Diversität der Cercozoa in terrestrischen und limnischen Habitaten.

Introduction

Protists

The term protist defines an informal grouping of unicellular eukaryotes that do not form tissues. Protists represent the vast majority of eukaryotic diversity of which only a small fraction is yet known. Despite their estimated 60.000 - 300.000 species (Foissner 2008; Mora et al. 2011) with a tremendous diversity in ecology and morphology, they were traditionally assigned to the same single eukaryotic kingdom, Protista in the Eukaryota, next to plants, animals and fungi (Haeckel 1866; Whittaker 1969). The Protista accommodated therefore organisms of fundamentally different lifestyles, like autotrophic and mixotrophic algae, heterotrophic bacterial grazers and parasites, but also organisms of diverse appearances, like ciliates, flagellates, naked amoebae and testate amoebae. That is why protists were shared as a field of research among zoologists, botanists and mycologists. The most animal-like protists (i.e. heterotrophic protists feeding by means of phagocytosis) were called protozoa (proto= first; zoa= animals) and studied by zoologists, whereas pigmented protists were adopted by the botanists as protophytes (phytes= plants) and fungi-like protists (like the fruiting body forming myxomycetes or the osmotrophic filamentous oomycetes) were studied by mycologists. Researchers were well aware that even morphological highly similar taxa (e.g. the dinoflagellates or euglenids) comprised heterotrophic protists and also photosynthetic algae, but were unable to resolve this issue in a widely accepted taxonomy. Since the 18th and 19th century where protists were most often studied by light microscopy, novel methods were established: In the early 20th century electron microscopical techniques were developed to acquire more detailed morphological data for protist taxonomy, in the late 20th century molecular methods enabled the comparison of genetic markers for phylogeny.

The molecular methods led finally to a widely accepted consensus in protist taxonomy (Adl et al. 2005, Adl et al. 2012, Baldauf 2008). Protists turned out to be paraphyletic, instead of being separated kingdoms, with multicellular eukaryotes nestled in between. Although ciliates show monophyletic origin (Lynn and Sogin 1988; Sogin and Elwood 1986), flagellates, naked and testate amoebae are dispersed all over the eukaryotic tree of life showing paraphyly and polyphyly. For instance, testate amoebae evolved independently in at least three different lineages, the Amoebozoa, Cercozoa and Stramenopiles (Kosakyan et al. 2016;

Nikolaev et al. 2005; Wylezich et al. 2002). The search for the last eukaryotic common ancestor is still ongoing and a highly discussed controversy. Currently it is only accepted that the eukaryotic ancestor was probably a heterotrophic flagellate of unknown affiliation, but concepts differ about the most basal protist taxon (Baldauf 2008; Stechmann & Cavalier-Smith 2002). Cavalier-Smith (1981) established the Archezoa, but this grouping of lineages he believed to be the most basal protists turned out to be an artifact of long branch attraction (Philippe and Germot 2000). Based on rare genetic events like gene fusions and fissions it is assumed that the eukaryotes are divided into two groups, the unikonts (Opisthokonts and Amoebozoa) and bikonts (the remaining eukaryotic diversity). However, still no consensus whether to root the eukaryotes in the unikonts or bikonts could be achieved (Baldauf 2008). Nevertheless, it is widely accepted that during eukaryotic evolution several lineages lost independently their flagella and evolved locomotion by amoeboid movement and/ or gained (or secondarily lost again) autotrophy by endocytobiosis, leading to the intermingled ecology and physiology, sometimes even between closely related protist taxa (Nowack 2014; Rogers et al. 2007; Stechmann & Cavalier-Smith 2002).

Heterotrophic protists in soil biology and their feeding types

Protists, especially in soil ecology, were commonly considered to represent the major bacterial grazers, thereby channeling the carbon flow to higher trophic levels (Bonkowski 2004, Crotty et al. 2011, de Ruiter et al. 1995, Hunt et al. 1987).

However, these assumptions were derived from simple model calculations, based on laboratory experiments with few selected species. Although molecular methods gradually revealed the enormous phenotypic and genetic diversity of heterotrophic protists, models on protist functional roles have not changed in soil biology (Holtkamp et al. 2011, Banašek-Richter et al. 2009). For instance Glücksman et al. (2010) and Weisse et al. (2001) showed that prey (bacteria) communities have been altered by protist grazing in a species-specific manner, but still by far most information on the impact of protist grazing have been obtained in studies either with whole microbial communities in which measured effects can not be traced back to the causing species or in abstract assemblages with just one or few selected organisms. Moreover, such studies were most often conducted only with exclusively bacterivorous protists. The non-bacterivorous protists have been addressed in very few, most often taxonomic studies (Bass et al. 2009; Berney et al. 2013; Foissner 1980; Petz and Foissner 1985). Their ecology and functional importance is often not known.

Heterotrophic feeding in general needs many evolutionary adaptations. The functional response of a heterotrophic protist is basically composed of search time, handling time, ingestion and digestion (Fig. 1). The presumed most widespread and maybe simplest method of preying (eukaryotic and prokaryotic) cells is the process of phagocytosis: this includes the incorporation of the whole prey into a food vacuole, adjusting the milieu to unsuitable conditions (e.g. increased or decreased pH; Laybourn-Parry, 1984) and bombarding this environment with a battery of enzymes degrading various compounds (Bowers and Korn 1973; Laybourn-Parry, 1984). The two most important advantages of this method are a probably low cost for degrading enzymes, as they do not have to be excreted, where they would be highly diluted and once ingested prey (usually) can be digested while the predator already can search for a novel food source.

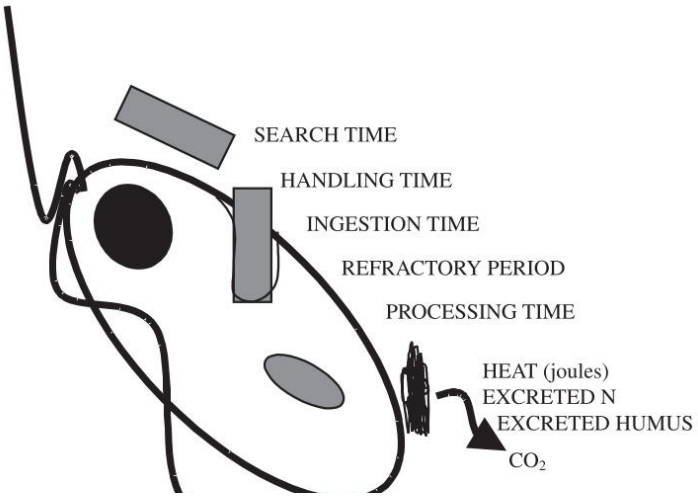

Figure 1: Simplified feeding process of a heterotrophic protist. Extracted from Adl and Gupta 2006

Feeding on eukaryotic cells can be viewed as an additional evolutionary hurdle, as it requires a series of adaptations: First of all (and maybe most important), food size relative to its own is a crucial factor. Many eukaryotic cells have evolved (sometimes inducible) ways to prevent their ingestion by increasing their size by the formation of colonies or filaments (Lampert et al. 1994; Van Donk et al. 2011).

The protection of the cell body by a rigid covering is another common defense strategy that repeatedly evolved. For instance such coverings are e.g. cell walls (usually completely enclosing the cells, composed of chitin or cellulose, being found mainly in green algae), tests (usually with one or two openings, most often built by scales or by agglutinated foreign materials, like sand grains embedded in an organic cement, most prominently known from testate amoebae) or frustules (two rigid, porous, silica-composed and overlapping sections covering the cell, found in diatoms). Those coverings may even (a) bear spines, rods or horns

which may also prevent the ingestion by predators or (b) be enveloped by mucilage which may prevent adhesion of predatory cells or even impede prey recognition (Van Donk et al. 2011).

Next to such morphological defense mechanisms, protists also evolved behavioral defense strategies that might be inducible and aggressor-specific, like (a) the production of chemical compounds, e.g. toxins; (b) flight, often aided by special organelles, like extrusomes or trichocysts, that can be 'fired' to the possible predator; (d) metabolic movement that enables already ingested prey to stretch and burst food vacuoles, like in *Euglena mutabilis* when being consumed by *Actinophrys* sp. or a yet undescribed species in the Leptophryidae (Hausmann 1978; Potin et al. 1999, Van Donk et al. 2011; Hess pers. comm.; own observations unpublished).

Some specialized predators co-evolved strategies to overcome such defense mechanisms. These adaptations are often highly specialized: Large cells, like filaments, even with a thick cell wall might be an easy prey if the predator is adapted by morphology and behavior, e.g. some ciliates (e.g. Grossglockneriidae; Foissner 1980, 1999) are equipped with a feeding tubus for the lysis of fungal cell walls; or some cercozoans (e.g. Vampyrellida; Viridiraptoridae; Hess et al. 2012, Hess & Melkonian 2013) are able to perforate algal or fungal cell walls by extracellular enzymatic degradation. Moreover, trichocysts or extrusomes might also be used by attacking cells (Hausmann 1978).

Research in protistology is astonishing and still full of surprises; we therefore focus in this thesis on heterotrophic protists that show interesting and unusual feeding types.

Culturing heterotrophic protists and its difficulties

In early times of protistology, protists were predominantly studied by direct observations of fresh sampled material. Marine and freshwater samples do not require any preparation thus being intensively studied (Hertwig & Lesser 1874; Penard 1890). Soils however, due to being opaque, can not be observed directly but need preparation like dilution. Diverse methods have been developed, like the most probable number (MPN; Darbyshire et al. 1974) or liquid aliquot method (LAM; Butler and Rogerson 1995), all of which have advantages and disadvantages. Still today, there is no method that enables an objective unbiased view on

soil microorganisms and soil protists are mainly studied by indirect observation of cultured material extracted from soil samples. Despite the abstract nature of such observations, culturing, i.e. the long term maintenance of single species or strains in an artificial and controlled environment, enabled researchers to study complex life cycles, for instance the amoebozoan genus *Pelomyxa* GREEFF 1874, comprises about 20 described species of which the validity is still highly discussed (Frolov et al. 2004, 2005, 2006 and 2011). It is currently not clear whether all described species are just different stages of the life cycle of *Pelomyxa palustris* (the type species of the genus; Chapman-Andresen 1978, 1982), or how many true *Pelomyxa* species exist (2004, 2005, 2006 and 2011). Another example for protists with complex life cycles are the fruiting-body forming amoebae where in many cases the identity of fruiting bodies and trophozoites can not be assigned to each other (Tice et al. 2016). Even in well studied amoebae, like the Acanthamoebidae, novel life history stages (like fruiting-bodies) can be discovered (Tice et al. 2016). Finally, cultures enable the conduction of laboratory experiments, such as an alteration of environmental variables, i.e. biotic or abiotic, enabling conclusions about species autecology, i.e. preferred food sources, necessity of certain chemical compounds in the environment and in general conclusions might be drawn about several biological aspects, such as their physiology and ecology.

For bacteria it is estimated that barely 1% of their diversity can be cultured (see for instance: Ferrer et al. 2009; Lee et al. 2010). Although many (bacterivorous) protist lineages are easily culturable by basically transferring single individuals into water with a carbon source, the vast majority has highly specific requirements (Page 1976). Therefore it would not be surprising to find a similar pattern for protists; and indeed culturing protists differs in the degree of difficulty and is strongly depended of the requirements of the targeted species.

Besides abiotic conditions like medium composition and temperature, it seems obvious that heterotrophic protists need a suitable food source in a sufficient concentration: e.g. fungivorous protists need fungi; bacterivorous protists need bacteria and so on. Culturing bacterivorous protists is often quite easy, often non-toxic strains of bacteria are added or maybe even easier, co-transferred bacteria are fed with solved carbon, the bacteria then grow fast, even under nutrient limited conditions and subsequently can be grazed by the protists.

In contrast to often easily culturable and fast growing bacteria as food source, the culturing of protists that have to be fed with comparatively slow growing and difficult to maintain eukaryotes as food source harbours additional difficulties: At the time of observation of the desired protist predator in a natural sample, the required food source has to be already available in a suitable concentration that does not only enable the growth of the predator but further the sustained growth of the food source. Since eukaryotes are more complex and usually larger than bacteria, they grow slow in comparison to many bacteria and can not be easily stored by drying, cooling or freezing (although research is currently making progress in that field). The amount of cultures maintained by single researchers are therefore very limited, this emphasises the importance of culture collections in protistology. Moreover, many eukaryotes, like algae and fungi (whether as food source or the desired cultured protist itself) produce metabolites that in cultures accumulate and finally may lead to extinction of one or more co-cultured eukaryotes; a frequent subcultivation is therefore necessary, increasing operating expenses (Andersen 2005).

However, according to own experiences, it is difficult to find a suitable food source. If the process of feeding in a natural sample is not observed, which especially in opaque environments like soils is difficult, researchers might not even know what the presumed eukaryvorous predator actually feeds on. Knowledge on protist taxonomy and phylogeny might enable a ‘good guess’ about the required needs of the desired protist, but often those needs are not only lineage specific but furthermore species or strain specific requiring trial and error. For instance there are generalists, like *Amoeba* spp. that feed on bacteria or various eukaryotes (Prescott and James 1955) and specialists, like the Vampyrellida, Viridiraptoridae or the endobiotic phytomyxids. For instance the SSU-sequences of the Vampyrellida reveal, whether the desired protist groups within fungivores, “filamentous algae feeders” or “unicellular algae feeders” clades and enables to draw conclusions about the feeding preferences of the sequenced Vampyrellida species (Hess et al. 2012); the parasitic phytomyxids show co-evolution with their hosts, although host shifts seem to occur (Neuhauser et al. 2014); and the monophagous *Hatena arenicola* feeds not only on a single species of *Nephroselmis* algae but even only on distinct genetic lineages (Okamoto and Inouye 2006; Yamaguchi et al. 2014).

However, long-time maintained cultures enable research on a professional basis and are therefore imperative for taxonomic and autecological research.

Cercozoa

The first discovered protist phylum Cercozoa CAVALIER-SMITH 1998, accommodates morphological and ecological divergent organisms that were found to be closely related based on molecular analyses. Together with the Radiolaria and Foraminifera, both with a more conserved morphology, they constitute the eukaryotic supergroup Rhizaria (Adl et al. 2012; Cavalier-Smith 1998a,b). The Radiolaria accommodate usually floating cells with radiating axopodia and mineral 'skeletons', the Foraminifera accommodate testate amoebae with organic tests and reticulate granofilopodia (Burki et al. 2010).

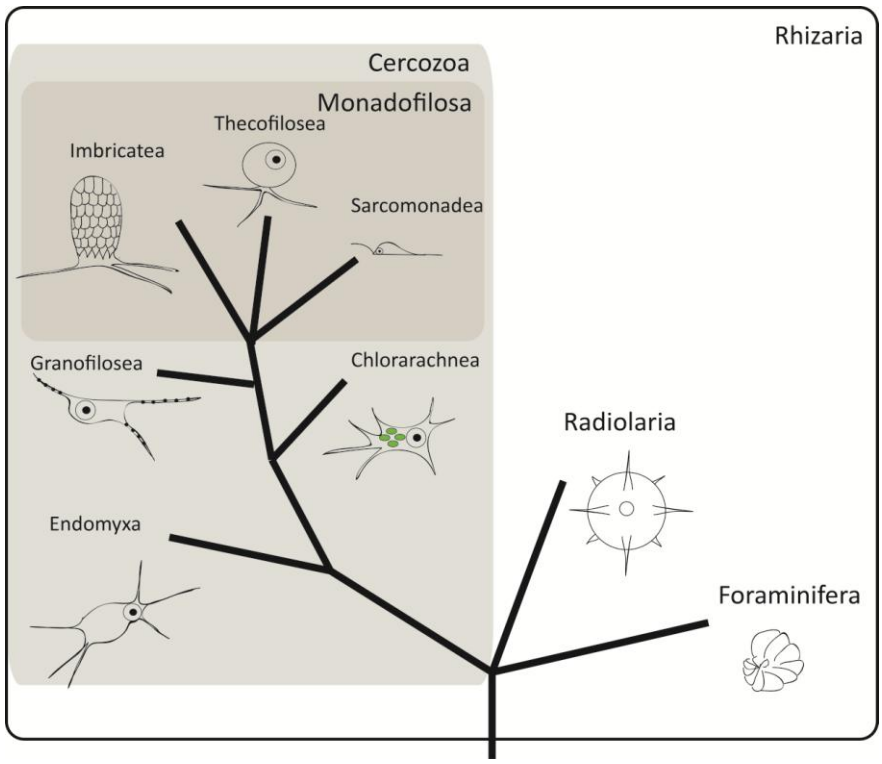

Figure 2: Hypothesized and strongly simpflified sketch of cercozoan evolution (after Cavalier-Smith and Chao 2003).

The divergent Cercozoa consist predominantly of naked amoebae, flagellates and amoeboflagellates (Bass et al. 2009a,b; Hess & Melkonian 2013; Hess et al. 2012). Nestling between those, it comprises several polyphyletic testate amoeba lineages: e.g. the order Euglyphida with tests made of siliceous plates (Cavalier- Smith 1998a, b), the family Rhizaspididae (being here renamed Rhogostomidae) with organic thecae (Howe et al. 2011) and the family Pseudodifflugiidae with tests composed of agglutinated foreign material embedded in an organic cement, the latter branching in the Thecofilosea (Wylezich et al. 2002). Environmental surveys of terrestrial, limnic and marine systems have shown a high genetic diversity of Cercozoa (and particular the little studied Thecofilosea) that can not yet

be connected to morphological and autecological data (Bass and Cavalier-Smith 2004), leading to our first hypothesis that there are still many lineages in Cercozoa to be discovered.

The Thecofilosea were established by Cavalier-Smith and Chao (2003) to accommodate thecate flagellates and amoebae. On a morphological basis it was assumed that the thecate amoeba family Chlamydophryidae might branch as well in the Thecofilosea, but molecular data was lacking (Howe et al. 2011).

The family Chlamydophryidae was established by de Saedeleer (1934) (then as a subfamily of Gromiidae) to accommodate genera like *Chlamydophrys* CIENKOWSKI, 1876 and *Lecythium* HERTWIG ET LESSER, 1874. Species of both genera are all filose amoebae with a hyaline test or theca, but they divide in different ways (Meisterfeld 2002; for a discussion see Chapter 3). The predominantly bacterivorous *Chlamydophrys* could easily be cultured and was therefore well characterized long ago (Belar 1921). In contrast, no *Lecythium* (Hertwig and Lesser 1874) was successfully cultured, leading to sometimes poorly described species and therefore a confusing taxonomy. We propose therefore the second and third hypotheses that *Lecythium*, which has been reported to feed on algae, but being unculturable, can be cultured with the right techniques and further groups on basis of molecular data in the Thecofilosea.

What we know about fungivores and hypothesized evolutional adaptations

A series of studies has been conducted by Chakraborty et al. (1982, 1983, 1985) showing that several omnivorous species of the Amoebozoa are able to feed on spores and yeasts and further are even able to reduce the colonization of root by mycorrhizal fungi, suggesting that fungi-feeding protists significantly affect the rhizosphere system.

Only few specialised fungivorous protists have been described (predominantly the Grossglockneriidae and terrestrial lineages in the Vampyrellida (Cercozoa); Bass et al. 2009; Foissner 1980). Morphology of those lineages is well characterized, but there are only few available SSU sequences and nearly nothing is known about their dispersal and feeding preferences.

The terrestrial Vampyrellida have been repeatedly reported from agricultural fields all over the world (predominantly of Germany, Australia and Japan but also reports of Iran and Canada; Hess et al. 2012; Homma et al. 1979; Homma and Ishii 1984). Data about their true nutrition in the field is scarce. In laboratories the amoebae could be cultured mainly on spores of fungi, predominantly with *Botryotinia fuckeliania, Gaeumunnomyces graminis* and *Cochliobolus sativus* and even on the oomycete *Pythium debaryanum* (Pakzad 2003; Pakzad and Schlösser 1998; Chakraborty and Old 1982; Chakraborty et al. 1983).

Feeding on hyphae, as they are large filaments that can not be completely enveloped by a food vacuole, seems more difficult to achieve. Next to the spore feeding Vampyrellida, some have been reported to feed on fungal hyphae, probably by external degradation as found in filamentous algae feeding Vampyrellida; Hess et al. 2012; Homma et al. 1979; Homma and Ishii 1984). The Grossglockneriidae is a family of ciliates with several described genera (*Grossglockneria, Mykophagophrys* and *Pseudoplatyophyra*) with specific oral structures enabling them to feed on filament forming fungi (Foissner 1980; Lynn et al. 1999; Dunthorn et al. 2008). They have been repeatedly studied from Austrian forest litters, out of twelve tested fungi *Grossglockneria* only fed on three: *Aspergillus* sp., *Mucor mucedo* and a not further determined Mucoraceae sp. (Foissner 1980; Petz et al. 1985). The latter two representing typical “sugar fungi”, those fast growing fungi are usually the first to colonize novel food sources and as a trade-off produce only few secondary metabolites (Frankland 1998; Torres et al. 2005).

Nevertheless, in comparison to the very few studies focussing on fungivores, there are more studies, old and recent, targeting especially algivorous protist lineages. The question arises why algivores are more intensely studied than fungivores.

There are two possible explanations: [1] fungivores are just rare, less abundant and less diverse than e.g. algivores; [2] soil hampers work with protists and prevents direct observation, thus fungivores are just not recognized. Resulting in the forth of our hypotheses: Protist functions are understudied in terrestrial systems; especially the functional roles of fungivores have been underestimated.

The few studied terrestrial relatives of algivorous protists most often show fungivorous feeding habits. It is therefore likely, that the shift from being a freshwater algivorous protist

to a terrestrial fungivorous protist (and maybe vice versa) is accompanied by few and/ or simple adaptations.

The shift from freshwater to terrestrial (soil) habitats might include the following adaptions:

(1) Since soils are often referred to as harsh environments with a huge variability in moisture and therefore salinity, protists should be able to cope with droughts. The ability to form cysts (a life history stage that usually is defined as a partial dehydration and the secretion of a more or less rigid cell envelope, usually composed of cellulose, chitin, proteins or a combination of those) is necessary in terrestrial protists as it enables the survival in dry periods or heatwaves that occur frequently (see Adl and Gupta 2006).

The development of tests was seen as an additional adaptation to dry conditions, since the test might reduce water loss and therefore represent an advantage to dry environments. Accordingly a smaller test opening should be of advantage in drier environments. Schönborn (1992) and Bobrov and Mazei (2004) have shown that the size of the opening of the test varies and can be adapted to drought within few generations. Series of physiological changes are likely, including an adaptation to rapid changing osmotic pressure and very different chemical compositions of the environment;

(2) As space is limited in soils many soil protists show a reduction in size compared to their freshwater relatives. For instance, *Parvularia* is smaller than its closest freshwater relative *Nuclearia* (Dirren et al. 2014; Torruella pers. comm.), terrestrial Glissomonads are smaller than the freshwater inhabiting Viridiraptoridae (Hess and Melkonian 2013). Behavioural adaptations, especially in locomotion, might be of advantage when changing to a space limited environment like soils. For instance the exclusively limnic *Vampyrella* predominantly float, whereas the closely related *Theratromyxa* and *Platyreta* mainly creep (Bass et al. 2009, Hess et al. 2012), similar evolutionary process seem to be found in *Nuclearia* and the closely related *Parvularia* although there is not much data yet (Dirren et al. 2014; Dirren and Posch 2016; Torruella pers. comm.), the described Viridiraptoridae show the ability to swim, a behaviour not known from the terrestrial glissomonads but one (i.e. *Proleptomonas faecicola,* see Hess & Melkonian 2013).

(3) Since most soil systems harbour less algae than fungi (Damon et al. 2012, Cutler et al. 2013), algivorous protists might take advantage of novel food sources like fungi (i.e. becoming fungivorous), this should lead to an alteration of the set of digestive enzymes, since fungi mainly build their cell walls from chitin, in contrast to (green-) algae that use mainly cellulose, it is likely that the composition of the enzyme cocktail needed to degrade cell walls changes either in concentrations of compounds, by evolving novel enzymes or by obtaining enzymes by lateral gene transfer from the prey itself (Adams 2004; Popper et al. 2011; Xu et al. 2016).

Accordingly we hypothesize that life in terrestrial habitats requires special adaptations in ‘aquatic‘ protists, and we may detect adaptations of terrestrial protists, for example in the predominantly algivorous Tectofilosida.

Aims

The central aim of this thesis was to give insight into eukaryvorous protists, in particular Cercozoa, in freshwater and terrestrial systems, in order to contribute to a deepened understanding of cercozoan evolution. We cultured, investigated and characterized selected 'unculturable' protists of the phylum Cercozoa in terms of morphology, behaviour and phylogeny.

The following hypotheses were proposed:

1. Many still unknown lineages exist in the Cercozoa.

2. The algivorous genus *Lecythium* groups in the Thecofilosea.

3. Significant culturing efforts are needed to link protist morphotypes and functions to environmental sequences.

4. Protist functions are understudied in terrestrial systems; especially the functional roles of fungivores have been underestimated.

5. Life in terrestrial habitats requires special adaptations in 'aquatic' protists, and we may detect adaptations in terrestrial protists that are lacking in closely related freshwater inhabitants, for example in the predominantly algivorous Tectofilosida.

Chapters in three parts

Part 1 - Characterization of the unusual bacterivorous amoeba *Kraken carinae*.

Chapter 1: A Novel Lineage of 'Naked Filose Amoebae'; *Kraken carinae* gen. nov. sp. nov. (Cercozoa) with a Remarkable Locomotion by Disassembly of its Cell Body.

Chapter 2: Cercomonad or archaic Imbricatea? On the hunt for the true taxonomy of the scale-bearing *Kraken* (*incertae sedis*, Cercozoa, Rhizaria): Combining ultrastructure data and a two-gene (SSU + LSU) phylogeny.

Part 2 - Eukaryvorous amoebae of the Thecofilosea, Cercozoa.

Chapter 3: Description of *Lecythium terrestris* sp. nov. (Chlamydophryidae, Cercozoa), a Soil Dwelling Protist Feeding on Fungi and Algae.

Chapter 4: A bowl with marbles: Revision of the thecate amoeba genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa, Rhizaria) including a description of four new species and an identification key.

Chapter 5: Shedding light on the polyphyletic genus *Plagiophrys*: The transition of some of its species to *Rhizaspis* (Tectofilosida, Thecofilosea, Cercozoa) and the establishment of *Sacciforma* gen. nov. (Cryomonadida, Thecofilosea, Cercozoa).

Chapter 6: Polyphyly in the thecate amoeba genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa), redescription of its type species *L. hyalinum*, description of *L. jennyae* sp. nov. and the establishment of *Fisculla* gen. nov. and Fiscullidae fam. nov.

Part 3 - Eukaryvorous protists, their capabilities and dispersal.

Chapter 7: What does it take to eat a fungus? A case study with the eukaryvorous amoeba *Fisculla terrestris*.

Chapter 8: The soil food web revisited: Diverse and widespread mycophagous soil protists.

Results

Part 1 - Characterization of the unusual bacterivorous amoeba *Kraken carinae*.

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Protist, Vol. 167, 268–278, June 2016
http://www.elsevier.de/protis
Published online date 28 April 2016

Protist

ORIGINAL PAPER

A Novel Lineage of 'Naked Filose Amoebae'; *Kraken carinae* gen. nov. sp. nov. (Cercozoa) with a Remarkable Locomotion by Disassembly of its Cell Body

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Submitted November 11, 2015; Accepted April 16, 2016
Monitoring Editor: Alastair Simpson

The term 'filose amoebae' describes a highly polyphyletic assemblage of protists whose phylogenetic placement can be unpredictable based on gross morphology alone. We isolated six filose amoebae from soils of two European countries and describe a new genus and species of naked filose amoebae, *Kraken carinae* gen. nov. sp. nov. We provide a morphological description based on light microscopy and small subunit rRNA gene sequences (SSU rDNA). In culture, *Kraken carinae* strains were very slow-moving and preyed on bacteria using a network of filopodia. Phylogenetic analyses of SSU sequences reveal that *Kraken* are core (filosan) Cercozoa, branching weakly at the base of the cercomonad radiation, most closely related to *Paracercomonas*, *Metabolomonas*, and *Brevimastigomonas*. Some *Kraken* sequences are >99% similar to an environmental sequence obtained from a freshwater lake in Antarctica, indicating that *Kraken* is not exclusively soil dwelling, but also inhabits freshwater habitats.

Key words: *Kraken*; Cercozoa; Filosa; protist; soil; cercomonads.

Introduction

Protists, especially small bacterivorous amoebae, are difficult to distinguish due to few morphological characters. Therefore many amoebae (or amoeboflagellates) have been lumped into morphotypes or morphospecies which often comprise high cryptic diversity, and in some cases morphologically defined taxa have been shown to be polyphyletic or paraphyletic (Bass et al. 2009b; Smirnov and Brown 2004; Smirnov 2011; Wylezich et al. 2002). Despite convergent morphological traits; the ecology of protists can differ enormously

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http://dx.doi.org/10.1016/j.protis.2016.04.002

(Poulíčková et al. 2008). Therefore it is of high interest to phylogenetically resolve amoeboid morphotypes as accurately as possible.

The informal term 'naked filose amoebae' has been used by protistologists for more than 150 years enabling a quick categorization of amoebae (Cienkowski 1865). Naked filose amoebae were placed in various taxonomic groups, such as Proteomyxidea (Lankester 1890) or Reticulosa (Cash and Hopkinson 1905; Rhumbler 1904). Molecular phylogenies have shown some such groups to be polyphyletic: many filose amoebae are now known to branch in various places in cercozoan phylogenies, but others are distributed across the eukaryotic tree of life, in the stramenopiles, opisthokonts, excavates, and Amoebozoa (Adl et al. 2012; Amaral Zettler et al. 2001; Bass et al. 2009a; Berney et al. 2015; Cavalier-Smith 1998a, b).

The Cercozoa were established as a phylum within the last 20 years (Cavalier-Smith 1998a, b; Cavalier-Smith and Chao 2003). Environmental DNA surveys of terrestrial, limnic and marine systems have shown a high hidden diversity of Cercozoa that needs to be described by morphological and ecological means (Bass and Cavalier-Smith 2004). Since then, many studies obtained molecular data of already described or previously unknown Cercozoa, showing that cercozoans are highly diverse in morphology and ecology, comprising e.g. bacterivorous flagellates, algivorous and mycophagous amoebae as well as endophytic biotrophs (e.g. Bass et al. 2009a, b; Dumack et al. 2016; Howe et al. 2009, 2011; Neuhauser et al. 2014).

Further species descriptions and taxonomic approaches combined with morphological as well as molecular data showed that even within Cercozoa, 'naked filose amoebae' are of polyphyletic origin. Granofilosean amoebae such as *Limnofila* have previously been misidentified as other taxa (*Gymnophrys* Cienkowski, 1876, *Biomyxa* Leidy, 1875) that most likely belong to Endomyxa or Retaria, the endomyxan *Filoreta* Cavalier-Smith and Bass, 2009 has been confused with the amoebozoan *Corallomyxa* Grell, 1966 (Bass et al. 2009a), and some vampyrellids (Hess et al. 2012; Berney et al. 2013) strongly recall variosean Amoebozoa, at least in still photographs (Berney et al. 2015). This problem is compounded by the difficulty of representing such variable forms in illustrations, leading to redundancy in taxon descriptions. An overlooked example of this is *Penardia*, (Cash 1904) as a probable synonym of *Chlamydomyxa* (Archer 1875), Cash's illustration of *Penardia* being the typical trophozoite of *Chlamydomyxa* (Eckhard Völcker pers. comm.). Recent studies conclusively show that intensive light microscopy combined with well-sampled molecular phylogenies are essential for accurate and robust species descriptions to enable an enduring protistan taxonomy, particularly for morphologically elusive lineages such as those under the umbrella of 'naked filose amoebae'.

In this study we describe *Kraken* gen. nov., a novel filosan cercozoan with possible phylogenetic affilities to the gliding biflagellate bacterivorous cercomonads (Cercomonadidae). We provide detailed description of six cultured strains, comprising four genotypes, and describe a new genus and species by differential interference contrast (DIC) high definition video microscopy and SSU rDNA phylogeny.

Results

Sampling and Abundance

Kraken cells were isolated from 6/129 screened soil samples (Table 1). In each of these six samples independent of sampling site, they were present at an abundance of 350–400 Individuals $\times$ g^{-1} dry weight soil as determined by the Liquid Aliquot Method (LAM).

Morphology

Trophozoite: The cell bodies of *Kraken carinae* (Fig. 1, Supplementary Material 2) were roundish with a length/width ratio of 1.0 ± 0.0 ($n = 41$). The diameter of 5 of the 6 obtained isolates was 7.75 ± 0.9 μm ($n = 41$) whereas clonal cells of one isolate (KD0248) were slightly larger: 8.75 ± 0.9 μm ($n = 43$, $F = 29.16$, $p < 0.001$). The cell body usually contained one nucleus, rarely two nuclei, with a single round nucleolus in the middle, a contractile vacuole, one large food vacuole and several small granules (Fig. 1 B, D). The nucleus diameter was approximately 3.4 ± 0.2 μm. The diameter of the nucleolus was about 1.6 ± 0.2 μm. A cell covering was not observed. Each cell usually formed one long, thin and narrowing filopodium that branched and anastomosed (Fig. 1 A, C). The filopodium originated from a localised point usually located at the basal end of the cell body (Supplementary Material 2). The filopodium usually branched close to this point multiple times and expanded in all directions up to a length of 200–300 μm, so the whole diameter of an active cell may be more than 500 μm wide. Usually only individuals cultured for several weeks without disturbance showed anastomosing, highly branching filopodia. When mechanically disturbed, *Kraken* individuals tended

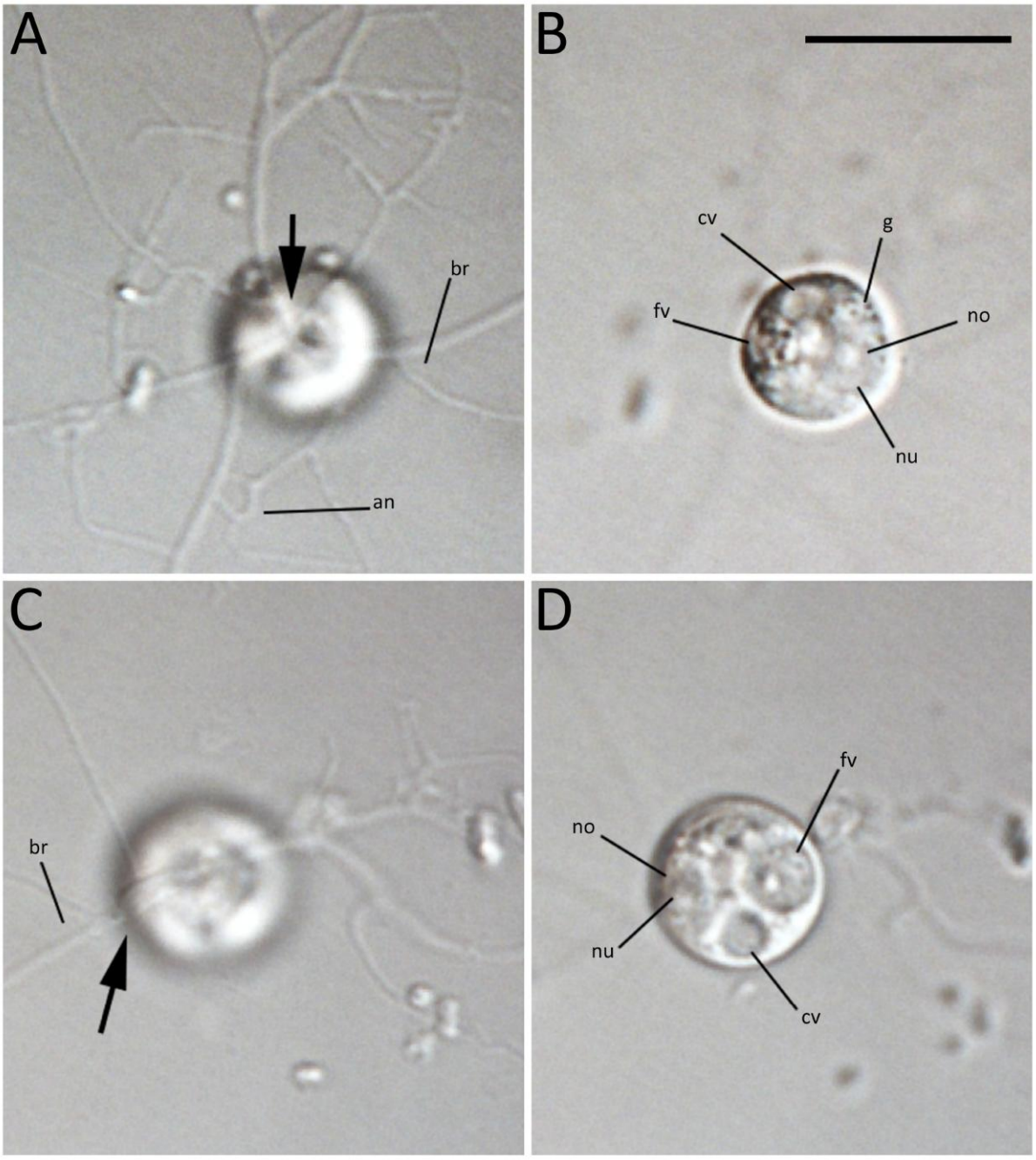


Figure 1. Cellular features of *Kraken carinae* (KJ0002) presented by two different individuals (**A**+**B**; **C**+**D**) by light micrographs (DIC). The filopodium might branch (br) or anastomose (an). The origin of the filopodium is usually located under the cell body and branches directly at least once (indicated by arrows). Cells contain one contractile vacuole (cv), one food vacuole with several particles (fv) and one round nucleus (nu) with one roundish nucleolus (no). Granules (g) are often observed but their number differs tremendously. For a three dimensional animation showing a ring-like structure around the base of the filopodium of the individual A+B see Supplementary Material 2. Measure bar indicates 10 μm.

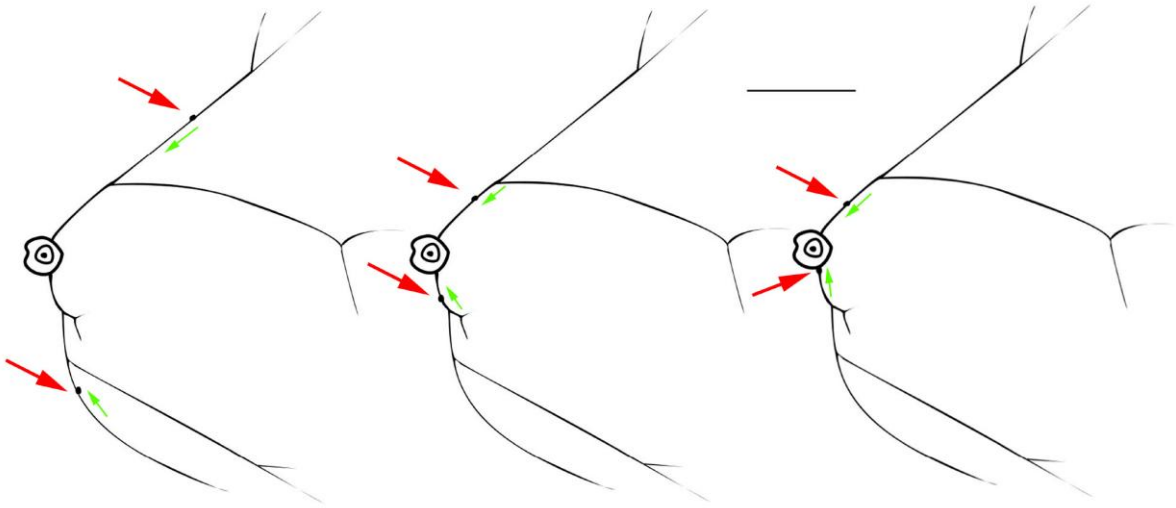


Figure 2. *Kraken carinae* (KD0092) with caught bacteria that are transported from the cell periphery to the cell body. For an animation of that process see Supplementary Material 1. Measure bar indicates 20 μm; red arrows indicate the position of the round structures; green arrows indicate movement direction.

to withdraw their filopodium to the cell body and stay inactive for several hours. Bacteria and masses of cell plasma (~1 μm diameter) could be observed gliding from the cell periphery through the filopodia to the cell body (Fig. 2). These masses occasionally moved for a short phase in the opposite direction. Movement: Cells were usually attached to the Petri dish surface and seemed highly immotile. The anastomosing filopodium formed a network that is used to slowly explore its surroundings. An active movement of the filopodia could only be seen in time-lapse films (Supplementary Material Video 1). The cell body only rarely moved. Different types of locomotion were noted: 1) a deeply branched filopodium gripped the substrate and carried the cell body above the surface with swaying movements of the branches similar to the locomotion of e.g. *Euglypha*, 2) by moving the cell body along the filopodium without distortion or collapse of cell body structure, so that the cell body changed position while the filopodium remained in its position (Supplementary Material Video 1), 3) the most striking locomotive form is characterized by a disassembly of the cell body, that is then transported in one or more masses of cell material through a filopodium and reassembled at another position, similar to *Bigelowiella longifila*, a chlorarachniophyte (Ota et al. 2007; Fig. 4; Supplementary Material Video 1). Floating, actively swimming cells, flagellated stages, or other cell forms could not be observed. Dormant stage: Cysts were quite frequent in cultures after 4–6 weeks' growth, but were never observed in cultures of shorter growth time (Fig. 3, Supplementary material 3). Cysts contained granular structures that sometimes formed a spherical structure inside the cyst wall (Fig. 3 B, D). Cell plasma sometimes filled the cyst completely. In culture most cysts were usually overgrown with bacteria. Division: Cells usually divided longitudinally by binary fission. Serial divisions were seen (Supplementary Material Video 1).

Phylogenetic Analyses

Nearly complete SSU rDNA sequences (Table 1) of the six cultured strains were determined and used for phylogenetic analyses (Fig. 5). Four different SSU rDNA genotypes were detected that differed in up to 11 nucleotides across the obtained ~1680 bp SSU fragments. The SSU rDNA sequences of *Kraken carinae* contain no intron.

The phylogenetic tree includes the two cercozoan subphyla – Filosa and Endomyxa. All six newly generated *Kraken* sequences form a maximally-supported clade in both Maximum Likelihood and Bayesian analyses together with one environmental sequence (AB695519) obtained by Nakai et al. (2012) from environmental sequencing of the eukaryotic community in moss of a freshwater lake in Antarctica. The phylogenetic position of *Kraken* within Filosa is unresolved, but it branches at the base of the cercomonad radiation in both ML and Bayesian phylogenies, sister (but with very low support)

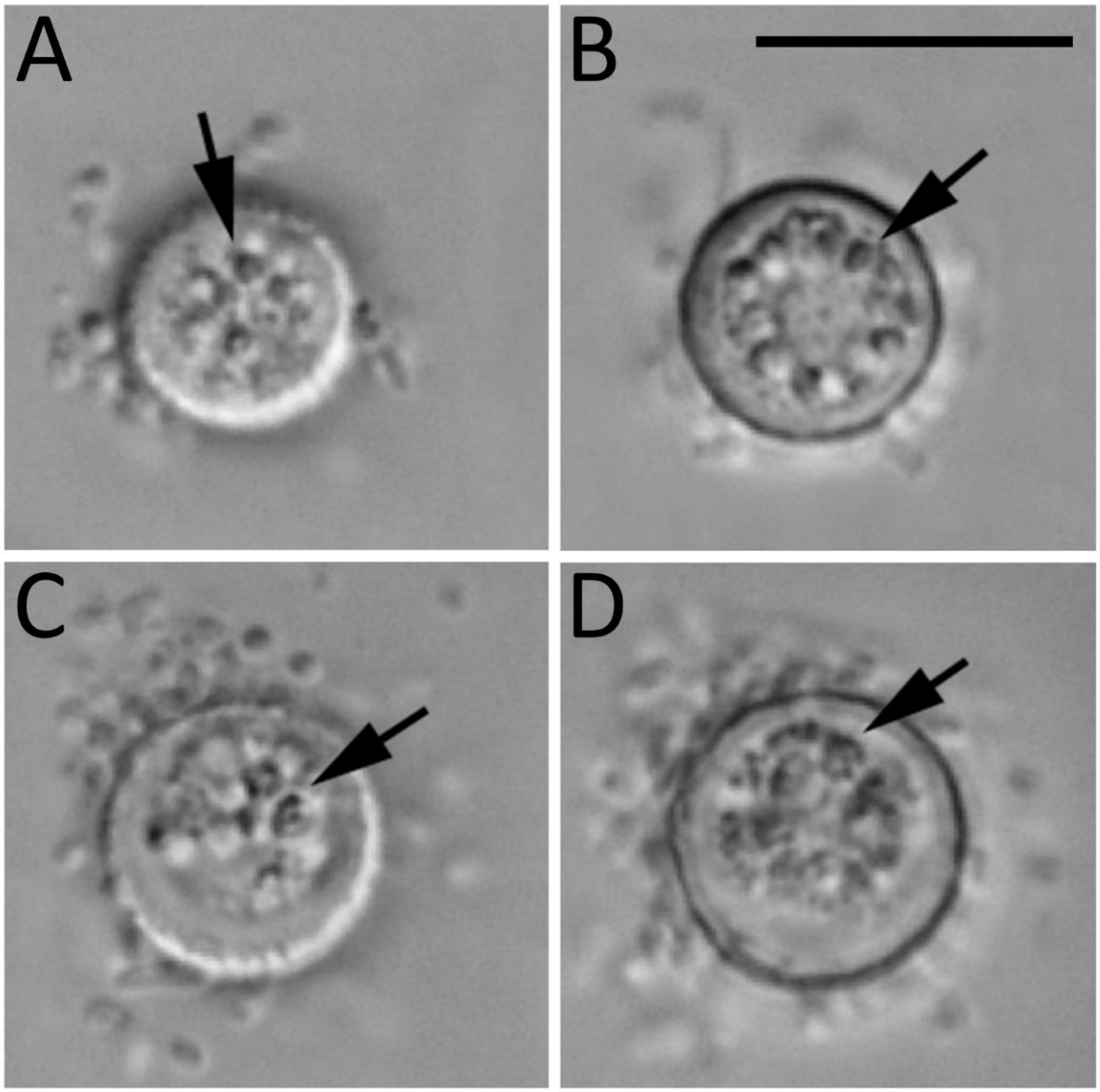


Figure 3. Cellular features of *Kraken carinae* (KJ0002) in its dormant stage (cyst) provided on two different individuals (**A**+**B**; **C**+**D**) by light micrographs (DIC). Cysts contain granular structures (some indicated by arrows) that may or may not form a spherical structure inside the cyst wall. Cell plasma may (**A**+**B**) or may not (**C**+**D**) fill the cyst completely. Cells usually are overgrown with bacteria. For a animation of the individual **A**+**B** see Supplementary Material 3. Measure bar indicates 10 μm.

to the maximally supported *Paracercomonas, Brevimastigomonas* and *Metabolomonas* clade 0.6 Bayesian PP/29% ML bootstrap.

Kraken Dumack, Schuster, Bass et Bonkowski, gen. nov.

Diagnosis: Very slow moving filose amoeba. Cell body roundish in shape. Usually a single highly branched filopodium originating between the cell body and the substrate through a ring-like structure sometimes visible by light microscopy. The filopodium branches and anastomoses, forming a network. Division longitudinal. Bacterivorous, prey is transported through the filopodium to the cell body. Cells contain one, rarely two nuclei with one

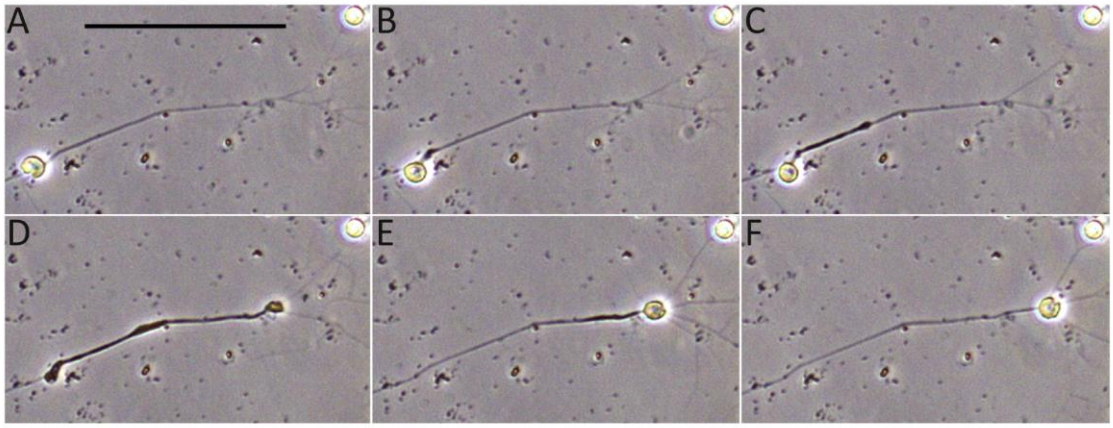


Figure 4. Movement of *Kraken carinae* exemplified on strain KD0248. (**A**) The cell body is round in shape, the filopodium elongated and highly branched in the cell periphery. (**B**) The cell body disassembles. (**C**+**D**) The cell body is transported in one mass (sometimes more masses) of cell material to its new position in the cell periphery. (**E**) The cell body reassembles. (**F**) The cell withdraws its posterior filopodium. The whole process of cell body transport took 45 minutes in this example. A video of the process is available as Supplementary Material Video 1, an animation as Supplementary Material 4. Measure bar indicates 100 μm.

round nucleolus, one contractile vacuole, and usually one food vacuole.

Etymology: krakōnan, n [Proto-Germanic] similar words present in several old nordic languages, Kraken refers to the monster Hafgufa (the Kraken) in norse mythology that catches its prey (ships, men, whales and "everything else") with its plentiful arms. Like the Kraken of the legend our isolates are characterized by a huge network of filopodia preying on bacteria that are then transported to the cell body for digestion.

Type species: *Kraken carinae*

Kraken carinae Dumack, Schuster, Bass et Bonkowski, sp. nov.

Diagnosis: *Kraken* as defined above. Cell body (longest axis) 5.5–11.5 μm.

Type material (hapantotype): A glass slide containing several fixed individuals is deposited as Inv. Nr. 2015/27 in the Upper Austrian State Museum Invertebrate Collection

Type-generating strain: KJ0002; deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ) under the accession number 244502210-KA

Sequence of type-generating strain (SSU rDNA): KP940376 Cercozoa sp. KD-2015b isolate KJ0002

Type location: Surface soil, agricultural field in northern Germany: N53°22′22.8″ E13°48′01.6″

Etymology: carina [Latin], noun = ship, nutshell; referring to the prey of the mythical Kraken. Additionally, this species is dedicated to Carina Platten in recognition of her support and encouragement.

Discussion

Taxonomic Placement of *Kraken* gen. nov. and Discussion of 'Naked Filose Amoebae' Phylogeny

Although naked filose amoebae have been studied for over 150 years their taxonomy remains poorly understood. By isolation and phylogenetic analyses of six strains of 'naked filose amoebae' we were able to establish a new genus in the phylum Cercozoa. Although its exact phylogenetic position within the Filosa is not strongly supported with SSU phylogeny, *Kraken* gen. nov. groups robustly within the subphylum Filosa, with weakly supported affinity to the Cercomonadida. Morphologically and phylogenetically, *Kraken* is consistent with other known Filosa, which comprise many filose testate amoebae or amoeboflagellates with "true filopodia", i.e. filopodia that may be used for locomotion (Albrecht-Buehler 1976) as it is shown for *Kraken* gen. nov.

Our observations of the cells do not specifically support an affinity with cercomonads, although the dimensions and general appearance of the cell is not inconsistent with the diversity of body forms within *Paracercomonas, Brevimastigomonas* and *Metabolomonas*. However, all other known

Table 1. Strains of *Kraken* spp. and corresponding data. Species isolated and cultured in this study are presented in bold. Type species for the genus and type strain for the species are underlined. The environmental clone MPE2-25 was obtained by Nakai et al. 2012.

Species	Strain	SSU rDNA accession	Culture accession	Sequence length (nt)	Country of origin	Coordinates of origin	Date of Isolation	Habitat
Kraken carinae	KD0248	KP940373	244501215-KA	1686	Spain	N40°20′1.95″ W3°53′0.02	May 2014	surface soil; agricultural; very dry; global change experiment
	KD0071	KP940375	244501930-KA	1699				
	KD0092	KP940374	244502023-KA	1640				
	KJ0001	KP940377	244502117-KA	1686	Germany	N53°22′22.8″ E13°48′01.6″	Aug 2014	surface soil; agricultural; close crop rotation wheat and corn
	KJ0002	KP940376	244502210-KA	1693				
	KJ0003	KP940378	244502341-KA	1695				
Kraken sp.	MPE2-25	AB695519	-	1722	Antarctica	S69°28′37″ E39°34′00″	-	freshwater lake, moss pillars

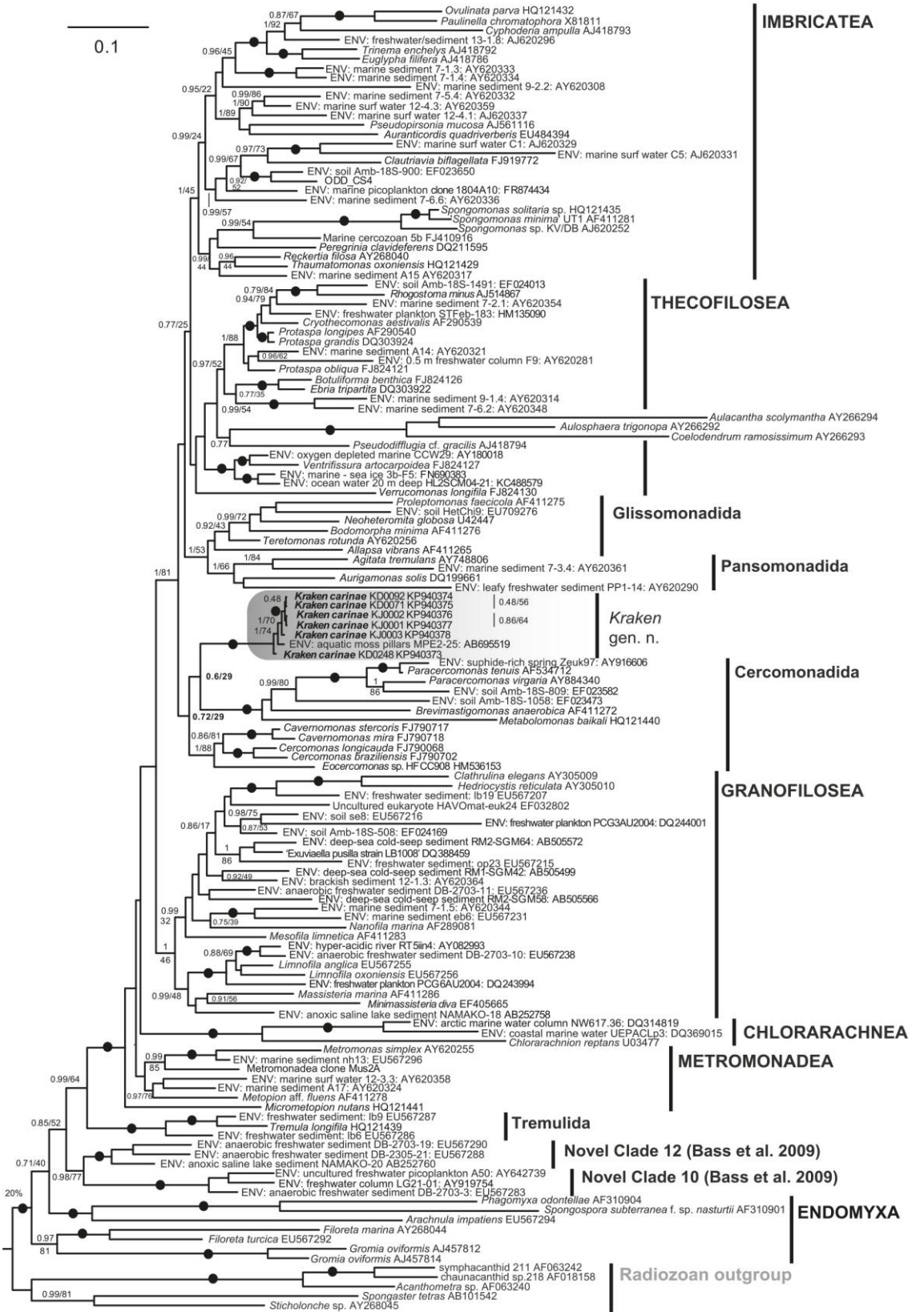
cercomonads are flagellate, whereas *Kraken* is not, and most have more metabolic and motile cells. The lack of granules in the filopodia of *Kraken* are consistent with a cercomonad than granofilosean relationship (Bass et al. 2009a, b).

Nevertheless morphological similarities might also been found in comparison to other filosan clades. Although we did not find evidence of a test surrounding the *Kraken* cells, it is possible that electron microscopical studies may reveal a thin, organic test. On first sight other filosan amoebae show morphological similarities to *Kraken carinae*, e.g. the testate amoebae *Rhogostoma spp*. in the Rhizaspididae (Howe et al. 2011) and small species within the Chlamydophryidae, like the rather small *Lecythium terrestris* (Dumack et al. 2016). The shape of the aperture has been shown to be a reliable character of the distinction of thecofilosean clades (Howe et al. 2011). The point of filopodia origin of *Kraken spp*. is round (Supplementary Material 2) and therefore more similar to the Chlamydophryidae, and not crack-like, as seen in Rhizaspididae.

To resolve the phylogenetic position of *Kraken* within Filosa and to investigate the relationship of the slightly smaller strain KD0248 to the other strains multi-gene analyses are required (e.g., initially 28S rDNA). KD0248 may turn out to be a separate species of *Kraken*, but more isolates of this lineage are required to statistically support any morphological distinction from *K. carinae*. The discovery of *Kraken* underlines the fact that highly distinct lineages (morphologically and phylogenetically) remain to be discovered even within groups such as Cercozoa and sarcomonads in particular that have been relatively well studied both with respect to environmental sequencing and culture-based surveys (Bass et al. 2009b; Brabender et al. 2012; Harder et al. 2016; Howe et al. 2009). Also noteworthy is that only one other *Kraken* sequence was found from a blastn-search of GenBank, demonstrating that culture-based studies remain crucial for discovery of novel protist lineages, and may reveal novel lineages within clades that are otherwise well represented by environmental sequences (e.g. Reticulamoeba; Bass et al. 2012).

Dispersal, Abundance and the Difficulty of Detecting *Kraken*

The liquid aliquot method (LAM) revealed the presence of *Kraken carinae* in only 6/125 screened samples. With 350–400 Individuals $\times$ g^{-1} dry weight we consider *Kraken carinae* to be rare in


0.1
IMBRICATEA
THECOFILOSEA
Glissomonadida
Pansomonadida
Kraken gen. n.
Cercomonadida
GRANOFILOSEA
CHLORARACHNEA
METROMONADEA
Tremulida
Novel Clade 12 (Bass et al. 2009)
Novel Clade 10 (Bass et al. 2009)
ENDOMYXA
Radiozoan outgroup
Ovulinata parva HQ121432
Paulinella chromatophora X81811
Cyphoderia ampulla AJ418793
ENV: freshwater/sediment 13-1.8: AJ620296
Trinema enchelys AJ418792
Euglypha filifera AJ418786
ENV: marine sediment 7-1.3: AY620333
ENV: marine sediment 7-1.4: AY620334
ENV: marine sediment 9-2.2: AY620308
ENV: marine sediment 7-5.4: AY620332
ENV: marine surf water 12-4.3: AY620359
ENV: marine surf water 12-4.1: AJ620337
Pseudopirsonia mucosa AJ561116
Auranticordis quadriverberis EU484394
ENV: marine surf water C1: AJ620329
ENV: marine surf water C5: AJ620331
Clautriavia biflagellata FJ919772
ENV: soil Amb-18S-900: EF023650
ODD_CS4
ENV: marine picoplankton clone 1804A10: FR874434
ENV: marine sediment 7-6.6: AY620336
Spongomonas solitaria sp. HQ121435
'Spongomonas minima' UT1 AF411281
Spongomonas sp. KV/DB AJ620252
Marine cercozoan 5b FJ410916
Peregrinia clavideferens DQ211595
Reckertia filosa AY268040
Thaumatomonas oxoniensis HQ121429
ENV: marine sediment A15 AY620317
ENV: soil Amb-18S-1491: EF024013
Rhogostoma minus AJ514867
ENV: marine sediment 7-2.1: AY620354
ENV: freshwater plankton STFeb-183: HM135090
Cryothecomonas aestivalis AF290539
Protaspa longipes AF290540
Protaspa grandis DQ303924
ENV: marine sediment A14: AY620321
ENV: 0.5 m freshwater column F9: AY620281
Protaspa obliqua FJ824121
Botuliforma benthica FJ824126
Ebria tripartita DQ303922
ENV: marine sediment 9-1.4: AY620314
ENV: marine sediment 7-6.2: AY620348
Aulacantha scolymantha AY266294
Aulosphaera trigonopa AY266292
Coelodendrum ramosissimum AY266293
Pseudodifflugia cf. gracilis AJ418794
ENV: oxygen depleted marine CCW29: AY180018
Ventrifissura artocarpoidea FJ824127
ENV: marine - sea ice 3b-F5: FN690383
ENV: ocean water 20 m deep HL2SCM04-21: KC488579
Verrucomonas longifila FJ824130
Proleptomonas faecicola AF411275
ENV: soil HetChi9: EU709276
Neoheteromita globosa U42447
Bodomorpha minima AF411276
Teretomonas rotunda AY620256
Allapsa vibrans AF411265
Agitata tremulans AY748806
ENV: marine sediment 7-3.4: AY620361
Aurigamonas solis DQ199661
ENV: leafy freshwater sediment PP1-14: AY620290
Kraken carinae KD0092 KP940374
Kraken carinae KD0071 KP940375
Kraken carinae KJ0002 KP940376
Kraken carinae KJ0001 KP940377
Kraken carinae KJ0003 KP940378
ENV: aquatic moss pillars MPE2-25: AB695519
Kraken carinae KD0248 KP940373
ENV: suphide-rich spring Zeuk97: AY916606
Paracercomonas tenuis AF534712
Paracercomonas virgaria AY884340
ENV: soil Amb-18S-809: EF023582
ENV: soil Amb-18S-1058: EF023473
Brevimastigomonas anaerobica AF411272
Metabolomonas baikali HQ121440
Cavernomonas stercoris FJ790717
Cavernomonas mira FJ790718
Cercomonas longicauda FJ790068
Cercomonas brazilliensis FJ790702
Eocercomonas sp. HFCC908 HM536153
Clathrulina elegans AY305009
Hedriocystis reticulata AY305010
ENV: freshwater sediment: lb19 EU567207
Uncultured eukaryote HAVOmat-euk24 EF032802
ENV: soil se8: EU567216
ENV: freshwater plankton PCG3AU2004: DQ244001
ENV: soil Amb-18S-508: EF024169
ENV: deep-sea cold-seep sediment RM2-SGM64: AB505572
'Exuviaella pusilla strain LB1008' DQ388459
ENV: freshwater sediment: op23 EU567215
ENV: deep-sea cold-seep sediment RM1-SGM42: AB505499
ENV: brackish sediment 12-1.3: AY620364
ENV: anaerobic freshwater sediment DB-2703-11: EU567236
ENV: deep-sea cold-seep sediment RM2-SGM58: AB505566
ENV: marine sediment 7-1.5: AY620344
ENV: marine sediment eb6: EU567231
Nanofila marina AF289081
Mesofila limnetica AF411283
ENV: hyper-acidic river RT5iin4: AY082993
ENV: anaerobic freshwater sediment DB-2703-10: EU567238
Limnofila anglica EU567255
Limnofila oxoniensis EU567256
ENV: freshwater plankton PCG6AU2004: DQ243994
Massisteria marina AF411286
Minimassisteria diva EF405665
ENV: anoxic saline lake sediment NAMAKO-18 AB252758
ENV: arctic marine water column NW617.36: DQ314819
ENV: coastal marine water UEPACLp3: DQ369015
Chlorarachnion reptans U03477
Metromonas simplex AY620255
ENV: marine sediment nh13: EU567296
Metromonadea clone Mus2A
ENV: marine surf water 12-3.3: AY620358
ENV: marine sediment A17: AY620324
Metopion aff. fluens AF411278
Micrometopion nutans HQ121441
ENV: freshwater sediment: lb9 EU567287
Tremula longifila HQ121439
ENV: freshwater sediment: lb6 EU567286
ENV: anaerobic freshwater sediment DB-2703-19: EU567290
ENV: anaerobic freshwater sediment DB-2305-21: EU567288
ENV: anoxic saline lake sediment NAMAKO-20 AB252760
ENV: uncultured freshwater picoplankton A50: AY642739
ENV: freshwater column LG21-01: AY919754
ENV: anaerobic freshwater sediment DB-2703-3: EU567283
Phagomyxa odontellae AF310904
Spongospora subterranea f. sp. nasturtii AF310901
Arachnula impatiens EU567294
Filoreta marina AY268044
Filoreta turcica EU567292
Gromia oviformis AJ457812
Gromia oviformis AJ457814
symphacanthid 211 AF063242
chaunacanthid sp.218 AF018158
Acanthometra sp. AF063240
Spongaster tetras AB101542
Sticholonche sp. AY268045

these samples. Nevertheless we were able to isolate them from two European countries with quite different climates, and an environmental sequence reveals the presence of the genus in a freshwater lake in the Antarctica. Therefore *Kraken* is possibly globally distributed, represented by closely related SSU sequence types.

The question arises why *Kraken* gen. nov. has not previously been found. Possible explanations for this are as follows. (a) The low abundance of *Kraken carinae* in our soil samples was in a range that exceeds the sensitivity of most general cultivation-based methods. Therefore *Kraken* individuals might simply not have been noticed in analyses conducted by other methods with higher detection thresholds, or when occurring in even lower abundance. (b) With a cell body size of less than 10 μm *Kraken* individuals are relatively small and therefore easily overseen. Although their filopodial networks can cover areas of at least 0.5 × 0.5 mm, these can be extremely difficult to observe when the surface is overgrown with bacteria. (c) *K. carinae* cells move rarely and very slowly, which makes them difficult to see in a mixed microbial culture. (d) *K. carinae* grows very slowly. To colonize the surface of a Petri dish an actively growing culture required several months under our culture conditions. Many protist screenings are performed only for some days or weeks. Within that time *Kraken* spp. would likely not be detected. (e) *K. carinae* is very fragile. Weak disturbance (i.e. movement of the water body in the Petri dish) leads to damage of the filopodia often followed by cell death. These factors make microscopical work with *Kraken* extremely difficult and time consuming. (f) With light microscopical observation and low magnification or low contrast *K. carinae* could be easily misidentified. Bacteria that are transported through the agranular filopodia of *Kraken* might be confused for granules leading to a misidentification as a granofilosean (e.g. *Mesofila* or *Limnofila)*.

Methods

Isolation and cultivation: In total 125 samples were screened of a climate change experiment that was conducted in plant pots in Mediterranean climate and nine samples were taken from three agricultural fields in northern Germany. Soil surface samples were collected (see Table 1) and stored for 3–6 months (Spain and Göttingen samples) or 3–6 days (Uckermark samples) at 4 °C until analysis. Protist abundance and community composition were assessed by a liquid aliquot method (LAM) according to Butler and Rogerson (1995). Briefly, 1 g of a homogenized soil sample was suspended in 250 ml of Neff's Modified Amoeba Salinae (NMAS; Page 1988) and shaken for 20 minutes. For incubation, the suspension was diluted by a factor of 4 and 20 μl of the suspension were added to 180 μl 0,15% Wheat Grass Medium (WG, Spanish soils) or 180 μl NMAS (German soils). The WG was made by adding dried wheat grass powder (Weizengras, Sanatur GmbH, D-78224 Singen) to PJ medium (Prescott and James, 1955). In total 144 wells per sample were stored at 15 °C in the dark. The plates were inspected for protists after 7 and 21 days using an inverted microscope (Nikon Eclipse TS100) at 100x and 400x magnification. Protist morphotypes were determined according to Jeuck and Arndt (2013), Bass et al. (2009a, b), Smirnov (2011) and Smirnov and Brown (2004). Monoclonal cultures were prepared by pipetting one individual into a sterile Petri dish filled with fresh WG medium. Cultures were stored at 15 °C in the dark.

Preparation of the hapantotype: For the preparation of the hapantotype, cultures were mixed 1:4 with Schaudinn's fluid for 5 minutes at 60 °C, followed by a serial dehydration in an ascending series of Ethanol. The preserved cells were mixed 1:2 with glycerine and transferred to a glass slide. A coverslide was glued on top.

Light microscopy: Time-lapse films of cultures were made with a Nikon digital sight DS-U2 and the Program: NIS-Elements 3.1, the camera was mounted on an inverse microscope (magnification: 100x–400x, type: Nikon Eclipse TE 2000-E) and an upright microscope (magnification: 600x, type: Nikon Eclipse 90i). For video records of the *Kraken* with an upright microscope the following method was used: cultures were grown on a cover slide inside a Petri dish filled with WG medium for a couple days. Directly before video recording, another cover slide was prepared with vaseline on the edges as distance holders and then gently pushed onto the other cover slide into the waterbody of the culture. If the procedure was performed too roughly the filopodia of the *Kraken* were easily destroyed. The slides were lifted with a forceps, and put onto a specimen holder and examined with differential interference contrast (DIC).

SSU sequencing: 15 μl aliquots of the monoclonal cultures were transferred to a sterile 200 μl Eppendorf tube and 13 μl ddH_2O were added. These samples were frozen at −20 °C to destroy the protist cells. The PCR was performed with a 50 μl reaction mixture containing 5 μl of 0.1 μM forward and reverse primer solution each, 5 μl dNTPs (200 μM), 5 μl reaction buffer and 1 U DreamTaq DNA-polymerase (Applied Biosystems, Weiterstedt, Germany). General eukaryotic primers were used including EukA, EukB (Medlin et al. 1988) and 590F (Quintela-Alonso et al. 2011) as well 25F and cercozoan specific 1256R (Bass and Cavalier-Smith 2004).

The PCR products were purified by adding 0.15 μl of Endonuclease I (20 U/μl, Fermentas GmbH), 0.9 μl Shrimp Alkaline Phosphatase (1 U/μl, Fermentas GmbH) and 1.95 μl water to 8 μl of the PCR products. The mixtures were then heated 30 min at 37 °C, and subsequently 20 min at 85 °C. The sequencing reaction was done by using the Big Dye Terminator Cycle sequencing kit and an ABI PRISM automatic sequencer.

Figure 5. Bayesian SSU rDNA phylogeny of Cercozoa. The support values of the Bayesian (Posterior Probabilities) and the Maximum Likelihood (bootstrap) analyses are shown (BPP/ML) if values were above BPP=0.95; ML = 85%, and/or if they are directly relevant to the interpretation of the phylogenetic position of *Kraken*. See Methods for further details.

The sequences were manually checked for sequencing errors and combined to consensus sequences that were deposited in the NCBI database (see Table 1) and used for BLASTn search that revealed cercozoan affiliation and an environmental sequence with high similarity that was included in our analyses.

Phylogenetic analyses: Sequence alignments were constructed using the e-ins-i algorithm in MAFFT (Katoh and Standley 2013) and refined and masked by eye where necessary. 1767 positions were retained for the analyses. Refined alignments were analysed in RAxML (Stamatakis 2006; 2014) BlackBox (GTR model + gamma; all parameters estimated from the data; one starting tree); bootstrap values were mapped onto the highest likelihood tree obtained (Stamatakis et al. 2008). Bayesian consensus trees were constructed using MrBayes v 3.2 (Ronquist et al. (2012) in parallel mode (Altekar et al. 2004) on the Cipres Science Gateway (Miller et al. 2010). Two separate MC^3 runs with randomly generated starting trees were carried out for 4 million generations each with one cold and three heated chains. The evolutionary model applied included a GTR substitution matrix, a four-category autocorrelated gamma correction and the covarion model. All parameters were estimated from the data. Trees were sampled every 100 generations. 1.2 million generations were discarded as "burn-in" (trees sampled before the likelihood plots reached a plateau) and a consensus tree was constructed from the remaining sample. The Bayesian topology was used for Figure 5, with Bayesian Posterior Probabilities and Maximum Likelihood bootstrap values mapped on to the nodes.

Acknowledgements

We would like to thank Marina Müller and Fernando Maestre Gil for providing the soil samples. This study was partly supported by grant (BO 1907/2-2) of the DFG (Deutsche Forschungsgemeinschaft) to MB within the Research Unit "Carbon flow in belowground food webs assessed with isotope tracers" (FOR 918) and NERC grant NE/H000887/1 to DB.

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2016.04.002.

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Chapter 2: Cercomonad or archaic Imbricatea? On the hunt for the true taxonomy of the scale-bearing *Kraken* (*incertae sedis*, Cercozoa, Rhizaria): Combining ultrastructure data and a two-gene (SSU + LSU) phylogeny.

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Title

Cercomonad or archaic Imbricatea? On the hunt for the true taxonomy of the scale-bearing *Kraken* (*incertae sedis*, Cercozoa, Rhizaria): Combining ultrastructure data and a two-gene (SSU + LSU) phylogeny.

Keywords

Sarcomonadea, Imbricatea, scales, flat cristae, cercomonads, electron mircoscopy

Abstract

The genus *Kraken* represents a morphologically distinct lineage of filose amoebae within the Cercozoa. Currently only a single species, *Kraken carinae*, has been described. SSU phylogeny showed an affiliation with the Cercomonadida, branching with weak support at its base, close to *Paracercomonas*, *Metabolomonas*, and *Brevimastigomonas*. Light microscopical analyses showed several unique morphological and behavioral features of the genus *Kraken*, but ultrastructure data was lacking. In this study, *K. carinae* has been studied by electron microscopy, this data conjoined with a concatenated SSU and LSU phylogeny was used to give more insight into *Kraken* taxonomy. The data confirmed the absence of flagella, but also showed novel characteristics, like the presence of extrusomes, osmiophilic bodies, mitochondria with flat cristae and, surprisingly, the presence of single-tier scales, which are carried by cell outgrowths, much of what is expected of the last common ancestor of the class Imbricatea. The phylogenetic analyses however confirmed previous results, indicating *Kraken carinae* as a sister group to *Paracercomonas* within the Sarcomonadea with an increased but still moderate support of 0.98/63. Based on the unique features of the *Kraken* we establish the Krakenidae fam. nov. that we, due to contradicting results in morphology and phylogeny, assign *incertae sedis*, Cercozoa.

Introduction

Recently, the genus *Kraken* was described as a new lineage of cercozoan filose amoebae inhabiting soil and freshwater (probably sediment) ecosystems (Dumack et al. 2016a). Small subunit (SSU) phylogeny indicated with low support an affiliation with Filosa (Cercozoa), in particular to *Paracercomonas*, *Metabolomonas*, and *Brevimastigomonas* (Paracercomonadidae, Cercomonadida). Cercozoa is a phylum of high morphological diversity, comprising naked amoebae (Hess et al. 2012), testate amoebae (Dumack et al. 2016b,c) but also flagellates and amoeboflagellates (Bass et al. 2009a,b; Hess and Melkonian 2013). The Cercomonadida are a taxon of relatively well studied amoeboflagellates with quite conserved morphology (Bass et al. 2009a). They are currently assigned to the Sarcomonadea, CAVALIER-SMITH, 1993. The Sarcomonadea are, next to the Thecofilosea and Imbricatea, one of the as yet known major classes of Filosa, Cercozoa. The latter two mainly accommodate test- or scale-bearing protists, whereas the Sarcomonadea were established to unite cercozoan free-living, heterotrophic and naked (amoebo-) flagellates (Cavalier-Smith 1993), although they might be of polyphyletic origin (Cavalier-Smith and Karpov 2012). They comprise the orders Pansomonadida, Glissomonadida and Cercomonadida (Cavalier-Smith and Karpov 2011). The latter two are very common, diverse and abundant in terrestrial and freshwater systems and are commonly referred to as the predominant protistan bacteria grazers (Glücksman et al. 2010).

Light microscopic observation of *Kraken* did not specifically support the affinity to cercomonads, although its dimensions and general appearance was not inconsistent with the diversity of body forms within the Cercomonadida (Dumack et al. 2016a). However, all as yet described species of the Cercomonadida are biflagellate, naked cells and some may be able to form short filopodia. The *Kraken* in contrast bears no flagella but has instead a huge network of filopodia that exceeds the size of the cell body by a factor of 50. *Kraken* differs therefore markedly from the currently known Cercomonadida. Since SSU phylogeny could not resolve its direct affiliation, ultrastructure as well as more genetic data, enabling a more detailed comparison with other Cercomonadida and other cercozoan amoebae or amoeboflagellates, are desirable.

Two-gene phylogenies have been shown to resolve low support in some questionable clades of single gene phylogenies (Chantangsi et al. 2010; Wylezich et al. 2010). Although there is a considerable amount of SSU data of cercozoans available, only few strains have been characterized by additional genes, mostly large subunit sequence (LSU) data (Chantangsi et al. 2010; Wylezich et al. 2010). Additionally various ultrastructural traits, obtained by SEM (e.g. overall cell shape, scales and tests) or TEM (e.g. flagellar apparatus) display group-specific patterns and may reveal valuable

morphological characteristics for comparison (Cavalier-Smith and Karpov 2012; Hess & Melkonian 2014).

We therefore decided to combine a concatenated SSU and LSU phylogeny with ultrastructure data to get more insight into *Kraken* taxonomy. In particular, two questions arise: Does a concatenated (SSU+LSU) phylogeny confirm the phylogenetic placement of *Kraken carinae* presented in Dumack et al. (2016a) and does it further provide an enhanced support? Will ultrastructure data show characteristics typical for cercomonads, its class Sarcomonadea or other cercozoans?

We were in particular interested to find a possible rudimentary flagellar apparatus, and to obtain data of the cell (surface) architecture. Our data show the ultrastructure of a very unusual filosan amoeba and may lead to a re-interpretation of cercozoan evolution.

Results

Ultrastructure of *Kraken carinae*

Cell shape and content

The light microscopical observations presented in Dumack et al. (2016a) correspond to the ultrastructural data. Briefly, the overall shape of the cell body is spherical with an invagination (Dumack et al. 2016a called it 'ring-like structure') at the basal end from which branching and anastomosing filopodia arise (Fig. 1A-E). Cysts and elongated cells (during cell body transport) have not been studied in detail and are not presented here. The fragile cells lost filopodia during centrifugation and therefore the cell body and filopodia are shown here separately.

The cells contain a single, eccentric nucleus (2.4 – 3.2 µm), usually located at the basal end of the cell body close to the invagination, with a homogenously granular nucleolus in its center (Fig2A-C). The invagination reaches usually into the cell body, deforming the nucleus spherical shape. The shape of the invagination can be seen in the selected serial sections of a single cell (Fig. 3A-F). Golgi stacks are found in association to the nucleus (Fig. 2I, 4A). The cells contain various amounts of spherical mitochondria (0.5 - 0.6 µm) with an electron-translucent central matrix (Fig.2A). The plate-like (flattened) cristae lie on the internal periphery of the mitochondria (Figs. 4B, 4C). Sections of tips of the cristae are sometimes roundish (Fig. 4C, arrow). The contractile vacuole has irregular cell walls during systole (Fig. 4D).

In the periphery of the cell body extrusomes are located (Fig. 4). The extrusomes are reminiscent of the kinetosomes of cercomonads and glissomonads (Mylnikov, 1988; Fig. 4G,I). They are enveloped by a small vesicle and consist of a capsule with an internal cylinder. After discharging the cylinder is partly exposed (Fig. 4J).

Additionally to the medium stained extrusomes, the cells contain a huge amount of small vesicles which are either electron-translucent, resembling reserve granules (Fig. 4L) or highly stained, resembling osmiophilic bodies (Fig. 2A, 2E) and bundles of microfilaments (Figs 5A,B). *Kraken* cells do not contain flagella, kinetosomes or a flagellar root system.

The filopodia are more electron-translucent than the cell body. Aggregations of osmiophilic amorphous material, small electron-translucent vesicles and microtubules were seen inside the filopodia (Figs 5D-G, selected serial sections). Additionally the filopodia contain small vacuoles with single bacteria (Fig. 4E). According to light microscopical observations on the feeding process of the *Kraken*, these bacteria were caught immediately before the fixation and were in the process of transportation to the cell body. Within the cell body one (rarely two) food vacuoles are located in the apical end (for the ejection of such a food vacuole during defecation, see Fig. 4F). No endocytobiotic bacteria were observed.

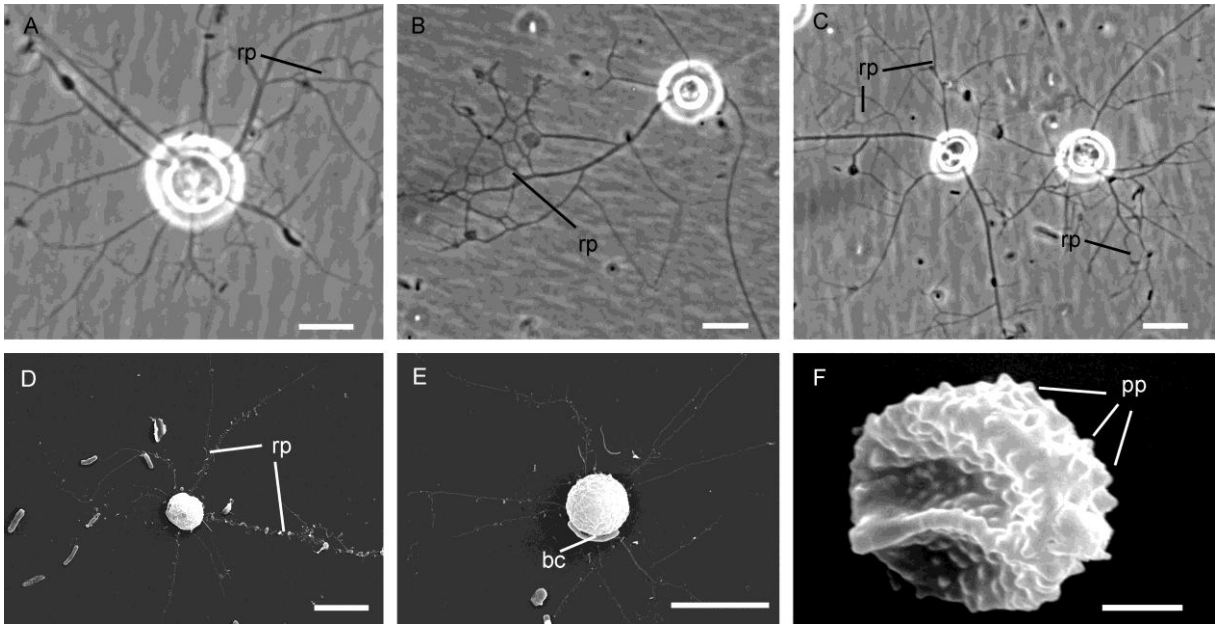

Fig. 1. General view of the cells. A-C – light microscopy pictures. Roundish cells forming anastomosing reticulopodia. D-F – scanning electron microscopy pictures; D-E cells form branching pseudopodia, F – the cell is covered papillae, bc – attached bacterium. Scale bars: A – 5 µm; B-E – 10 µm; F – 1 µm

Cell surface and scales

The surface of the cell body appears at smaller magnification rough (Figs 1F, 2A-2D). At higher magnification pyramidal papillae (outgrowths) covering the cell surface are observable. Every papilla bears a thin unadorned and oval scale (0.3-0.35 µm in width and 0.6 µm in length) and is densely filled by ribosomes (Figs 2D-2G). The scales are evenly distributed over the cell body and rarely overlap, but were never observed on the filopodia. Probably, the scales are formed in cisternae of the Golgi apparatus (Fig. 2I), which lies close to the nucleus and from which they are transported in vesicles to the cell surface (Fig. 2H).

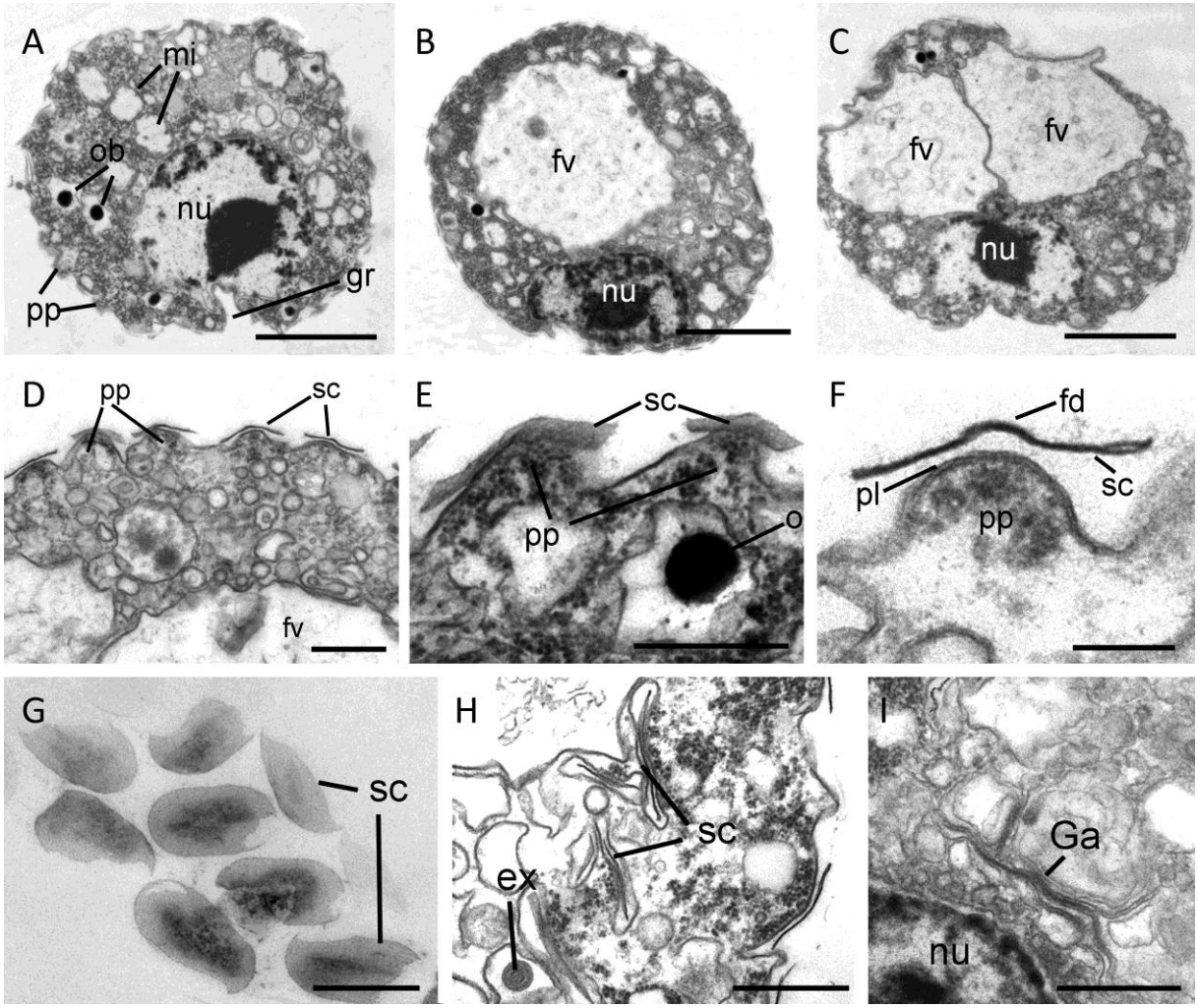

Fig. 2. Cell sections (TEM). A-C – sections of whole cell, nucleus (nu) lies eccentric, close to the basal invagination, 1-2 food vacuoles (fv), roundish mitochondria (mi) and osmiophilic bodies (ob) are seen. D-E – arrangement of the papillae (pp) and single-tier scales (sc) on the cell surface. F, G - shape of the scales (sc). H – location of young scales and extrusomes inside vesicles in cytoplasm. I – possible formation of the scale inside cisterns of Golgi apparatus (Ga). Scale bars: A-C- 2 µm; D, G, H, I - 0.5 µm, E -0.4 µm; F - 0.1 µm

Phylogenetic analyses

The maximum likelihood tree shows the position of the *Kraken* within the cercozoan subphylum Filosa (Fig. 6). The Filosa comprises currently published sequences of the Monadofilosa and Granofilosea and as an outgroup the Chlorarachnea. The Monadofilosa are comprised of the Sarcomonadea, Imbricatea and Thecofilosea. As in previous analyses using SSU and LSU rDNA sequences for phylogeny, the chlorarachnean outgroup was highly supported, the Granofilosea were of moderate support and the Monadofilosa were highly supported (Chantangsi et al. 2010).

The Sarcomonadea, accommodating the *Kraken* sequences, separate from the Thecofilosea and Imbricatea with full support. Within the Sarcomonadea the three following well known clades were resolved: (1) the Glissomonadida HOWE, BASS VICKERMAN CHAO & CAVALIER-SMITH, 2009; (2) the Cercomonadidae KENT, 1880; (3) the Paracercomonadidae CAVALIER-SMITH, 2011; all with maximal support. The Cercomonadida were not monophyletic in our analysis, similar to previous studies in which they were either not monophyletic (Chantangsi et al. 2010) or the monophylum of little support (Wylezich et al. 2010). Within the Sarcomonadea, *Kraken* forms a sistergroup to the Paracercomonadidae with moderate support of 0.98/63. The genus *Kraken* is represented by the three currently known (and cultured) different genotypes of the species *Kraken carinae*. All strains formed a monophylum with maximal support. Similar to the SSU phylogenetic analyses (Dumack et al. 2016a) the morphologically similar strains KJ0003 and KD0092 grouped closely to each other, and the morphologically slightly different (smaller cell body) strain KD0248 basal to both.

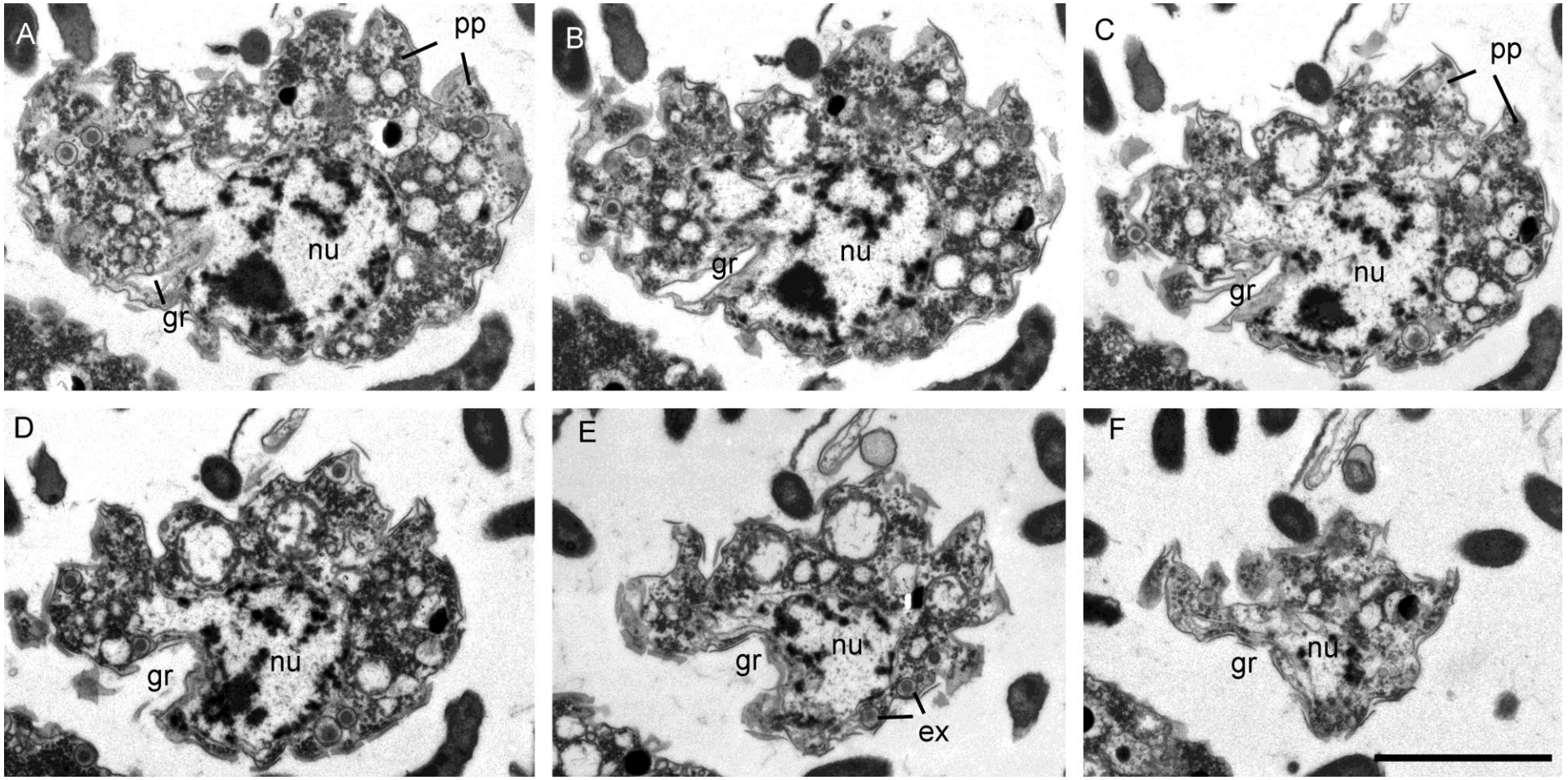

Fig. 3. Selected serial sections (TEM) of the same cell showing the location of the groove (gr) as invagination of the nucleus (nu) and cell surface, also papillae (pp) and extrusomes (ex) are seen. Scale bar 2 µm.

Discussion

Ultrastructure data

Kraken carinae has very unique ultrastructure. Important ultrastructure characteristics for the taxonomy of protists (if present) are: flagella, the flagellar apparatus and the structure of scales (Cavalier-Smith & Karpov 2012; Hess and Melkonian 2014; Scoble and Cavalier-Smith 2014). However, the complete loss of flagella and the flagellar apparatus in *K. carinae* prevent the comparison with Sarcomonadea.

The loss of flagella is likely a derived character, since *Kraken* cells usually feed as a sit-and-wait predator and move rarely. We suggest that *Kraken* inhabits small soil (or sediment) pores and only emerges from that with their long filopodia. *Kraken* has a peculiar way of movement by disassembling its own cell body (thereby reducing its cell diameter), possibly used when moving form pore to pore. A similar way of movement is known from the chlorarachniophyte *Bigelowiella* (Ota et al. 2007), leading to the question whether this is an ancestral trait or an adaption to life in sediments that evolved independently.

Due to is unique form of the locomotory structures *Kraken* can not be compared to the group specific ‘tails’ of ‘other’ Cercomonadida (Bass et al. 2009a).

Mitochondria with tubular cristae are common in Filosa (Bass et al. 2009a; Cavalier-Smith and Karpov 2012; Hess and Melkonian 2014; Shiratori et al. 2014). In contrast, *K. carinae* bears flat cristae. Only very few Cercozoa differ from tubular cristae, e.g. anaerobic Cercomonadida with cristae-lacking mitochondria or for instance some granofiloseans, such as the genus *Limnofila* which groups more basal in the Cercozoa and bears, similar to the *Kraken,* flat cristae (Bass 2009b; Cavalier-Smith and Chao 2003, Mikrjukov and Mylnikov 1995). *Limnofila* further shows some remarkable similarities with *Kraken* on the morphological level. Apart from flat cristae, both taxa are slow-moving filose amoebae with a huge filopodial network and a uninuclear cell body (Mikrjukov and Mylnikov 1998). Moreover the extrusomes which this study found in the *Kraken,* show similarity to the extrusomes of *Limnofila* which also contain a cylinder that is being exposed when the extrusome is charged (Mikrjukov and Mylnikov 1995). However distinct differences of *Limofila* compared to the *Kraken* are: A less defined form of the naked cell body of *Limnofila*, granules within the filopodia and flagella (or flagellate stages), apart from its well defined phylogenetic position (Bass 2009b).

Moreover the *Kraken* bears scales, a character unknown from any Sarcomonadea or Granofilosea species.

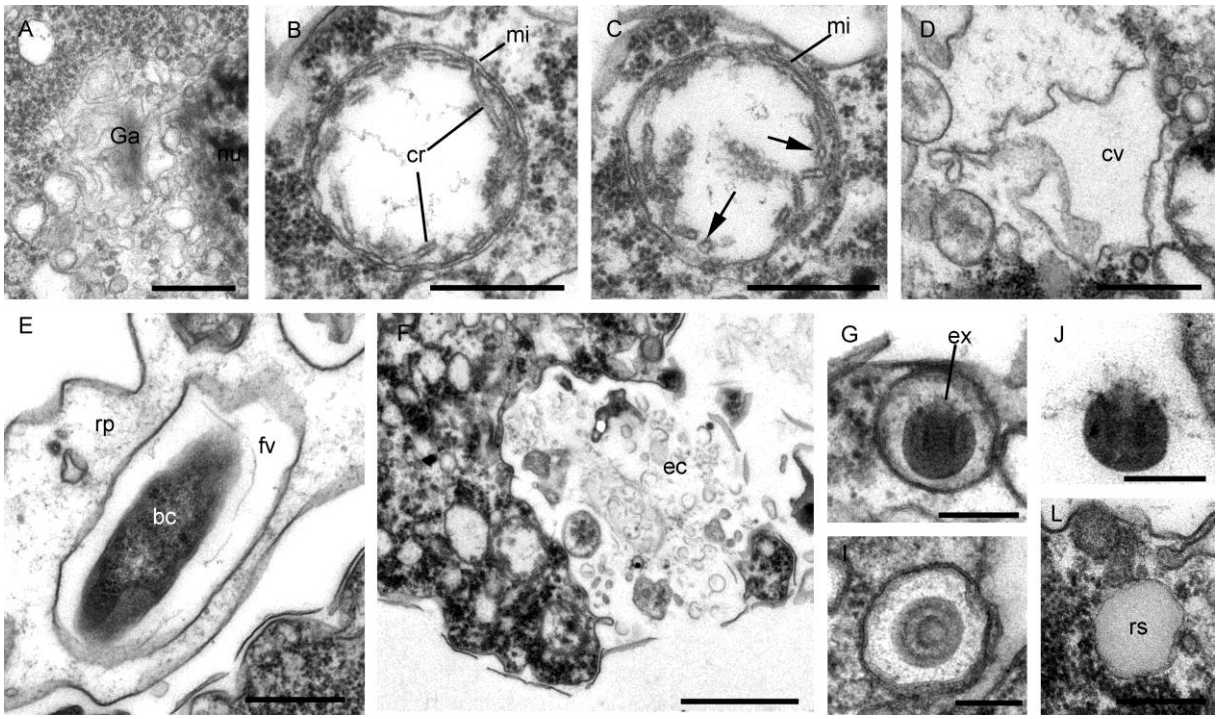

Fig. 4. Some organelles of the cell (TEM). A – Golgi apparatus (Ga) lies close to the nucleus (nu), B, C – mitochondria with plate-like cristae (cr), sometimes the roundish profile of the crista tip is seen (arrow). D – contractile vacuole (cv) of stage systole. E – small food vacuole (fv) contains bacterium (bc) inside reticulopodium. F – ectoproct (ec) eject the content of the food vacuole. G-J – extrusomes (ex) (G and I - longitudinal and cross sections, J – extrusome outside of the cell after its discharging). L – reserve substance. Scale bars: A, D, E – 0.5 µm; F – 1.0 µm ; B, C – 0.4 µm; G-I - 0.1 µm; L – 0.2 µm

Scales however are predominantly known from the Imbricatea, where the two major orders, the Thaumatomonadida and Euglyphida, both (usually) build scales. The Thaumatomonadida bear two-tier scales, the Euglyphida build whole rigid tests of overlapping single-tier silica scales (for an extensive and detailed discussion see Scoble and Cavalier-Smith 2014). However, due to their absence in some lineages, e.g. *Esquamula* or the Spongomonadida, they are considered as a paraphyletic character (Scoble and Cavalier-Smith 2014; Shiratori et al. 2012). Whole tests are considered as polyphyletic, as they are found in distant related cercozoan taxa, indicating that the development of a protective test has evolved multiple times in Cercozoa (Dumack et al. 2016 b,c; Kosakyan et al. 2016; Wylezich et al. 2002).

Scoble and Cavalier-Smith (2014) assumed that the Imbricatean ancestor probably bore unadorned oval single-tiered scales; much as the scales of the *Kraken*. Accordingly, the *Kraken* might resemble an archaic ancestor of the Imbricatea. Since tests apparently evolved multiple times in the Cercozoa, it is not unlikely that also scales evolved multiple times. It seems unlikely that the last common

ancestor of the Filosa already carried scales, the Thecofilosea may have developed from those their organic tests and the Imbricatea further developed two-tier scales (Thaumatomonadida) or whole rigid tests composed of scales (Euglyphida), but were lost in most Sarcomonadea, except in the possible sarcomonadean *Kraken.*

Phylogenetic analyses

The phylogeny of the Sarcomonadea is still controversial (see Cavalier-Smith & Karpov 2012 for a detailed discussion). It is still unclear whether Sarcomonadea are monophyletic, and especially the phylogenetic position of the Paracercomonadidae (extremely long branch, changing position in trees) contributes to this uncertainty. Since *Kraken* groups with SSU and concatenated SSU+LSU phylogeny next to *Paracercomonas*, this long branch might (a) interfere with good support or (b) may even indicate questionable results due to long branch attraction. Nevertheless the constructed two-gene phylogeny confirms the SSU phylogeny published in Dumack et al. (2016a). Although still moderate, the phylogenetic support was significantly enhanced (from 0.6/29 to 0.98/63) in this study. Since only few cercozoan LSU sequences are currently available we were not able to achieve a highly supported phylogeny. With an increase of (a) taxon sampling of Cercozoa and especially the Paracercomonadidae (in particular *Metabolomonas* and *Brevimastigomonas*) or (b) gene sampling, in particular whole transcriptomes the phylogenetic positions of the *Kraken* and Paracercomonadidae within Cercozoa might be resolved.

The *Kraken* remains *incertae sedis*

The ultrastructure data give some indications that the *Kraken* resembles a direct descendant of the scale-bearing last common ancestor of the Imbricatea. The two-gene phylogeny indicates, however with moderate support, an affiliation with the Cercomonadida in the Sarcomonadea. Due to these contradicting results we are not able to clearly assign the *Kraken* to either the class Sarcomonadea or Imbricatea.

The characters of the *Kraken* have not been found in any other cercozoan family. We therefore establish a new family Krakenidae fam. nov., but still hesitate to establish a novel order that should be called Krakenida if it will be shown to be necessary in future studies. To resolve this issue, ultrastructure data (of e.g. *Discomonas*), SSU sequencing data (of e.g. *Zoelucasa*; Nicholls 2012), or large scale multi-gene transcriptome analyses are needed in future studies.

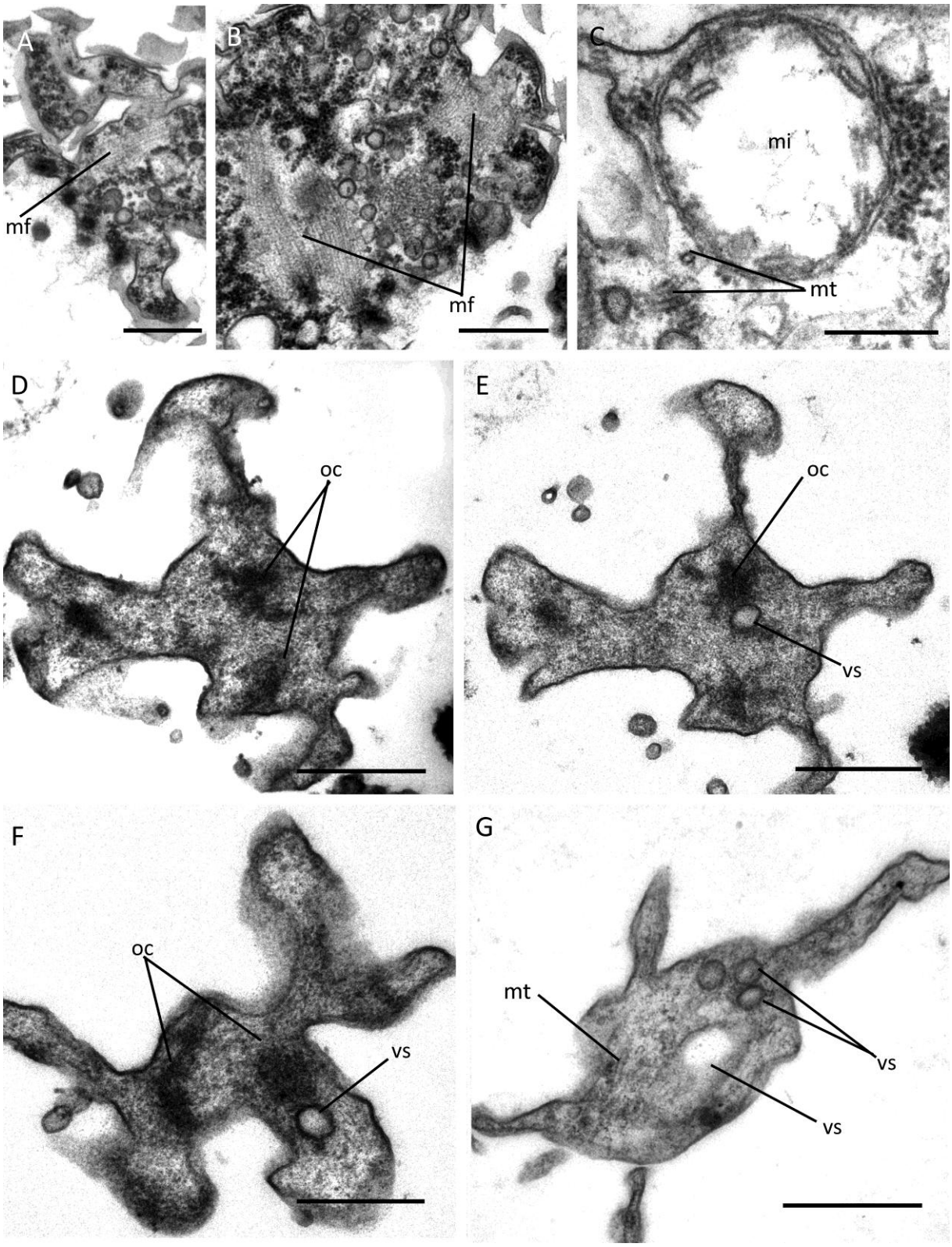

Fig. 5. Microfilaments and microtubules in the cells (TEM). A, B – arrangement of the microfilament bundles (mf) in the cytoplasm, C – microtubules (mt) inside cell, D-F – arrangement of the osmiophilic concentration of amorphous material (oc) inside reticulopodia, G – microtubules (mt) inside reticulopodia. Scale bar: C – 0.2 µm; A, B, D – G – 0.5 µm

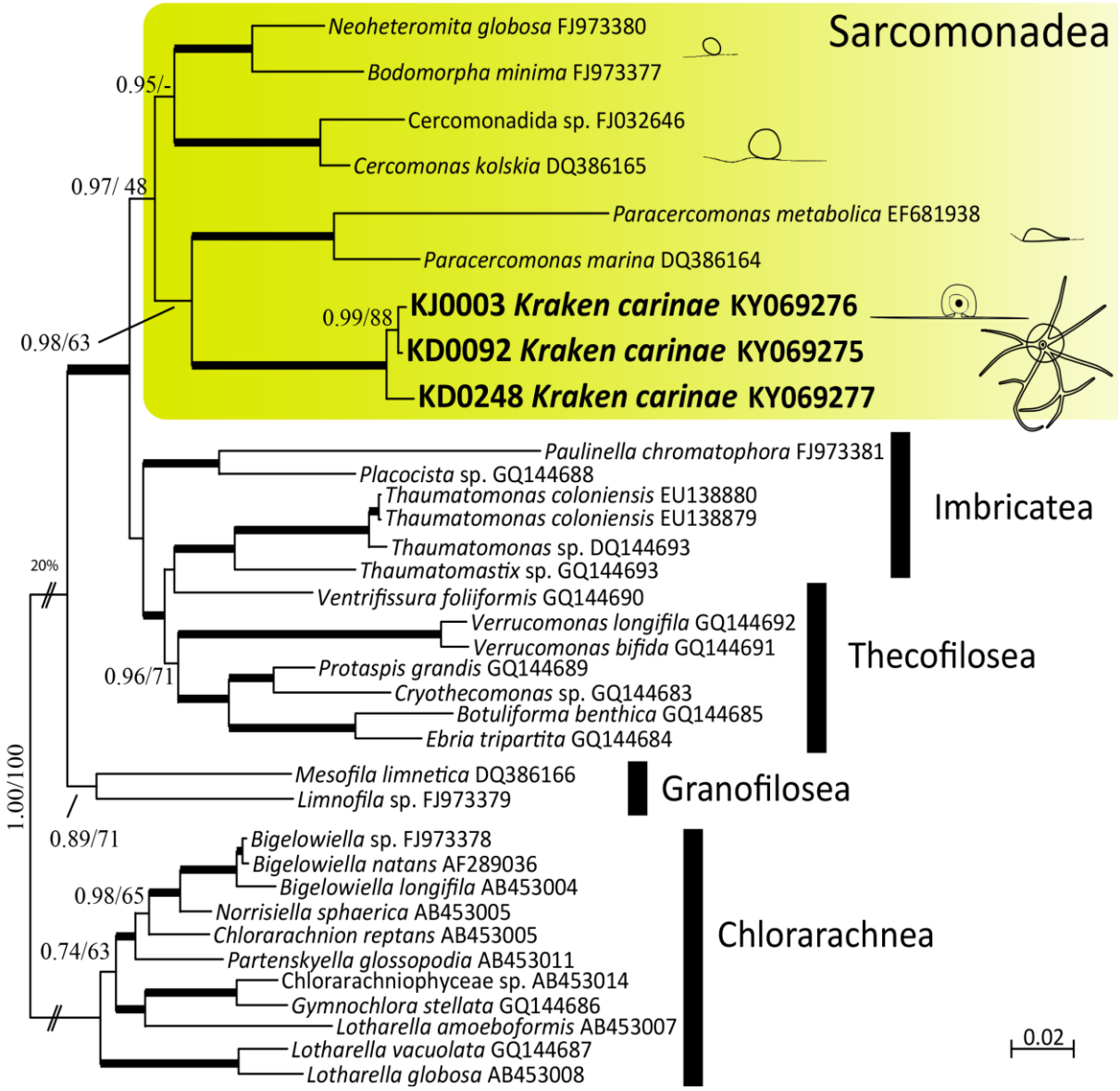

Fig. 6. Phylogeny of the Filosa, Cercozoa with Chlorarachnea sequences as outgroup based on concatenated SSU rDNA and LSU rDNA sequences. Classes are indicated by vertical bars, the Sarcomonadea are highlighted in colour, newly obtained sequences are in bold. The support values of the Bayesian and PhyML analyses are shown on the respective branches (BI/ML). Support values <50% (bootstrap values) and <0.95 (posterior probabilities) are only shown if necessary for interpretation of direct Kraken relationship, otherwise not shown (-). Branches with bootstrap values >95% are presented in bold. Interrupted branches (//) show 20% of their original length. The scale bars represent 0.02 nucleotide substitutions per site. Given accession numbers refers to the strains LSU sequences.

Krakenidae Dumack, Mylnikov and Bonkowski fam. nov.

Diagnosis. Limnic or terrestrial; Cells in the trophic phase form a large web of surface attached filopodia (irregular branched, elongate, anastomosing, network-forming). Filopodia thin and tapering; originating from the basal end of the cell body; flagella absent. Cell body covered by non-overlapping oval and unadorned single-tier scales; these are carried by small papillae (outgrowths); usually not overlapping. Mitochondria with plate-like cristae in their inner periphery. Extrusomes with unknown function contain a capsule and an internal cylinder present. Phagotrophic; bacterivorous; bacteria are caught with filopodia at the point of contact and then transported in a vacuole through the filopodia to the cell body for digestion. Cysts present, spherical.

Etymology: Name derived by the type genus.

Type genus: *Kraken* Dumack, Schuster, Bass et Bonkowski, 2016

Other genera included: none

Material and Methods

For detailed description of the source of samples, the isolation process and culture conditions of *Kraken carinae*, see Dumack et al. (2016). Briefly, *K. carinae* was isolated from agricultural soils of two European countries and cultured in low nutrient medium with co-cultivated bacteria. The cells are extremely fragile and die when exposed to slightest mechanical stress, i.e. movement of water body by lifting the Petri dish.

Sequencing

Phylogenetic analyses were done on one representative strain for each of the three known (and cultured) *Kraken* SSU genotypes; i.e. KJ0003, KD0092 and KD0248 (DSMZ- Registration numbers: 244502341-KA; 244502023-KA and 244501215-KA, respectively). 15 µl aliquots of the monoclonal cultures were transferred to a sterile 200 µl Eppendorf tube and 13 µl ddH2O were added. These samples were frozen at −20°C to destroy the protist cells. The PCR was performed with a 50 µl reaction mixture containing 5 µl of 0.1 µM forward and reverse primer solution each, 5 µl dNTPs (200 µM), 5 µl reaction buffer and 1 U DreamTaq DNA-polymerase (Applied Biosystems, Weiterstedt, Germany). General eukaryotic primers 184F, 1126R, 1105F and 2018R were used (Van der Auwera et al. 1994). The PCR products were purified by adding 0.15 µl of endonuclease I (20 U/µl, Fermentas GmbH), 0.9 µl shrimp alkaline phosphatase (1 U/µl, Fermentas GmbH) and 1.95 µl water to 8 µl of the PCR products. The mixtures were then heated 30 min at 37°C, and subsequently 20 min at 85°C.

The sequencing reaction was done by using the Big Dye Terminator Cycle sequencing kit and an ABI PRISM automatic sequencer.

The sequences were manually checked for sequencing errors, combined to sequence contigs and deposited in the NCBI database (KY069275, KY069276 and KY069277).

Phylogenetic analyses

Publications were screened for SSU and LSU sequences that were obtained from the same strain of as many cercozoan lineages as possible. These sequences were manually aligned in SeaView (V4.5.3, Gouy et al. 2010). An alignment with 34 sequences and 4,091 unambiguously aligned sites of which 67.8% were without polymorphisms was used for phylogenetic analyses. The program jmodeltest (V.2.1.5, Darriba et al. 2012) was used to determine the best fitting model: GTR+I+G, which was selected among 88 models (settings: Substitution schemes 11; add base frequencies +I+G rate variation nCat=4, ML optimized NNI as base tree). Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). The support values of the PhyML and the Bayesian analyses are given as (ML/BI).

Maximum likelihood phylogenetic analyses were run in PhyML V3.1 (Guindon and Gascuel 2003) with the following settings: GTR model; a proportion of invariable sites and a gamma-shaped distribution of the substitution rates across variable sites (GTR+I+G), with four rate categories; BIONJ distance-based starting tree with all model parameters estimated from the data. The Bayesian analyses were run using MrBayes v.3.2 (Altekar et al. 2004; Ronquist and Huelsenbeck 2003) with the following settings: five million generations, trees sampled every 100 generations, convergence of the two runs was estimated every 500 generations with a final average of the standard deviation of split frequencies of <0.01 at the end of the run. Of the sampled trees, 25% were discarded as burn-in.

Electron microscopic analyses

Electron microscopy was performed on a culture of *Kraken carinae* (strain KJ0003; DSMZ-Registration number 244502341-KA). Cells were maintained in Pratt medium (0.1 g/L KNO_3, 0.01 g/L $MgSO_4 \cdot x$ 7 H_2O, 0.01 g/L $K_2HPO_4 \cdot x3$ H_2O, 0.001 g/L $FeCl_3 \cdot x6$ H_2O, pH = 6.5-7.5) with the bacterium *Pseudomonas fluorescens,* MIGULA 1895, as food source.

Light microscopic observations were made with an AxioScope A1 (Carl Zeiss, Germany) using phase contrast, 70x water immersion objectives and an AVT HORN MC-1009/S video camera. Video clips were digitized using a Behold TV 409 FM tuner.

For scanning electron microscopy (SEM), cells from lag and exponential growth phase growing on the surface of cover slips were fixed with 2% glutaraldehyde (final concentration) for 15 min at 20 °C. The specimens were taken through a graded ethanol dehydration followed by a graded transfer to acetone. After critical point drying, the dried cover slips were mounted on aluminum stubs, which were coated with gold-palladium and then studied with a JSM-6510LV.

For transmission electron microscopy (TEM), cells were centrifuged to produce high density pellets. The pellets were fixed in a cocktail of 0.6% glutaraldehyde and 2% OsO_4 (final concentration) for 20-60 min at 1 °C and dehydrated in an alcohol series and then in rising acetone concentrations (30, 50, 70, 96 %; 10-20 minutes in each step). Subsequently, cells were embedded in a mixture of araldite and epon (Luft, 1961). Ultrathin sections were obtained with the LKB ultramicrotome. The TEM observations were made by using a JEM-1011 (Jeol, Japan) electron microscope. The chemical composition of the scales was not studied.

Acknowledgements

We like to thank Dr. Frank Nitsche for providing the LSU primers. This study was partly supported by grant (BO 1907/2-2) of the DFG (Deutsche Forschungsgemeinschaft) to MB within the Research Unit "Carbon flow in below- ground food webs assessed with isotope tracers" (FOR 918). This work (A.P.Mylnikov, ultrastructure observation) was supported by the Russian Foundation for Basic Research (15-04-05841, 14-04-00550). A.P. Mylnkov thanks Prof. S.A. Karpov for fruitful discussion on ultrastructural data.

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Part 2 - Eukaryvorous amoebae of the Thecofilosea, Cercozoa.

Chapter 3: Description of *Lecythium terrestris* sp. nov. (Chlamydophryidae, Cercozoa), a Soil Dwelling Protist Feeding on Fungi and Algae.

Protist, Vol. 167, 93–105, April 2016
http://www.elsevier.de/protis
Published online date 14 January 2016

Protist

ORIGINAL PAPER

Description of *Lecythium terrestris* sp. nov. (Chlamydophryidae, Cercozoa), a Soil Dwelling Protist Feeding on Fungi and Algae

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Submitted January 30, 2015; Accepted January 6, 2016
Monitoring Editor: Alastair Simpson

Testate amoebae have been frequently studied by protistologists, but still little information is available on some groups like the Chlamydophryidae. These amoebae are difficult to culture and therefore quantitative information on their morphology, phylogeny and ecology is scarce. We isolated and cultured a small testate amoeba from an agricultural field at Müncheberg near Berlin, Germany. Morphological analyses revealed it to be a new species of the genus *Lecythium*. We describe *Lecythium terrestris* sp. nov. and present its morphology, mycophagous and algivorous feeding habits and its ability to form cell aggregates by fusion. Using small-subunit ribosomal RNA gene phylogeny, we could confirm the phylogenetic position of the genus *Lecythium* among the Cercozoa where it groups closely to Pseudodifflugiidae (Tectofilosida).

Key words: *Chlamydophrys*; Tectofilosida; fungi; *Fusarium*; mycophagous yeast.

Introduction

Testate amoebae are frequently studied by protistologists, due to the often easily identifiable and distinct morphological characters of their tests (De Saedeleer 1934; Meisterfeld 2002). Within the last twenty years a number of molecular studies have been published that give insight into testate amoeba phylogeny (Cavalier-Smith and Chao 2003; Howe et al. 2011; Wylezich et al. 2002). Nevertheless, it is still the case that many testate amoebae, especially those with an organic test, lack molecular data and therefore a phylogenetic placement.

One of these groups are the Chlamydophryidae, for which we lack molecular data and have little knowledge of their life history, intraspecific morphological variation and ecology. De Saedeleer (1934) established the Chlamydophryinae (then as a subfamiliy of the Gromiidae), for genera like *Chlamydophrys* and *Lecythium*, and placed them in the Testaceafilosa, an assemblage of filose testate amoebae since then revealed to be polyphyletic (Howe et al. 2011; Wylezich et al. 2002). Later on some genera (e.g. *Rhogostoma* and

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http://dx.doi.org/10.1016/j.protis.2016.01.001

Capsellina) were added to this family (Meisterfeld 2002) but were subsequently removed and placed in a separate family Rhizaspididae (Howe et al. 2011). Clearly, molecular data are necessary to resolve the confusing phylogeny of all these testate amoebae. The Cercozoa, were established as a phylum after phylogenetic analyses (Cavalier-Smith 1998a, b). They include several independent groups of testate amoebae, e.g. the Euglyphida with tests made of siliceous plates (Cavalier-Smith and Chao 2003), the Rhizaspididae with organic tests (Howe et al. 2011) and the Pseudodifflugiidae that form tests by agglutinating foreign materials like sand grains (Wylezich et al. 2002). Based on their filose pseudopodia Howe et al. (2011) suggested the Chlamydophryidae also belong to the Cercozoa, but molecular data have so far been lacking to verify this.

We isolated a small testate amoeba with a thin and flexible test from an agricultural field at Müncheberg near Berlin, Germany. Morphological determination revealed it to be a new species of *Lecythium*. This report presents the morphology and behaviour of *L. terrestris* sp. nov. and further establishes its phylogenetic position within the Cercozoa.

Results

General Morphology and Life History Stages

The amoeboid organism is characterized by a thin and hyaline test that is usually oval or pyriform in shape and carried in an upright position (Figs. 1 and 2). The tests range from 12-17 μm in length to 10-16 μm in width with a length/width ratio of 1-1.27 (n= 22; Fig. 2). The aperture is usually positioned between folds of the test. These folds are variable in size and shape; in most individuals they are bent towards the outside of the cell, but in some cases towards the cell inside (Fig. 2).

The cell body contains one nucleus (about 4.5± 0.2 μm), which contains a single roundish nucleolus (about 2.1± 0.1 μm) and is usually located in the apical end of the cell (e.g. opposing the aperture). Contractile vacuoles are difficult to observe but cells usually contain one, rarely up to three. Granula (up to 3 μm) are quite frequent in the cell body but were never observed in the filopodia. They are usually dispersed randomly in most individuals, but sometimes accumulated in the centre and/ or the apical end.

Most filopodia of an actively moving individual extend in the direction of movement and seem to drag the cell body over the substrate, while filopodia of non-moving cells are formed in all directions (Fig. 1). The filopodia are hyaloplasmatic, variable in size and often branch and might anastomose, but rarely extend longer than 60 μm from the cell body. The filopodia were usually thin in diameter and long, but broad, striated lamellipodia could also be observed (Fig. 3). At several occasions a "loss" or "detachment" of a filopodium was observed. This could be also triggered by manual destruction of the filopodia. In most cases *L. terrestris* subsequently moved to the detached cell material and fused with it (Supplementary Material Video 1).

L. terrestris is able to form multinucleate cell aggregates (Figs. 1 and 3). These aggregates occurred rarely at constant food availability, but with a decrease of food concentration both the numbers of inactive uninucleate cells and the formation of aggregates increased. The life history of *L. terrestris* was reconstructed from time-lapse observations of cultures over 5 days with additional observation of starving cultures for several weeks (Fig. 4).

Stage I: Under constant availability of food (e.g. *S. cerevisiae*), *L. terrestris* engulfed whole prey cells. This led to an expansion of the cell size and, in the case of large food items, to a deformation of the test and cell body (e.g. when feeding on a banana-shaped *Fusarium* spore). A short digestion phase was followed by the excretion of non-digestable material (e.g. cell wall compounds) and then a longitudinal cell division (Fig. 5A, B and Supplementary Material Video 3).

Stage II: When food resources were exhausted ca. 95% of individuals formed inactive stages that were defined by complete withdrawal of filopodia, passive cell floating and gradual shrinkage of the cytoplasm (Fig. 5C, D). A cyst wall could not be detected. If no food sources were added, the cells died within about 4 months.

Stage III: Under food limitation ca. 5% of the individuals formed aggregates by fusion of usually less than 7 uninucleate individuals (Fig. 3), but occasionally aggregates formed by more than 20 individuals were observed. These multinucleate stages were characterized by an increased size (40-70 μm), longer and wider filopodia and high motility. Two types of aggregates could be observed: (A) regular aggregates are defined as a concentric arrangement of cells. Every fused cell retains its nucleus and cell shape. Those units might be organised (from the periphery to the centre) by 1-3 contractile vacuoles followed by a layer

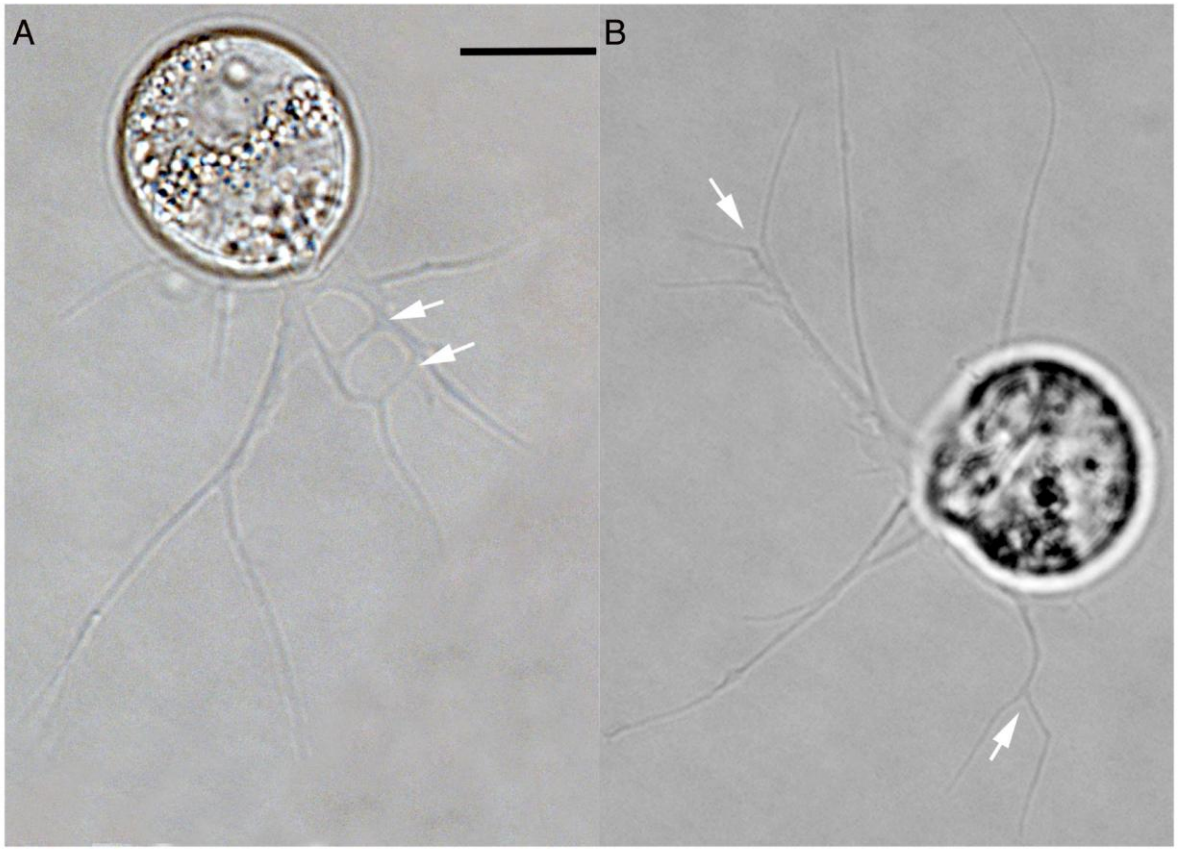

Figure 1. Filopodia of two active individuals of *Lecythium terrestris*. Arrows indicate anastomosing filopodia (**A**) and branched filopodia (**B**). Magnification bar indicates 10 μm.

of granula, followed by the nucleus, followed by another layer of granula; (B) irregular aggregates consist of deformed cells that lead to one big test enclosing several nuclei, sometimes resulting in a complete reduction of the tests of some fused cells.

Feeding Behaviour and Food Range

L. terrestris engulfed its prey by a siphon-like structure (Fig. 6; Supplementary Material Video 2). Feeding experiments revealed predation on a range of algae, unicellular fungi (yeasts) and spores of hyphae-forming fungi. No evidence of ingestion of bacterial cells could be found (Table 1). Depending on the supplied food organism the ingestion rate of *L. terrestris* differed, but was not quantified. Nevertheless all accepted algae, yeasts and fungal spores led to a sustainable growth of *L. terrestris*. The suitability of the prey appeared to be mostly depending on the motility of the prey and its size. For example, (A) the alga *Gloeochrysis apyrenigera* was readily ingested, but only when inactive (i.e. not moving) and (B) prey items usually had to be <25 μm long and <7 μm wide, e.g. *L. terrestris* was not able to ingest whole *Euglena mutabilis* cells due to their large size. Nevertheless, *L. terrestris* attached to dead *E. mutabilis* cells, and engulfed some of the cell contents (Fig. 6; Supplementary Material Video 2). However, some food sources (e.g. *Kirchneriella* sp., *Chromulina nebulosa* and all of the added bacteria) did not trigger active grazing behaviour and were therefore not ingested.

Phylogenetic Analysis

Almost the complete SSU sequence of *L. terrestris* (1728 bp) was obtained (GenBank accession number: KP728379). No intron could be found in the sequence. The reconstructed phylogeny is shown in Figure 7. The currently sequenced filose testate

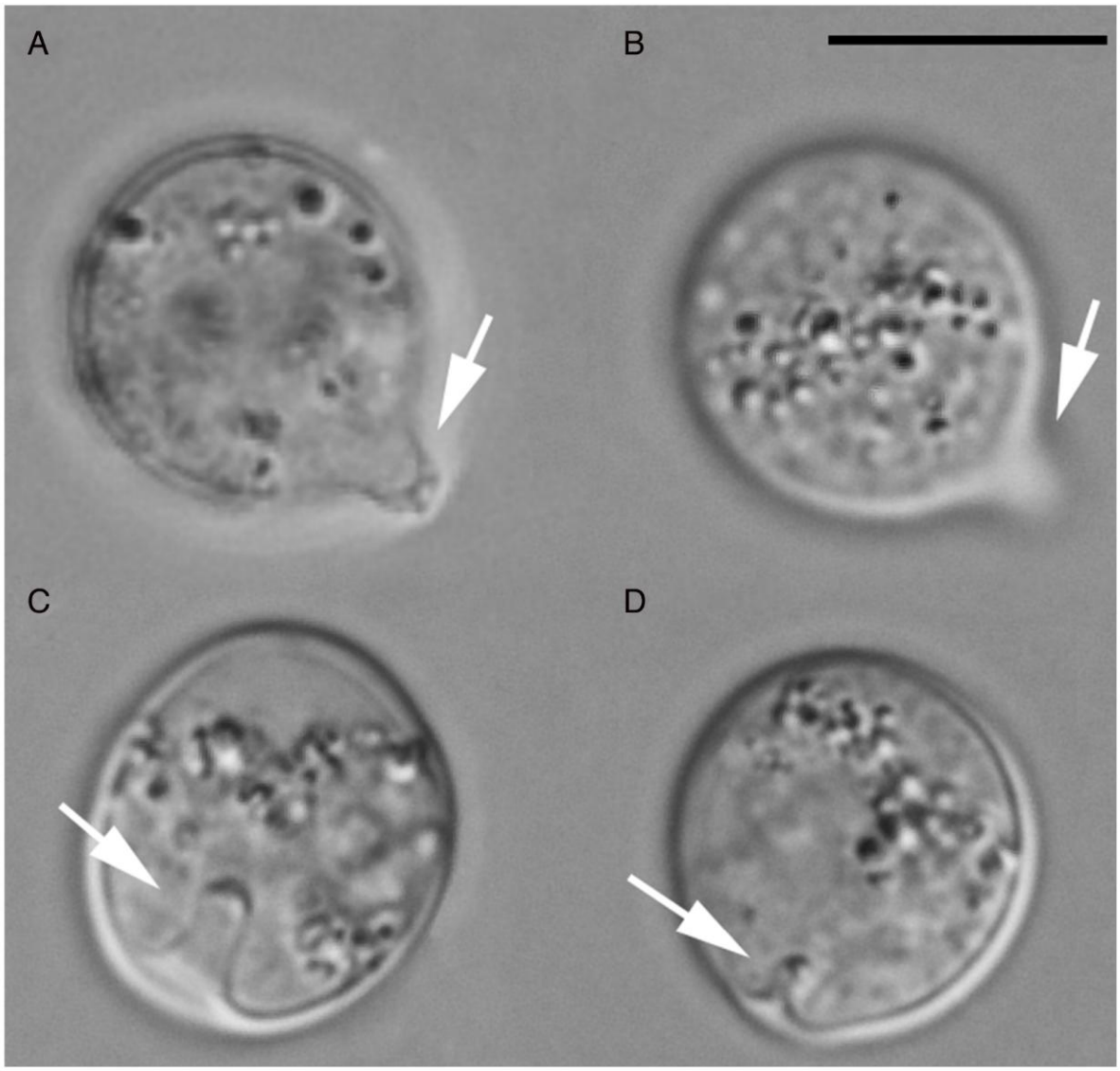

Figure 2. Tests of *L. terrestris* in differential interference contrast (DIC) at 600x magnification. The variation of the test opening is indicated by arrows. Micrographs **A** and **B** are taken from the same individual with its folds bent to the outside. **C** and **D** show two different individuals with folds bent to the inside. Magnification bar indicates 10 μm.

amoebae are highlighted. *L. terrestris*, *Pseudodifflugia cf. gracilis* and a group of environmental sequences clustered in a clade corresponding to the Tectofilosida with a bootstrap support value of 90% and Bayesian posterior probability of 1.0. In agreement with current evolutionary hypotheses, the Tectofilosida branched close to several genera like *Cryothecomonas, Protaspa, and Rhogostoma* in a clade corresponding to class Thecofilosea (Cavalier-Smith and Chao 2003; Howe et al. 2011; Yabuki and Ishida 2011). However, support for the basal branches of the Filosa was low, leaving open the exact position of Tectofilosida among cercozoans, in particular with respect to members of the class Imbricatea (Cavalier-Smith and Chao 2003; Howe et al. 2011; Yabuki and Ishida 2011).

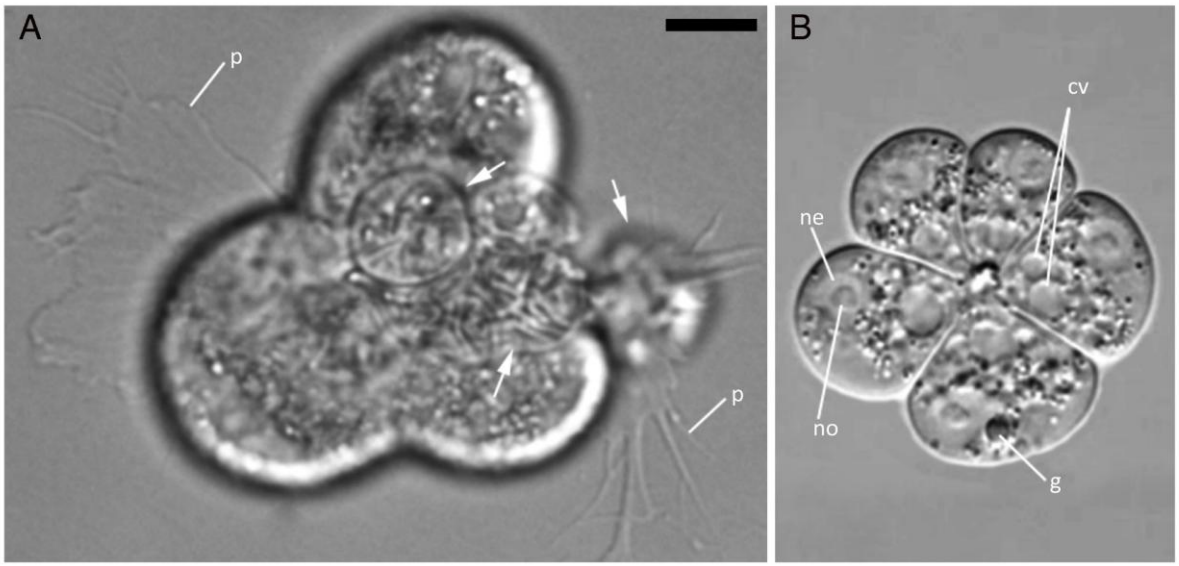

Figure 3. Cell aggregates of *L. terrestris*. (**A**) An irregular cell aggregate with broad, striated lamellipodia (p) in focus, arrows indicate deformed remains of tests of fused cells. The cell contained 10 nuclei (not shown). (**B**) A symmetrical cell aggregate. Each unit of the fused cell aggregate contains one nucleus (ne) with one roundish nucleolus (no), usually one, rarely two contractile vacuoles (cv) and several granula (g) of various size. Cells might be organised (from the the center to the periphery) by 1-3 contractile vacuoles, a layer of granula followed by the nucleus followed by another layer of granula. Magnification bar indicates 10 μm.

Diagnosis

Lecythium terrestris sp. nov.

Diagnosis: Colourless, testate amoebae. Test: round or pyriform, 12-17 μm (length), 10-16 μm (width), ratio 1-1.27 (length/width), colourless, hyaline, highly flexible, due to ingested material maybe deformed. Nucleus: round, about 4.5±0.2 μm, usually located in the apical end of the cell. Nucleolus: round, about 2.1±0.1 μm; central to nucleus. Locomotion: actively creeping with filopodia (which may anastomose and rarely extend longer than 60 μm); inactively by floating forms, these are formed during digestion and under unavailability of food material, the cytoplasm of starving cells shrinks during starvation. Cell aggregates: formed in older cultures with decline of food density, regular (concentric arrangement of cells where every fused cell retains its nucleus and cell shape) or irregular (deformed cells that lead to one big test enclosing several nuclei). Size depends on the number of fused individuals (usually <7 rarely more). Prey: immotile, unicellular fungi and algae <25 μm, no ingestion of bacteria observed. Prey gets ingested by a siphon-like pseudopodium, highly flexible and expandable (<20 μm). Cell division: longitudinal, binary.

Type material (hapantotype): A glass slide containing several fixed individuals is deposited in the Upper Austrian State Museum Invertebrate Collection as Inv. Nr. 2015/25; this material constitutes the name-bearing type of this species.

Type generating strain: CCAP 1943/1

Sequence of type generating strain (SSU rDNA): KP728379

Type locality: agricultural research field (V327) of the Leibniz Centre for Agricultural Landscape Research (ZALF) at Müncheberg near Berlin, Germany; N52°31′04.46″ E14°07′21.96″

Etymology: terrestris [Latin], pertaining to the earth or land. Referring to the habitat it was isolated from and with that the first described terrestrial *Lecythium* species.

Discussion

Assignment of *L. terrestris* sp. nov. to the Genus *Lecythium* and the Nomination of a New Species

Testate amoebae with hyaline and organic tests are poorly studied. Being a small filose testate amoeba with a hyaline, thin, flexible test without any scales

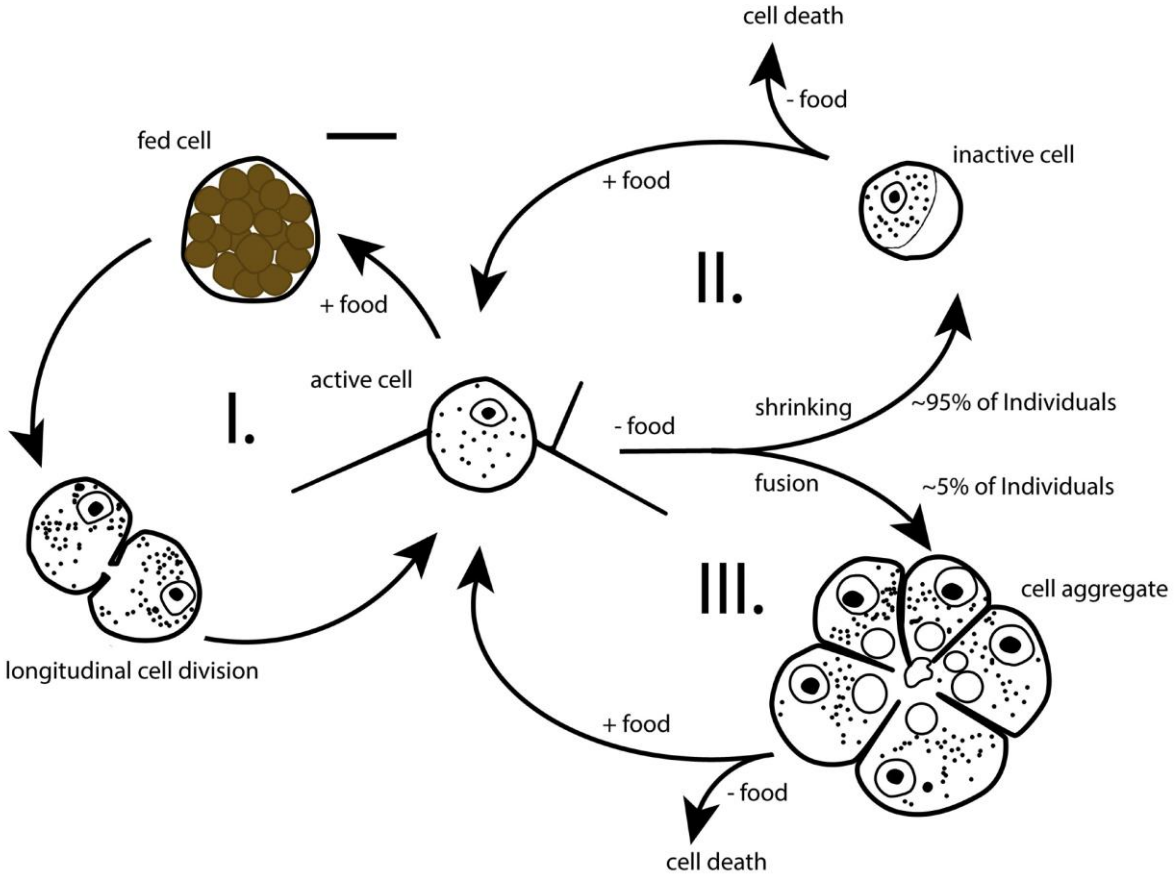

Figure 4. Behavioural response of *L. terrestris* to food supply (I.) or food limitation (II. and III.); -/+ food indicates the absence (-) or presence (+) of food in the culture. (I.) under food supply the cell feed and then divide longitudinally; under food limitation the cells might either (II.) shrink and rest or (III.) form active foraging cell aggregates by fusion (here of 5 individuals). Magnification bar indicates 10 μm.

or spines, our isolate could be morphologically identified either as a species of *Chlamydophrys*, *Lecythium* or *Leptochlamydophrys*, all of which belong to the Chlamydophyridae (De Saedeleer 1934; Meisterfeld 2002).

Since molecular data of these genera are lacking we made a thorough morphological analysis of our isolate and compared it with already described species of these genera. However, the differentiation of these genera is confusing and difficult. *Chlamydophrys* was described by Cienkowski (1876) with *Chlamydophrys stercorea* as type species and *Lecythium* was established as a genus by Hertwig and Lesser (1874) with *Lecythium hyalinum* as type species. These genera have in common that they are filose amoebae with a hyaline test, but we focus in our discussion on differences of the type species as well as later described species of those genera.

Chlamydophrys stercorea was described as an amoeba with numerous granules that are restricted to a median band and division by "budding", meaning that the cell divides through the aperture into two cells, one of which keeps the test, while other is an initially naked amoeba that builds a test de novo (Cienkowski 1876). Belar (1921) described bacterivory in all the *Chlamydophrys* species he observed (*C. stercorea*, *C. schaudinni*, *C. parva*, *C. major* and *C. minor*) as well as cannibalism, during which one individual ingested the other and sometimes even died, presumably due to the large size of the prey. Cienkowski (1876) and Belar (1921) gave very detailed descriptions for the formation of the cell aggregates (Cienkowski called them colonies,

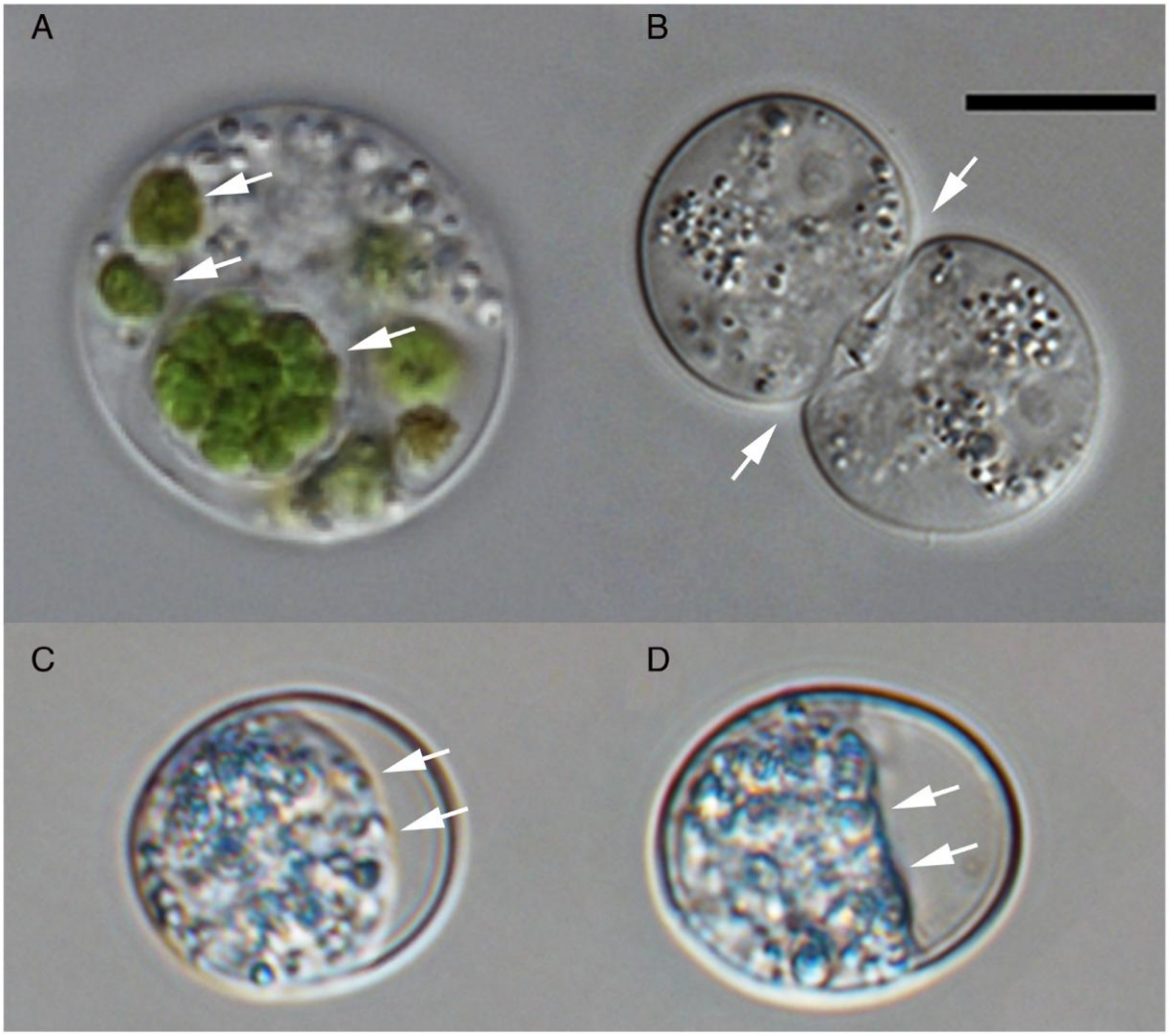

Figure 5. Different life history stages of *L. terrestris* in differential interference contrast (DIC) at 600x magnification. (**A**) a fed *L. terrestris* cell, arrows highlight some food vacuoles; (**B**) the longitudinal cell division, arrows indicate the axis of constriction (**C**, **D**) two starving cells, arrows indicate the cell body shrinking inside the test. Magnification bar indicates 10 μm.

Belar 'Plasmogams') in *C. stercorea* and *C. minor*. In those two species they were formed due to an incomplete cell division.

In the case of *Leptochlamydophrys* only the type species, *Leptochlamydophrys grata*, is known. This species was described by Breuer (1917) as *Chlamydophrys grata*, but was renamed by Belar (1921), because unlike *Chlamydophrys* it showed longitudinal division. However, morphological characters are not clear in the original description. *Leptochlamydophrys* was defined as similar to *Chlamydophrys*, but with longitudinal division. Breuer wrote that his observations were not based on a monoclonal culture, and since no findings have been reported ever since (Breuer 1917), these results might be questionable.

Lecythium hyalinum was also described with a zoning of cell contents, but granules were not restricted to a median band (Hertwig and Lesser 1874). Hertwig and Lesser (1874) gave no information about cell division, but subsequent protistologists reported longitudinal cell division in *L.*

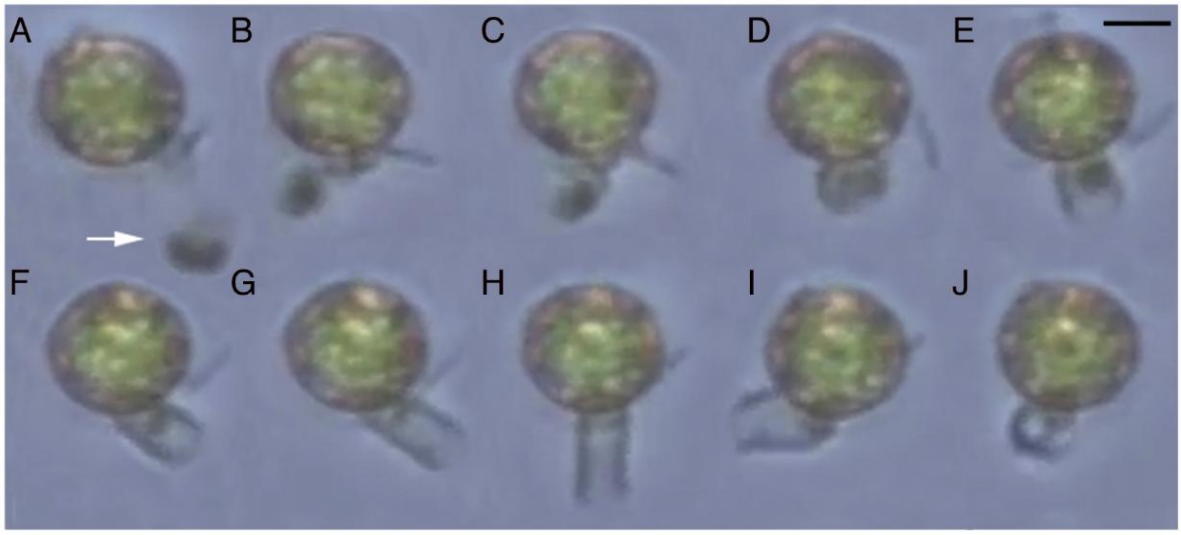

Figure 6. After ingestion of cell contents of *Euglena mutabilis*, the *Lecythium* cell seeks remaining prey cell content (indicated by a white arrow). With its filopodia, it draws the food particle in direction of the cell body (**A-C**); ingests it with its feeding-siphon (**D-F**); and seems to search for more cell content by moving its feeding-siphon into multiple directions (**G-I**) until the feeding siphon gets retracted (**J**). Magnification bar indicates 10 μm.

hyalinum, meaning that the mother cell splits up into two equal sized daughter cells (Belar 1921; De Saedeleer 1934). We did not find any report about bacterivory, cannibalism or detailed descriptions on the process of the formation of cell aggregates by *Lecythium*.

In the individuals observed in our clonal culture, granules were occasionally found in a median band, but in most cases they were dispersed all over the cell body, indicating, that this character is apparently not suitable for genus definition. We consider therefore the mode of cell division in *Chlamydophrys* and *Lecythium* as the discriminating character between the two genera. In *L. terrestris* the division is clearly longitudinal. Furthermore, it did not feed on bacteria and we never observed cannibalism. The aggregates were not formed due to an unfinished division, but by fusion. These differences compared to *Chlamydophrys stercorea* let us assign the isolate to the genus *Lecythium*.

Most *Lecythium* species are characterized by a main cell body larger than 30 μm (e.g. *L. hyalinum*, *L. granulatus, L. avidus* and *L. mutabilis*; De Saedeleer 1934; Hertwig and Lesser 1874; Leidy 1879; Schulze 1875). These species are by far too large to represent our isolate. Further, these species were found in freshwater habitats whereas *L. terrestris* was isolated from soil. Only two small *Lecythium* species have been described: *L. minutum*, the only described marine *Lecythium*, with a size of 9.3-11 μm, and *L. kryptosis* that is only poorly described with a size of 9-10 μm (Chardez 1971; De Saedeleer 1934). Both species are however far too small to represent our isolate. We therefore conclude that our isolate represents a new species in the genus *Lecythium.*

The Phylogenetic Position of the Chlamydophryidae and the Polyphyly of Filose Testate Amoebae

Based on morphology, De Saedeleer (1934) established the Testaceafilosa to unite filose testate amoebae such as the Chlamydophryidae, Pseudodifflugiidae and the Euglyphida. The advent of molecular phylogenies was necessary to shed light on the relationships between these amoebae, which have been shown to represent multiple independent lineages within Cercozoa.

Wylezich et al. (2002) and Cavalier-Smith and Chao (2003) showed in their phylogenetic analyses the polyphyly of the Testaceafilosa, with a sequence of *Pseudodifflugia cf. gracilis* (AJ418794) grouping in the Thecofilosea and several sequences of species of the Eugylphida grouping in the Imbricatea. Nikolaev et al. (2003) reported a sequence (AJ514867) that was annotated as "*Lecythium sp.*". This sequence does group in the Thecofilosea in our phylogenetic analysis, but does not show

Table 1. Results of the feeding experiments. √ food source was suitable for sustained culture growth ▲ big individuals could not be ingested; ▼ increased activity of *L. terrestris*, dead prey cells were partly ingested; – no ingestion could be observed;* shows GFP-tagged organisms; ○ refers to the natural bacteria community co-isolated with *Lecythium terrestris.*

Functional group	Phylum	Strain Number	Potential food source	Feeding
Yeasts	Ascomycota		*Candida rancensis*	√
			Metschnikowia reukaufii	√
			Saccharomyces cerevisiae	√
	Basidiomycota		*Cryptococcus laurentii*	√
			Cryptococcus victoriae	√
			Sporobolomyces roseus	√
			Sporobolomyces ruberrimus	√
Spores	Ascomycota		*Fusarium culmorum*	√
			Fusarium sp.	√
	Zygomycota		*Rhizopus sp.*	√
			Mucor sp.	√
Algae	Charophyta	CCAC 3009	*Cylindrocystis sp.*	-
	Chlorophyta	-	*Chlorella sp.*	√
		CCAC 3271 B	*Chlorella vulgaris*	√
		CCAC 3883	*Kentrosphaera sp.*	√▲
		CCAC 3525 B	*Kirchneriella sp.*	-
		CCAC 2315 B	*Neochlorosarcina sp.*	√
		CCAC 1896 B	*Stichococcus bacillaris*	√▲
	Euglenozoa	CCAC 0003	*Euglena mutabilis*	√▼
	Heterokontophyta	CCAC 4403 B	*Chromulina nebulosa*	-
		CCAC 4292 B	*Gloeochrysis apyrenigera*	√
Bacteria	Proteobacteria	50090	*Pseudomonas fluorescens* * *(filamentous)*	-
		ARQ1	*Pseudomonas fluorescens* * *(non filamentous)*	-
	Undetermined		Bacteria mixture○	-

a specific relationship with *L. terrestris*. Parfrey et al. (2010) showed a close relationship of that "*Lecythium sp.*" to *Capsellina sp.* (ATCC 50039; GenBank accession number GQ377676). Later Howe et al. (2011) obtained several sequences of *Rhogostoma spp.* that showed a close relationship to *Capsellina sp.* and "*Lecythium sp.*" Howe et al. (2011) assumed that the sequence obtained by Nikolaev et al. (2003) probably belongs to a misidentified *Rhogostoma minus*. Therefore the phylogenetic position of *Lecythium* remained still unknown.

The sequence of *L. terrestris* clustered together with *Pseudodifflugia cf. gracilis* in our phylogenetic analyses, with robust support values of 90%/1.0. We therefore confirm a close relationship of *Pseudodifflugia* and the genus *Lecythium* which is currently still grouped within the Chlamydophryidae and state that the Chlamydophryidae sensu Meisterfeld (2002), comprising e.g. *Lecythium* as well as *Rhogostoma*, should be considered as polyphyletic.

Accordingly, the filosan testate amoebae are currently grouped into three distinct phylogenetic clades, the Euglyphida (Imbricatea), the Rhizaspididae and the Tectofilosida (both Thecofilosea). The Rhizaspididae and the Tectofilosida show remarkable similarities in morphology. Both include genera with agglutinated tests (i.e., *Pseudodifflugia* in the Tectofilosida and *Capsellina* in the Rhizaspididae) as well as genera with hyaline tests (i.e. *Lecythium* in the Tectofilosida and *Rhogostoma* in the Rhizaspididae). Howe et al. (2011) provided a revised diagnosis of the Rhizaspididae emphasizing their bilateral symmetry, which according to them is unlike that of the Chlamydophryidae and other Tectofilosida. Therefore they supposed that the Chlamydophryidae might belong to the Tectofilosida, as we have now confirmed with phylogenetic analyses.

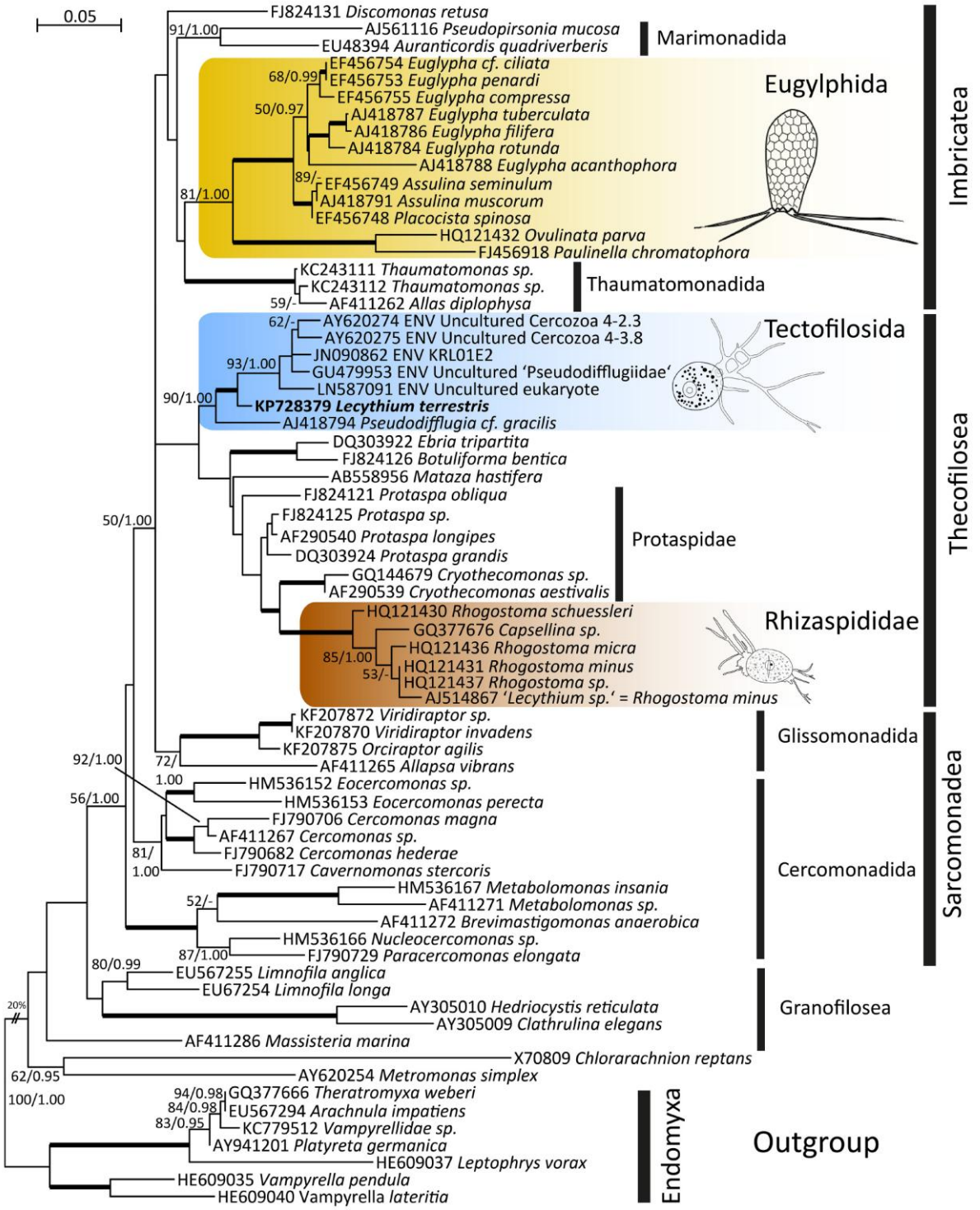

Figure 7. Reconstructed phylogeny for the Cercozoa with selected Endomyxa as outgroup. Shown is the maximum likelihood tree obtained by the PhyML GTR+I+G analyses including 69 sequences and using 1437 aligned sites. The support levels of the PhyML and the Bayesian analysis are shown on the respective branches (ML/BI)

Food Selection Experiments and Behavioural Response of *L. terrestris*

L. terrestris showed a broad nutritional range for eukaryotic food items that rarely exceed a maximum size of about 25 µm. Algae, unicellular fungi as well as fungal spores were digested and led to a sustained growth of *L. terrestris*. In most cases size and movement seemed to restrict the ingestion of the material, but some eukaryotes and all tested bacteria did not induce active grazing of *L. terrestris*. This indicates that *L. terrestris* might be able to sense and differentiate between possible prey organisms. Food choice experiments should therefore be performed to answer this question.

L. terrestris showed distinct morphological and behavioural changes in relation to food availability. The cell aggregates showed more and larger pseudopodia, as well as a higher motility compared to the small uninucleate cells. We consider these cell aggregates as exploratory stages for dispersal that might enable *L. terrestris* to migrate more easily to new patches of food. Also sexual reproduction might occur in these cell aggregates, but this could not be verified.

Methods

Isolation and establishment of monoxenic cultures: Soil was sampled at an agricultural research field (V327) of the Leibniz Centre for Agricultural Landscape Research (ZALF) at Müncheberg near Berlin, Germany. The agricultural site has been used to grow wheat and maize in a close crop rotation. An enrichment suspension was established by mixing 20 µl of a soil suspension (1 g fresh soil in 1 l Neff's Modified Amoebae Salinae "NMAS" (Page 1976), shaken for 10 minutes), 80 µl of a suspension containing *Fusarium culmorum* spores (8 Spores * μl^{-1}), 80 µl of a suspension containing *Saccharomyces cerevisiae* cells (332 cells * μl^{-1}) and 1 ml NMAS filled in six 24-well-plates. The plates were checked for protist growth after 7 and 21 days with an inverted microscope (Nikon eclipse TS 100, Japan). After 21 days *L. terrestris* showed high abundance and the yeast cells and spores had been consumed. *L. terrestris* was isolated by pipetting one single individual with a glass micropipette to a yeast suspension (200 µl of 332 cells * μl^{-1}+10 ml NMAS). The culture medium was later changed to Waris-H (McFadden and Melkonian 1986). Subsequently a bacteria-free culture of *L. terrestris* was established by sorting single individuals of *L. terrestris* to an axenic culture of the yeast *Cryptococcus laurentii*, using a BD FACSVantage SE (Becton-Dickinson Mountain View, CA, USA) with a 100 µm jet nozzle as described by Hess and Melkonian (2013). The food range experiments including the GFP-tagged *Pseudomonas fluorescens* (strains DSM 50090 and ARQ1; Jousset et al. 2006) were performed with bacteria-free cultures, all other experiments were performed with bacteria present.

Microscopic investigation and video recording: Cultures were continuously filmed (camera: Nikon digital sight DS-U2; program: NIS-Elements V4.13.04) for several days with an inverse microscope (100x-400x magnification, phase contrast, type: Nikon Eclipse TE 2000-E) and an upright microscope (600x magnification, DIC, type: Nikon Eclipse 90i).

Preparation of the Hapantotype: Culture material was mixed 1:4 with Schaudinn's fluid for 5 minutes at 60 °C, followed by a serial dehydration in an ascending series of ethanol mixtures. The fixed cells were mixed 1:2 with glycerine and transferred to a glass slide. A coverslide was glued on top.

Food choice experiments: Eleven fungal cultures were grown on Potato Glucose Agar (PGA) according to manufacturer's instructions (Sigma-Aldrich). Yeasts and algae were directly transferred to Waris-H before use. The fungal spores were suspended by transferring a 4cm^2 agar block into Waris-H. The suspension was shaken vigorously for 30 seconds to separate the spores from the agar block and subsequently the agar block was removed. The 10 algal strains were grown at 15 °C; light regime of 14 h light to 10 h dark with a light-intensity of about 5 µmol photons /m^2/sec. *Chlorella vulgaris* and *Kentrosphaera sp.* were grown in BBM + 3 V, *Chromulina nebulosa* and *Gloeochrysis apyrenigera* were grown in DY-V, all other algae were grown in Waris-H whereas *Stichococcus bacillaris* and *Euglena mutabilis* had an addition of 1% and 10% BSM respectively (for media composition see http://www.ccac.uni-koeln.de/textfiles/media.htm). The organisms were separately distributed in 5 cm diameter Petri dishes. Feeding success was determined by observations of ingestion, and cultures were checked for sustained growth and morphological changes of protist cells after 1, 4 and 12 days respectively with an inverted microscope. The GFP-tagged *Pseudomonas fluorescens* (strains DSM 50090 and ARQ1) were grown in LB Medium according to the manufacturer's instructions (AppliChem) as an overnight culture. 20 µl of bacteria suspension were given onto a glass slide to a 70 µl *L. terrestris* suspension and incubated for 1 hour. Subsequently protists in the suspension were checked for ingested bacteria with an upright microscope (600x magnification, type: Nikon Eclipse 90i) at fluorescent light with according filters.

SSU sequencing and phylogenetic analyses: 15 µl aliquots of monoclonal cultures were starved for 2 weeks and then transferred to a sterile 200 µl Eppendorf tube, and 13 µl ddH_2O were added. These samples were frozen at -20 °C to destroy the protist cells. The PCR was performed with a 50 µl reaction mixture containing 5 µl of 0.1 µM forward and reverse primer solution each, 5 µl dNTPs (200 µM), 5 µl reaction buffer and 1 U DreamTaq DNA-polymerase (Applied Biosystems, Weiterstedt, Germany). General eukaryotic primers for the small-subunit ribosomal RNA gene were used including EukA, EukB (Medlin et al. 1988) and 590F (Quintela-Alonso et al. 2011). 8 µl of the PCR product were purified by adding 0.15 µl of Endonuclease I (20 U/µl, Fermentas GmbH), 0.9 µl Shrimp Alkaline Phosphatase (1 U/µl, Fermentas GmbH) and 1.95 µl water. The mixture was heated 30 min at 37 °C, and

if values were above ML=50%; BI=0.95. If the maximum likelihood analysis showed support over 95%, the corresponding branches are shown in bold. The root is shortened to 20% and the groups of filose testate amoebae (i.e. the Euglyphida, Tectofilosida and the Rhizaspididae (in the Cryomonadida)) are highlighted in colour and with one simplified illustration each; pictures not to scale.

subsequently 20 min at 85 °C. The sequencing reaction was done by using the Big Dye Terminator Cycle sequencing kit and an ABI PRISM automatic sequencer.

The partial sequences were manually checked for sequencing errors and combined to one sequence contig. The sequence was deposited in the NCBI database under the accession number KP728379 and used for BLASTn search that revealed an affiliation of our isolate to the Cercozoa. Accordingly a Filosa (Cercozoa) alignment was established in SeaView (V4.5.3; Gouy et al. 2010) with Endomyxa as outgroup by manually aligning 64 selected SSU sequences of representatives of all major filosan groups, as well as 5 environmental sequences with a similarity of >95% to the sequence contig. The final alignment was manually trimmed to 1437 well-aligned sites and then used for phylogenetic analyses. The program jmodeltest (V2.1.5; Darriba et al. 2012) was used to find the best fitting model (GTR+I+G) by testing 88 different models (settings: Substitution Schemes 11; add Base frequencies +I+G rate variation nCat= 4 resulting in 88 models, ML optimized NNI as base tree). The GTR+I+G model was then used in a maximum likelihood (ML) analysis using PhyML (V3.1) (Guindon and Gascuel 2003). Statistical support for internal nodes was assessed with 100 bootstrap replicates. In addition, a Bayesian analysis was performed using MrBayes (V3.2.3) with a burn in of 25% and final split frequencies of less than 0.01 (settings: mcmcngen= 1000000 sample freq= 500 print freq= 500 diagn freq= 5000; Altekar et al. 2004; Ronquist and Huelsenbeck 2003).

Acknowledgements

This study was supported by the German Research Foundation (DFG) within the Research Unit 'Carbon flow in below-ground food webs assessed by isotope tracers' (FOR 918, subproject BO1907/2-2). We thank the Culture Collection of Algae at the University of Cologne (CCAC; http://www.ccac.uni-koeln.de/) for providing 9 of the 10 used algal strains, Dominik Bergerow (University of Bochum) for providing 5 of the 7 used yeast strains and Ferry Siemensma for exchange of expertise and helpful support with literature resources.

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2016.01.001.

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Chapter 4: A bowl with marbles: Revision of the thecate amoeba genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa, Rhizaria) including a description of four new species and an identification key.

Protist, Vol. 167, 440–459, November 2016
http://www.elsevier.de/protis
Published online date 12 August 2016

Protist

ORIGINAL PAPER

A Bowl with Marbles: Revision of the Thecate Amoeba Genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa, Rhizaria) Including a Description of Four New Species and an Identification Key

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Submitted January 26, 2016; Accepted August 2, 2016
Monitoring Editor: Alastair Simpson

Although testate amoebae have attracted interest of protistologists for more than 150 years, some groups especially those with a hyaline, organic test (= theca) are still poorly known. One of those is the genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa, Rhizaria), first described by Hertwig and Lesser in 1874. Only old, sometimes obscure, species descriptions were available until only recently a new species of *Lecythium* was described and a small ribosomal subunit RNA gene (SSU) sequence was provided. To shed light on the phylogeny and taxonomy of *Lecythium*, we (a) cultured six isolates of five *Lecythium* species and provide morphological as well as ecological observations, (b) obtained six new SSU sequences and conducted phylogenetic analyses of the Tectofilosida, showing that *Lecythium* splits into terrestrial and freshwater clades, and (c) did an intensive literature research on testate amoebae with a theca and provide an illustrated identification key focusing on *Lecythium*. For the first time, the presence of cysts in the genus *Lecythium* is reported and we compared those to the cysts of the presumed closely related *Chlamydophrys stercorea*. Our results suggest that still many undescribed *Lecythium* species will be found in terrestrial and freshwater habitats.

Key words: *Pamphagus*; *Chlamydophrys*; protist; fungivorous; algivorous; testate amoebae.

Introduction

Among amoebae those bearing a test present the advantage of being often easily identifiable. Tests, especially those containing silica or agglomerated sand grains, are persistent, which allows their recovery from dried or old environmental samples without the need of culturing; therefore they have long been focussed on by protistologists and ecologists (Mitchell et al. 2008). Taxonomy of testate amoebae was for a long time confusing and therefore often changed. Molecular

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http://dx.doi.org/10.1016/j.protis.2016.08.001

phylogeny finally revealed that testate amoebae are of polyphyletic origin (Cavalier-Smith 1998a, b; Nikolaev et al. 2005). Many testate amoeba lineages have been found to belong to the morphologically and ecologically highly diverse phylum Cercozoa (Cavalier-Smith 1998a, b), which consists predominantly of naked amoebae (Dumack et al., 2016a), amoeboflagellates (Bass et al. 2009) and flagellates (Howe et al. 2011). Cercozoan testate amoebae include order Euglyphida with tests made of siliceous plates (Cavalier-Smith 1998a, b), the family Rhizaspididae in order Cryomonadida with organic thecae (Howe et al. 2011), the families Pseudodifflugiidae with tests formed by agglutinating foreign material with organic cement (Wylezich et al. 2002) and, as was recently found, the Chlamydophryidae (De Saedeleer 1934), the latter two families belonging to the order Tectofilosida (Cavalier-Smith and Chao 2003; Dumack et al. 2016b).

The family Chlamydophryidae was established by de Saedeleer (1934) (then as a subfamily of Gromiidae) to accommodate genera like *Chlamydophrys* and *Lecythium*. Species of both genera are all filose amoebae with a hyaline test or theca, but they divide in different ways (Dumack et al. 2016b; Meisterfeld 2002). *Chlamydophrys* could easily be cultured and was therefore well characterized long ago (Belar 1921). In contrast, no *Lecythium* (Hertwig and Lesser 1874) were successfully cultured until recently, leading to sometimes poorly described species and therefore a confusing taxonomy. *Lecythium* includes species formerly erroneously assigned to e.g. *Pamphagus, Trinema* or *Gromia*. Recently, Dumack et al. (2016b) established a culture of *Lecythium terrestris* by providing yeasts and algae as a food source - bacteria having proven to be unsuitable. This triggered us to look for other *Lecythium* species to culture in a similar way.

To clarify the taxonomic status of the genus *Lecythium* and shed light on how the freshwater *Lecythium* are related to the soil derived *Lecythium terrestris* we obtained six new isolates. For each isolate we provide a detailed morphological description and a SSU sequence. By combining phylogenetic analyses and an extensive literature survey we are confident that four of the studied isolates are species new to science, which we herein describe.

[2] List of abbreviations: a = aperture; nu = nucleus; no = nucleolus; g = granules; bf = branched filopodia; af = anastomosing filopodia; fv = food vacuole; cv = contractile vacuole; fo = folds; vg = vacuole with granule; vh = hyaline vacuole; lg = large granule; sg = small granule; c = cyst wall; t = theca

Results[2]

Sampling and Culturing

Eight different *Lecythium*-like amoebae were observed from ca. 40 samples (Table 1), of which six were successfully isolated, two from terrestrial (*L. nemoris* and a novel strain of *L. terrestris*) and four from limnic samples (*L. siemensmai, L. asini, L. margaritae, L.* cf. *margaritae*). *L. hyalinum* and *L. mutabilis* did not grow under our culture conditions and the culture of *L.* cf. *margaritae* (KD1005) died shortly after we obtained a SSU sequence, but before we could preserve and mount any cells on a microscope slide.

Microscopical Observations

All isolated *Lecythium* species were filose amoebae with a highly flexible and hyaline theca, with sizes ranging from 12 to 63 μm (Fig. 1 and Table 2). The thecae were carried in an upright position and in most individuals were round, sometimes pyriform or deformed by folds in the theca or an aperture protruding or depressing. Inside the cells, refractive granules and vesicles were gathered at several layers. When forming aggregates (Figs 2–6C), the granules often spread equatorially and small granules formed a thin layer between nucleus and the theca at the apical end. In *L. margaritae* and *L.* cf. *margaritae*, the latter layer comprised hyaline vacuoles or vacuoles containing sometimes refractive granules (Figs 5, 6E), possibly lipid droplets. All observed *Lecythium* species were surface grazers, with highly branched and anastomosing filopodia (Figs 2–6D). Nevertheless some were able to form floating stages; in cultures of *L. terrestris* and *L. nemoris* floating cells with retracted filopodia were frequent, and individuals of *L. asini* and *L. hyalinum* formed floating stages with extended filopodia.

The nuclei of *L. asini, L. margaritae* and *L.* cf. *margaritae* appeared grainy (Figs 4–6) in contrast to *L. terrestris, L. siemensmai* and *L. nemoris* with a transparent nucleus (Figs 2, 3; *L. terrestris* not shown, see Dumack et al. 2016b). *L. nemoris* (Fig. 2), *L. siemensmai* (Fig. 3), *L. terrestris* (not shown, see Dumack et al. 2016b) and *L. asini* (Fig. 4) possessed a large and round nucleolus. However, in contrast to all other cultured *Lecythium* spp. with visible nucleoli, *L. margaritae* and *L.* cf. *margaritae* lacked a clearly visible one.

Cell aggregates were formed in older cultures with declining of food density, as explained for *L. terrestris* in Dumack et al. (2016b). A video of *L. asini*

Table 1. Information about the found *Lecythium* spp.; Strain and SSU sequence accession numbers, sampling location, food source in the sample (if observed).

Species	Strain	SSU rDNA accession	Sequence length	Coordinates	Isolation date	Habitat	Food source in sample
L. terrestris	KD1004	KT809356	1751 nt	52.517906, 14.122767	Apr 15	Agricultural field; surface soil	unknown
L. nemoris	KD1009	KT809361	1738 nt	50.861914, 7.195836	Jul 15	Moss growing on a withered tree stump in a deciduous forest	unknown
L. siemensmai	KD1008	KT809360	1722 nt	50.925411, 6.936010	Jul 15	Small pond at the greenhouses of the biocentre, Cologne	unknown
L. asini	KD1003	KT809357	1725 nt	51.081443, 6.788283	Apr 15	Large puddle with many green algae inside a donkey paddock	Diatoms, probably yeast
L. hyalinum	–	–	–	51.066492, 6.753195	Apr 15	Small natural pool next to a spring inside a forest, shadowy, much foliage	unknown
L. margaritae	KD1007	KT809359	1735 nt	50.979543, 6.990030	Apr 15	Riverbed of the Rhine, isolated from the first 2 cm of sediment consisting sand and stones (approx. 1–2 cm diameter)	Diatoms <50 μm
L. cf. *margaritae*	KD1005	KT809358	1739 nt	50.992730, 7.063243	Apr 15	Flooded area next to the Mutzbach, slow waterflow	Diatoms <20 μm
L. mutabilis	–	–	–	51.066492, 6.753195	Jul 15	Small natural pool next to a spring inside a forest, shadowy, much foliage	Diatoms >50 μm

in an early stage of aggregation with fusing filopodia is provided as Supplementary Material Video S1.

Cysts of *L. asini*

The cysts of *L. asini* were built inside the theca and showed condensed cell contents and a cyst wall surrounded by the theca (Fig. 7). Each granule seemed to be attached to a smaller granule; this could not be observed in active cells (Fig. 7C). *L. asini* cysts excysted after an incubation of about two weeks, when the culture medium was changed and fresh food was added, resuming normal behavior and reproduction.

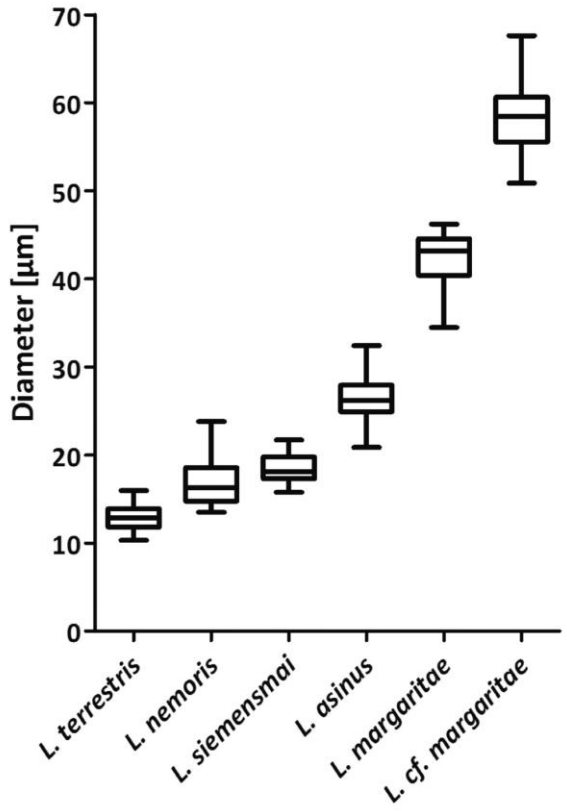


Figure 1. Cell body diameter in µm of the cultured strains and their distribution around the mean of the obtained cultures, displayed as boxplot graphs. The box indicates lower quartile, the median, and higher quartile, from bottom to top. The whiskers represent the range of the remaining data points.

Selective Feeding Experiment

Ten food sources were offered to the isolates of *Lecythium* spp., consisting of various eukaryotes (diatoms, one green alga and one yeast species) and some co-isolated not further determined bacteria (Table 3). Since the thecae of *Lecythium* spp. are transparent, feeding success could be directly monitored by light microscopy. The largest food source tested, the diatom *Nitzschia acicularis* (60 µm in length), was exclusively ingested by *L. margaritae*, the largest tested *Lecythium* species. The second largest diatom *Nitzschia amphibia* (45 µm in length) was ingested by *L. margaritae* and *L. asini*, the largest and second largest *Lecythium* species. The medium sized *Nitzschia communis* strains (~30 µm in length) were ingested by all tested *Lecythium* species. The undetermined very small pennate diatom (~7 µm), was a suitable food for all *Lecythium* species except *L. nemoris* and *L. margaritae*. The cylindrical and floating diatoms *Cyclotella meneghiniana* and *Stephanodiscus binderanus* gave different outputs. *Cyclotella meneghiniana* was not suitable as a food source at all, while *Stephanodiscus binderanus* was consumed by all *Lecythium* species but *L. asini*. The green alga *Characium* sp. was not ingested by *L.* cf. *margaritae.* The yeast *Saccharomyces cerevisiae* was not suitable for *L. margaritae* and *L.* cf. *margaritae.* No ingestion of bacteria was observed in any of our cultures.

Lecythium Phylogeny

Nearly full-length SSU sequences were obtained, ranging from 1,722 to 1,751 nucleotides (Table 1).

Table 2. Morphological measurements, and floating ability of the identified *Lecythium* spp.

Species	Strain	Cell size	Size of granules	Nucleus size	Nucleolus	Floating ability
L. terrestris	KD1004	12–17 µm	0.8–2.5 µm	~5 µm	yes	yes (filopodia withdrawn)
L. nemoris	KD1009	13–21 µm	0.4–1.8 µm	4.8–8 µm	yes	yes (filopodia withdrawn)
L. siemensmai	KD1008	16–22 µm	0.5–1.5 µm	5.5–7.3 µm	yes	–
L. asini	KD1003	19–32 µm	0.4–2.5 µm	11–16.5 µm	yes	yes (filopodia extended)
L. hyalinum	–	~40 µm	–	–	yes	yes (filopodia extended)
L. margaritae	KD1007	34–45 µm	1.2–4.6 µm	14–20 µm	no	–
L. cf. *margaritae*	KD1005	52–63 µm	1–8 µm	20–27 µm	no	–
L. mutabilis	–	~120 µm	–	–	–	–

Table 3. Results of the selective feeding experiment. – = no ingestion; ○ = some individuals ingested, no culture growth; ● =food source led to sustainable culture growth, ingestion rates differed but were not quantified; 0 not tested, ▲ marks food source used to culture the corresponding strain, note that some isolates only grew well with two food sources.

Food source			*L. terrestris*	*L. nemoris*	*L. siemensmai*	*L. asini*	*L. margaritae*	*L.* cf. *margaritae*
Species	Strain	Length [µm]						
Diatoms								
Nitzschia amphibia	CCAC 5733 B	~45 µm	–	–	–	●	●	0
Nitzschia communis	CCAC 1762 B	~30 µm	●	●	●	●▲	●	●▲
Nitzschia communis	CCAC 5737 B	~30 µm	●	●	●	●	●	0
Nitzschia acicularis	CCAC 5734 B	~60 µm	–	–	–	–	●	0
unknown pennate diatom	CCAC 5738 B	~7 µm	●	–	●	●	–	0
Cyclotella meneghiniana	CCAC 5735 B	~22 µm	–	–	–	–	○	0
Stephanodiscus binderanus	CCAC 5736 B	~12 µm	●	●	●	○	●	0
Green Alga								
Characium sp.	–	~15 µm	●	●	●▲	○	●▲	–
Yeast								
Saccharomyces cerevisiae	–	~5 µm	●▲	●▲	●▲	●▲	○	–
Bacteria								
Co-isolated not determined environmental bacteria	–	~1 µm	–	–	–	–	–	–

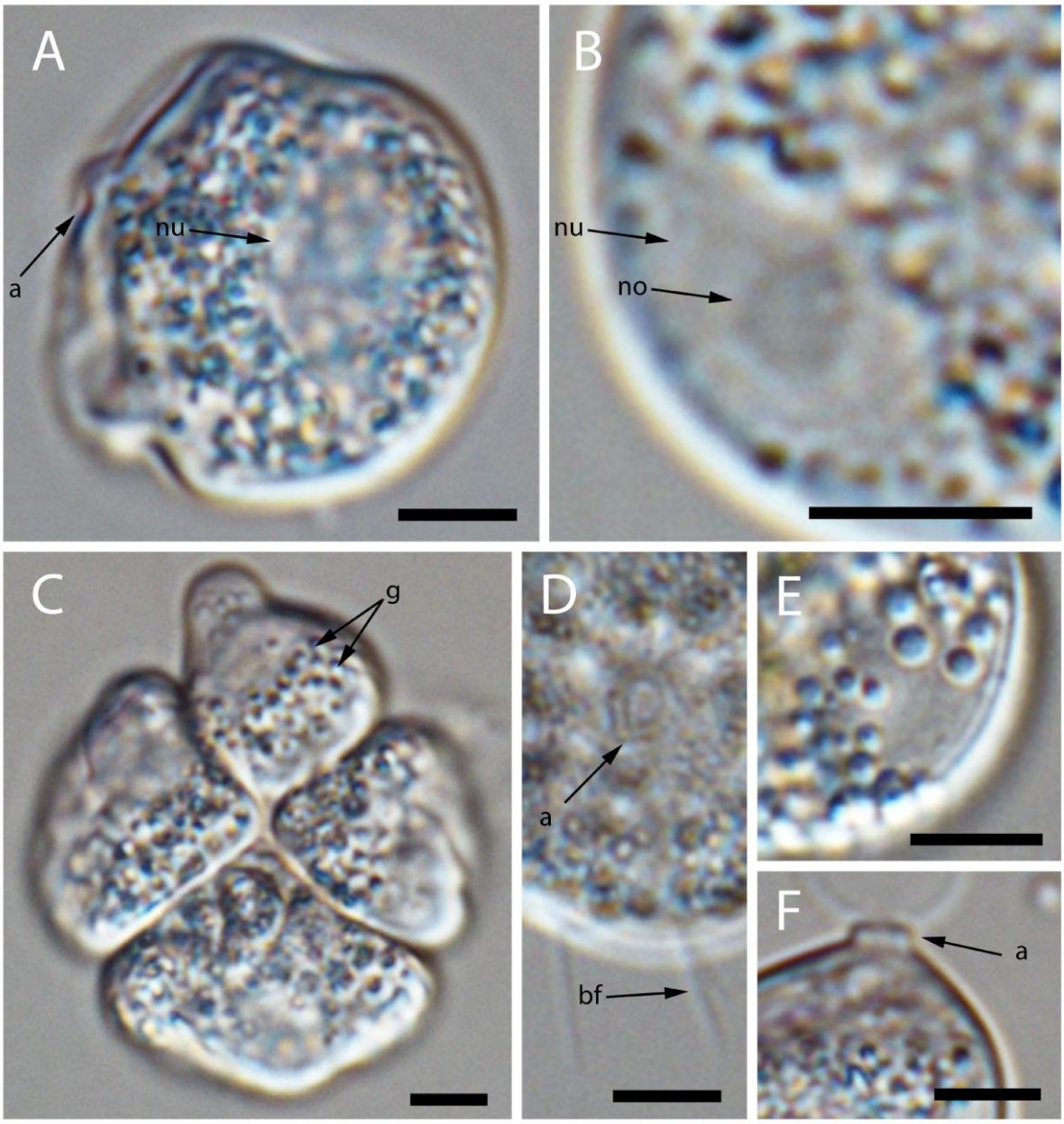


Figure 2. Cellular features of *Lecythium nemoris* (KD1009). Scale bars indicate 5 μm, pictures were taken with DIC. **A**: Side view, notice that the theca is wrinkled. **B**: The clear nucleus with the nucleolus. **C**: Aggregate consisting of four deformed individuals of similar size. **D**: A picture from the basal end of an active cell. **E**: Close-up of some granules. **F**: A slightly compressed individual with a salient aperture (arrow).

No introns were found. The reconstructed phylogeny is shown in Figure 8. All sequenced isolates formed a highly supported monophyletic group with the recently described *L. terrestris* (KP728379; Dumack et al. 2016b), but separated into two distinct clades. These are referred to as (a) the moderately supported terrestrial clade (75% ML bootstrap support; 1.00 posterior probability), comprising both *L. terrestris* isolates, *L. nemoris* and the terrestrial derived

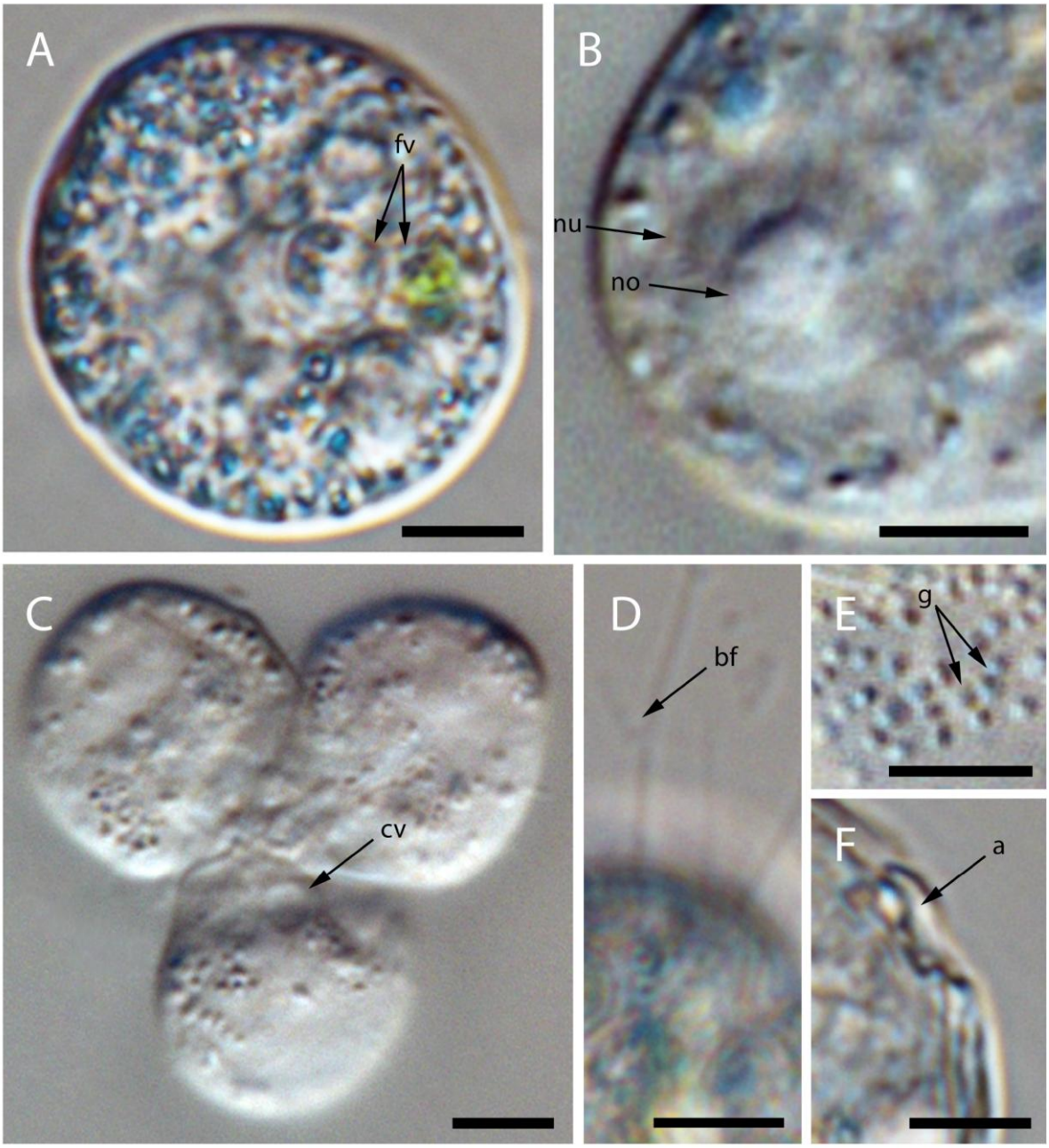


Figure 3. Cellular features of *Lecythium siemensmai* (KD1008). Scale bars indicate 5 μm, pictures were taken with DIC. **A**: Overview of a cell containing several yeast cells and a green alga. **B**: The nucleus with a clearly visible, round nucleolus inside. **C**: Cell aggregate consisting of three individuals. **D**: A picture from the basal end of an active cell. **E**: Close-up of some granules. **F**: The aperture seen from the side, slightly bent to the outside with wrinkles (arrowhead) extending from it. A video of *L. siemensmai* is available as Supplementary Material Video S2.

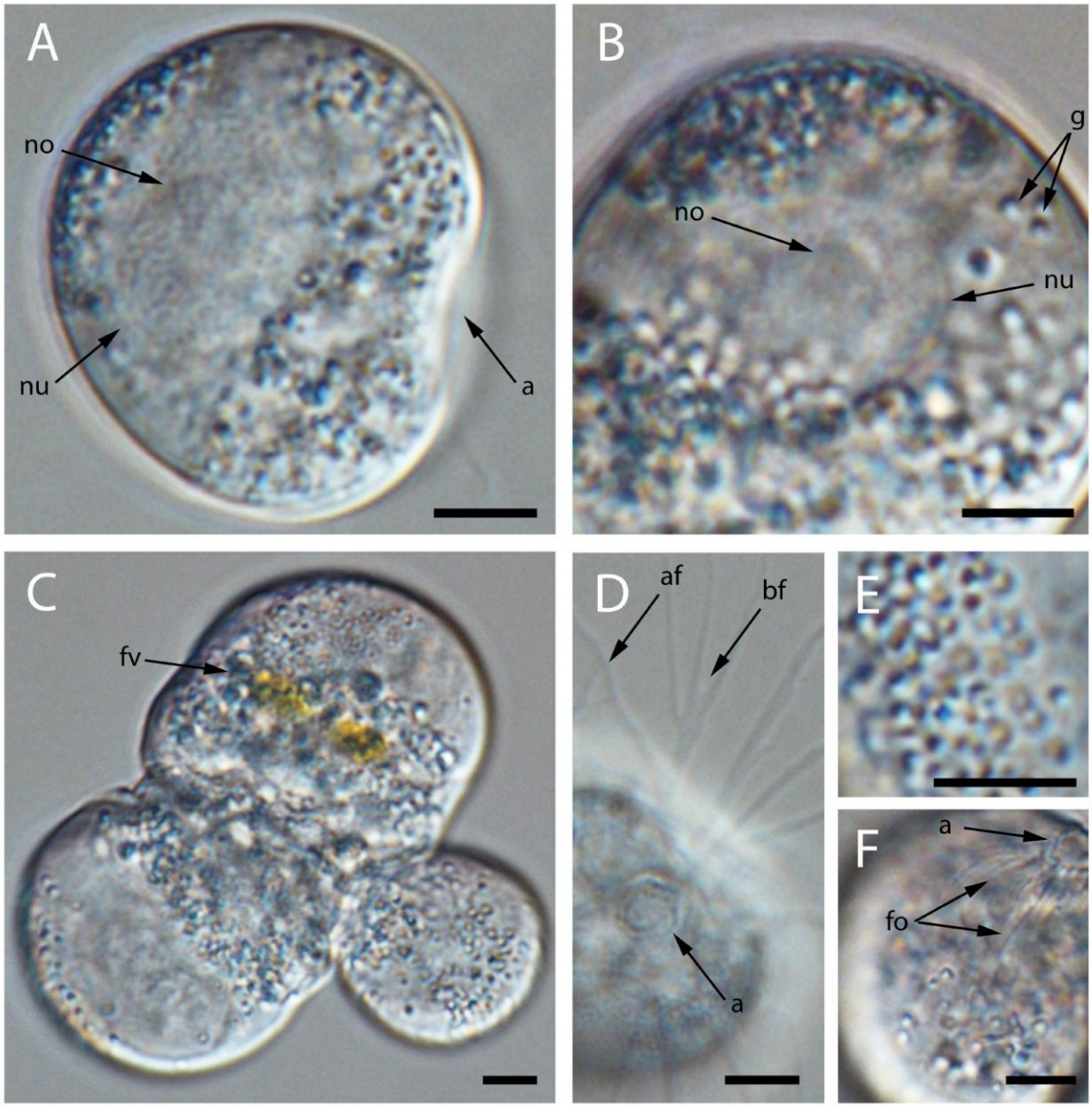


Figure 4. Cellular features of *Lecythium asini* (KD1003). Scale bars indicate 5 μm, pictures were taken with DIC. **A**: Overview image. **B**: Close-up of the fine grained nucleus (arrow) and the nucleolus. **C**: Three aggregated individuals of different sizes. **D**: A picture from the basal end of an active cell. **E**: Close-up of some granules. **F**: Hyaline vacuoles near the aperture in the upper right corner and wrinkles in the theca, expanding from it.

environmental sequence LN587091 (Scott et al. unpublished) and (b) the slightly better supported freshwater clade (83/1.00) that comprises *L. margaritae*, *L.* cf. *margaritae, L. siemensmai*, *L. asini* and the freshwater derived environmental sequence AY620274 (Bass and Cavalier-Smith 2004).

Diagnoses

Genus *Lecythium* Hertwig et Lesser, 1874 emend

Emend. diagnosis: Filose radially symmetric amoebae. Cell bodies covered by a colourless, thin, flexible (i.e. stretchable), organic theca, mostly

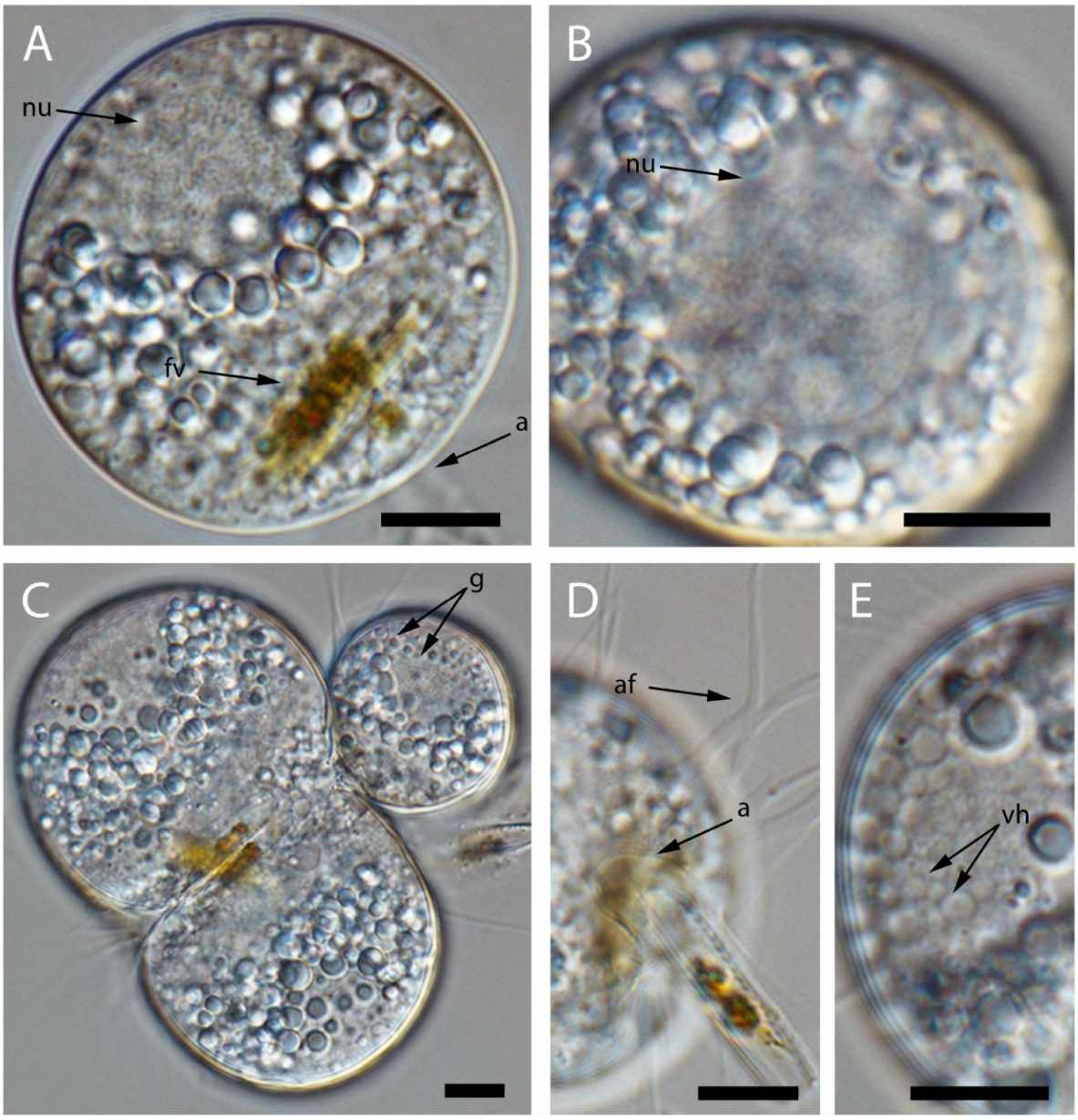


Figure 5. Cellular features of *Lecythium margaritae* (KD1007). Scale bars indicate 10 μm, pictures were taken with DIC. **A**: Overview image. **B**: Close-up of the grainy nucleus. **C**: Cell aggregate consisting of three cells. **D**: A picture from the basal end of an active cell while expelling an empty *Nitzschia communis* frustule. **E**: Thin layer of hyaline vacuoles between the nucleus and the apical end of the theca.

pyriform or spherical; often deformed by ingested food, sometimes wrinkled or folded. Theca has one basal opening through which granule-free filopodia emerge; at least *L. hyalinum* also has emerging vacuolated cytoplasm. Apical ellipsoid or round nucleus, clear or grainy; round nucleolus sometimes visible by light microscopy. One or several contractile vacuoles (hard to see) and food vacuoles, usually basal. Granules vary in amount, sometimes in layers, sometimes spread evenly. Cells creep by filopodia, which may branch or anastomose; most carry the cell body upright. Can form

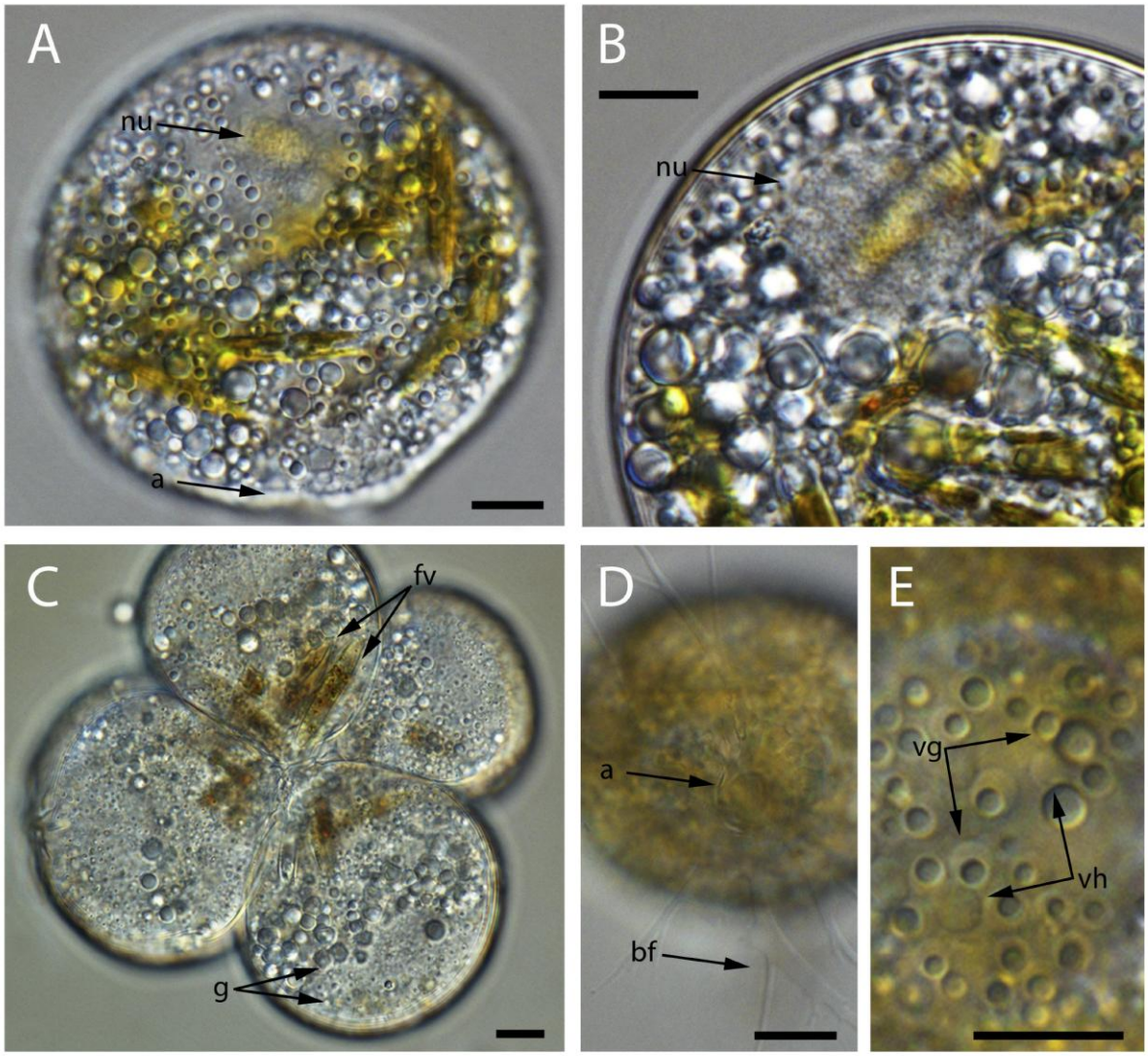


Figure 6. Cellular features of *Lecythium* cf. *margaritae* (KD1005). Scale bars indicate 10 μm, pictures were taken with DIC. **A**: Overview, with ingested diatoms. **B**: Close-up on the fine-grainy nucleus. **C**: Four individuals forming a regular aggregate. Note the wrinkles in the apical end of the theca. **D**: A picture from the basal end of an active cell. **E**: Hyaline vesicles of which some contain smaller, refractive granules, observed in a thin layer between the nucleus and the apical end of the theca.

floating stages with retracted or expanded filopodia. Cysts observed only in *L. asini*. Cells may aggregate basolaterally in a ring, each retaining its nucleus and individual shape, or apparently partially fuse basally within one multinucleate lobed theca. Eukaryotic prey (yeasts, algae) caught by filopodia; dragged to thecal opening for ingestion. No bacterial ingestion observed. At least *L. terrestris* can form a tubular feeding siphon. Most freshwater or terrestrial; one marine species. Division longitudinal, binary. Sexual or flagellate stages unknown.

Type species: *L. hyalinum* (Hertwig and Lesser 1874)

Lecythium nemoris Baumann, Dumack et Bonkowski, sp. nov.

Diagnosis: Cell body: round, diameter 13–21 μm. Ellipsoidal clear nucleus; long axis 4.8–8 μm.

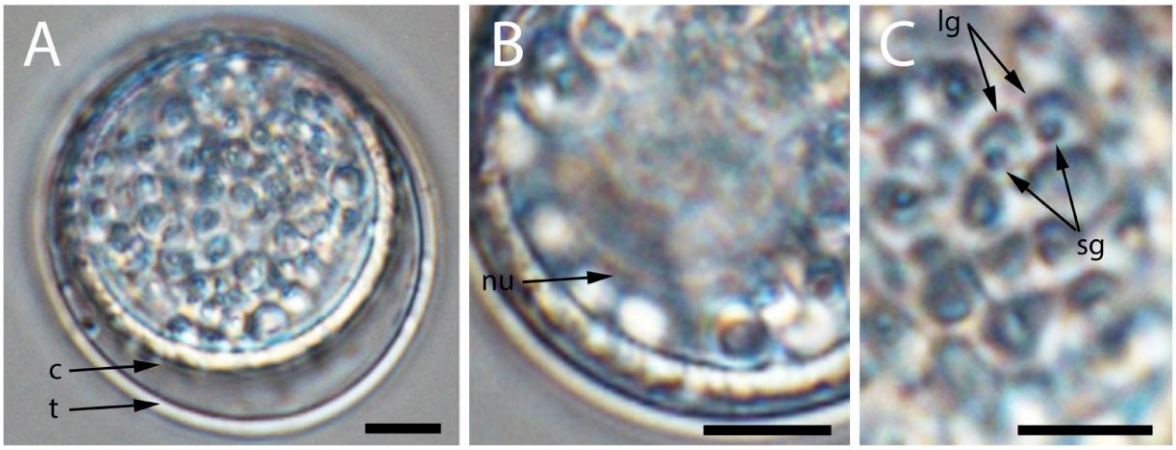


Figure 7. Cyst of *Lecythium asini*; scale bars indicate 5 μm, pictures were taken with DIC. **A**: Overview of the granule-filled cyst surrounded by two walls the outer theca and the inner cyst wall. **B**: Close-up on the nucleus in lateral position. **C**: The granules each seem to have a smaller granule attached. For animations of this cyst and a second individual see Supplementary Material Videos S3, S4.

Central round nucleolus. May form floating stages with retracted filopodia during or after digestion of food. Older food-depleted cultures have clumps of 2–7 basolaterally adhering cells, sometimes basally partially fused.

Type material (hapantotype): A glass slide containing several fixed individuals is deposited in the Upper Austrian State Museum Invertebrate Collection as Inv. Nr. 2015/776; this material constitutes the name-bearing type of this species.

Type generating strain: deposited in the Culture Collection of Algae and Protozoa, accession number CCAP 1943/2

SSU sequence of type generating strain: KT809361

Illustrations of type generating strain: Fig. 2

Type locality: Germany; patch of moss growing on a withering tree stump in a deciduous forest; coordinates: N 50.861914, E 7.195836.

Etymology: *nemoris* [Latin], genitive of *nemus, m* [Latin] = grove, forest. Refers to the sampling spot.

Lecythium siemensmai Baumann, Dumack et Bonkowski, sp. nov.

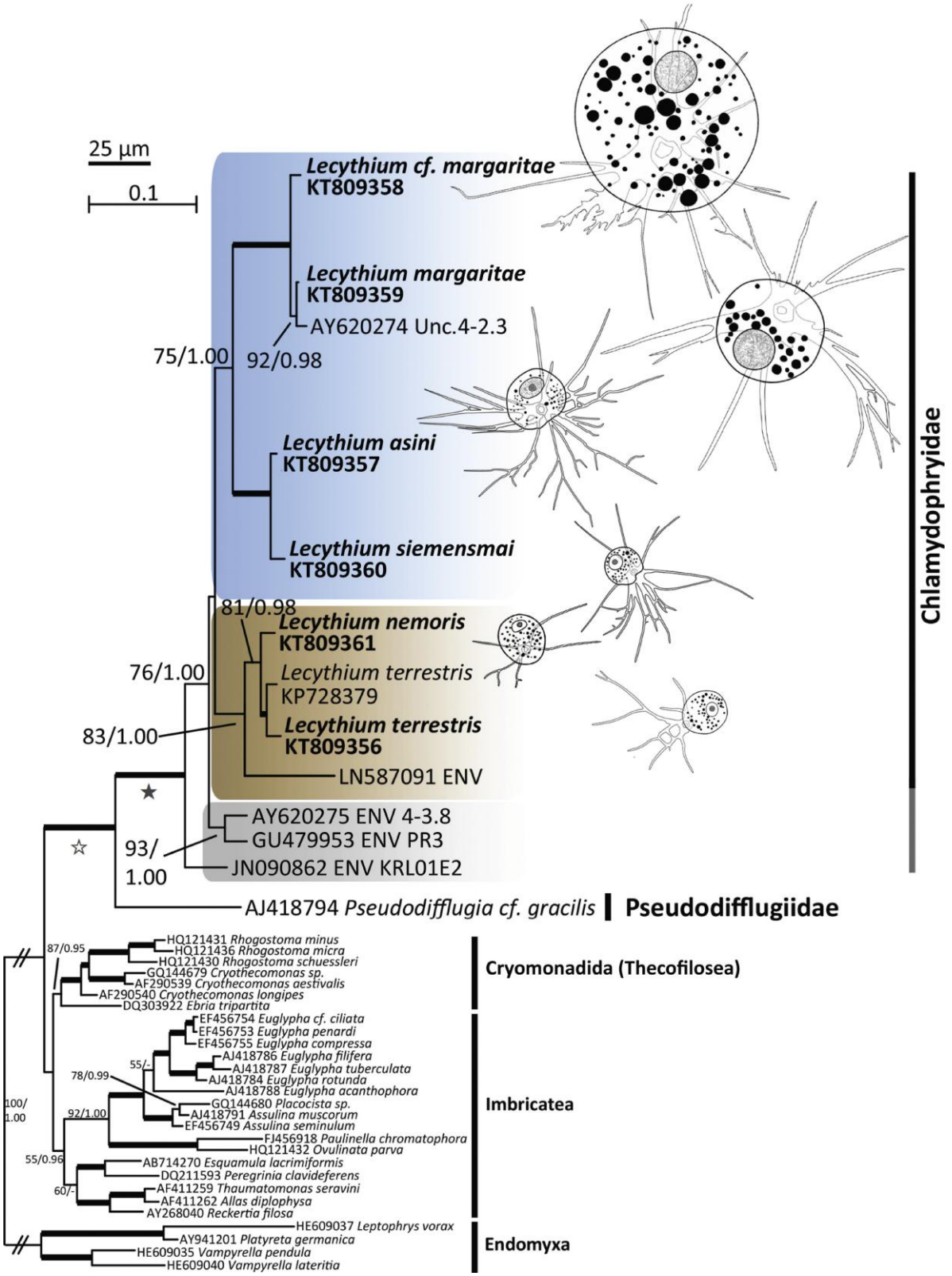
Diagnosis: Cell body: round, diameter 15–22 μm. Round clear nucleus; 5.5–7.3 μm. Central round nucleolus. No floating stages observed. Cell aggregates as in *L. nemoris*.

Type material (hapantotype): A glass slide containing several fixed individuals is deposited in the Upper Austrian State Museum Invertebrate Collection as Inv. Nr. 2015/777; this material constitutes the name-bearing type of this species.

Type generating strain: deposited in the Culture Collection of Algae and Protozoa, accession number CCAP 1943/4

SSU sequence of type generating strain: KT809360

Figure 8. Phylogeny of the genus *Lecythium* with a selection of thecofilosean, imbricatean and endomyxan sequences as outgroup based on SSU rDNA sequence. Shown is the best maximum likelihood tree obtained by PhyML analyses. Families and orders are indicated by vertical bars, newly obtained sequences are in bold. According to their sampling sites, three groups are marked in colour (brown: terrestrial derived *Lecythium* sequences; blue: fresh water derived *Lecythium* sequences; grey: three previously published environmental sequences that we provisionally group in the Chlamydophryidae, but were not colored since they might not resemble *Lecythium* species). The support values of the PhyML and the Bayesian analyses are shown on the respective branches (ML/BI). Support values <50% (bootstrap values) and <0.95 (posterior probabilities) are not shown (-), whereas branches with bootstrap values >95% are presented in bold. Interrupted branches (//) show 25% of their original length. Sequences not belonging to the Tectofilosida have been reduced in pixel size by 50% (includes branch length). The scale bars represent 25 μm and 0.1 nucleotide substitutions per site, respectively.


25 µm
0.1
Lecythium cf. margaritae
KT809358
Lecythium margaritae
KT809359
AY620274 Unc.4-2.3
75/1.00
92/0.98
Lecythium asini
KT809357
Lecythium siemensmai
KT809360
81/0.98
Lecythium nemoris
KT809361
76/1.00
Lecythium terrestris
KP728379
83/1.00
Lecythium terrestris
KT809356
LN587091 ENV
AY620275 ENV 4-3.8
GU479953 ENV PR3
93/
1.00
JN090862 ENV KRL01E2
AJ418794 Pseudodifflugia cf. gracilis
Pseudodifflugiidae
Chlamydophryidae
87/0.95
HQ121431 Rhogostoma minus
HQ121436 Rhogostoma micra
HQ121430 Rhogostoma schuessleri
GQ144679 Cryothecomonas sp.
AF290539 Cryothecomonas aestivalis
AF290540 Cryothecomonas longipes
DQ303922 Ebria tripartita
Cryomonadida (Thecofilosea)
EF456754 Euglypha cf. ciliata
EF456753 Euglypha penardi
EF456755 Euglypha compressa
AJ418786 Euglypha filifera
55/-
AJ418787 Euglypha tuberculata
78/0.99
AJ418784 Euglypha rotunda
AJ418788 Euglypha acanthophora
100/
1.00
GQ144680 Placocista sp.
92/1.00
AJ418791 Assulina muscorum
EF456749 Assulina seminulum
FJ456918 Paulinella chromatophora
HQ121432 Ovulinata parva
55/0.96
AB714270 Esquamula lacrimiformis
DQ211593 Peregrinia clavideferens
60/-
AF411259 Thaumatomonas seravini
AF411262 Allas diplophysa
AY268040 Reckertia filosa
Imbricatea
HE609037 Leptophrys vorax
AY941201 Platyreta germanica
HE609035 Vampyrella pendula
HE609040 Vampyrella lateritia
Endomyxa

Illustrations of type generating strain: Fig. 3
Type locality: small pond of the Institute of Zoology of the University of Cologne in the botanic garden next to the Cologne Biocenter, Cologne, Germany; stagnant water (small pond) with water lilies; coordinates: N 50.925411, E 6.936010.
Etymology: siemensma –i, this species was dedicated to Ferry J. Siemensma, a hobby-microscopist who created a very useful website on amoebae (www.arcella.nl), being an excellent guide for students new to amoeba taxonomy.

Lecythium asini Dumack, Baumann et Bonkowski, sp. nov.

Diagnosis: Cell body: round, diameter 19–32 μm. Ellipsoidal grainy nucleus; long axis 11–16.5 μm. Central round nucleolus. May form floating stages with extended motionless filopodia that expand to all directions. Cell aggregates usually with 2–5 cells. Smooth-walled dense cysts form within theca. Granules in the cyst seem to have another, smaller, granule attached.
Type material (hapantotype): A glass slide containing several fixed individuals is deposited in the Upper Austrian State Museum Invertebrate Collection as Inv. Nr. 2015/778; this material constitutes the name-bearing type of this species.
Type generating strain: deposited in the Culture Collection of Algae and Protozoa, accession number CCAP 1943/3
SSU sequence of type generating strain: KT809357
Illustrations of type generating strain: Fig. 4+7
Type locality: Big puddle in a donkey paddock of a deer park in Dormagen, Germany; coordinates: N 51.081443, E 6.788283.
Etymology: *asini -i, m* [Latin] = donkey. Refers to the sampling spot inside a donkey paddock of the deer park "Tierpark Tannenbusch".

Lecythium margaritae Dumack, Baumann et Bonkowski, sp. nov.

Diagnosis: Cell body: round, diameter 34–45 μm. Round grainy nucleus; 14–20 μm. Nucleolus not visible using light microscopy. With a thin layer of vacuoles (some hyaline, some filled with refractive granules, possibly lipid droplets) in the apical end of the cell. No floating stages were observed. Cell aggregates usually with 2–4 cells.
Type material (hapantotype): A glass slide containing several fixed individuals is deposited in the Upper Austrian State Museum Invertebrate Collection as Inv. Nr. 2015/775; this material constitutes the name-bearing type of this species.
Type generating strain: deposited in the Culture Collection of Algae and Protozoa, accession number CCAP 1943/5
SSU sequence of type generating strain: KT809359
Illustrations of type generating strain: Fig. 5
Type locality: bank of the river Rhine in Cologne, Germany; shallow river sediment; sand and pebbles <3 cm; coordinates: N 50.979543, E 6.990030.
Etymology: *margarita -ae, f* [Latin] = pearl. Refers to its appearance of a bag filled with glittering pearls.

Figure 9 provides an illustrated identification key for the up to now known *Lecythium* species.

Discussion

Phylogeny and Morphological Diversity of *Lecythium*

With the molecular analyses of 6 strains of *Lecythium* species, we shed further light on the phylogeny of the Tectofilosida. We could maximize the support to 100/1.0 of the Chlamydophryidae forming a sister group to the Pseudodifflugiidae (compared to 90/1.0 in Dumack et al. 2016b). Further, all sequenced isolates grouped closely to each other and formed a monophyletic clade.

Within the genus *Lecythium* we were able to establish terrestrial and freshwater subclades, both with moderate support of (75/1.0) and (83/1.0), respectively. All terrestrial isolates were rather small compared to the large cells of most freshwater isolates (and the other known freshwater *Lecythium* spp.). Adl and Gupta (2006) stated that soil protists often are smaller compared to freshwater relatives, likely due to the physical constraints of the soil structure. Soil pores might function as an environmental filter for the evolution of reduced cell size of its inhabitants. Our observations support these hypotheses, with the large limnic *L. asini, L. margaritae and L.* cf. *margaritae*; however the freshwater derived *L. siemensmai* is only marginally larger than the species in the terrestrial group. The difference in floating behavior might also be an adaptation to the habitat. More data about the evolution of Tectofilosida and their ecology are needed.

Lecythium Taxonomy

Lecythium spp. have been described under different genus names (e.g. *Pamphagus*, *Gromia* and *Trinema*). Most old descriptions of *Lecythium*-like amoebae were assigned to '*Pamphagus*', an invalid

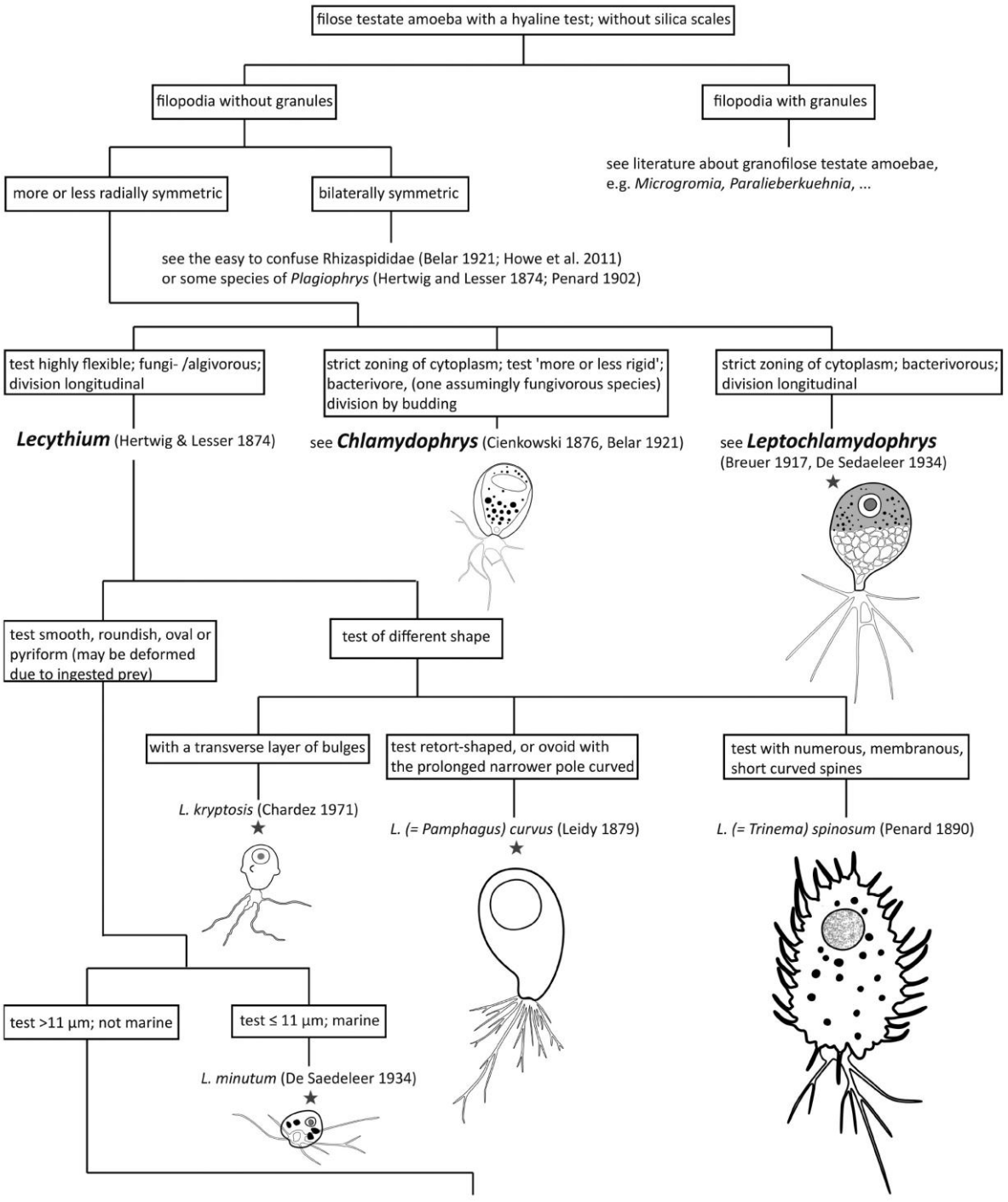


Figure 9. Identification key for filose amoebae with a hyaline test and focus on *Lecythium*. Genus names that are currently grouped into the Chlamydophryidae are in bold. The nucleus was drawn clear or granulated when data could be found. A nucleolus was only added to the drawings when it was reported in the original descriptions. Species marked with a star have not been reported since their original description. Drawings to scale.

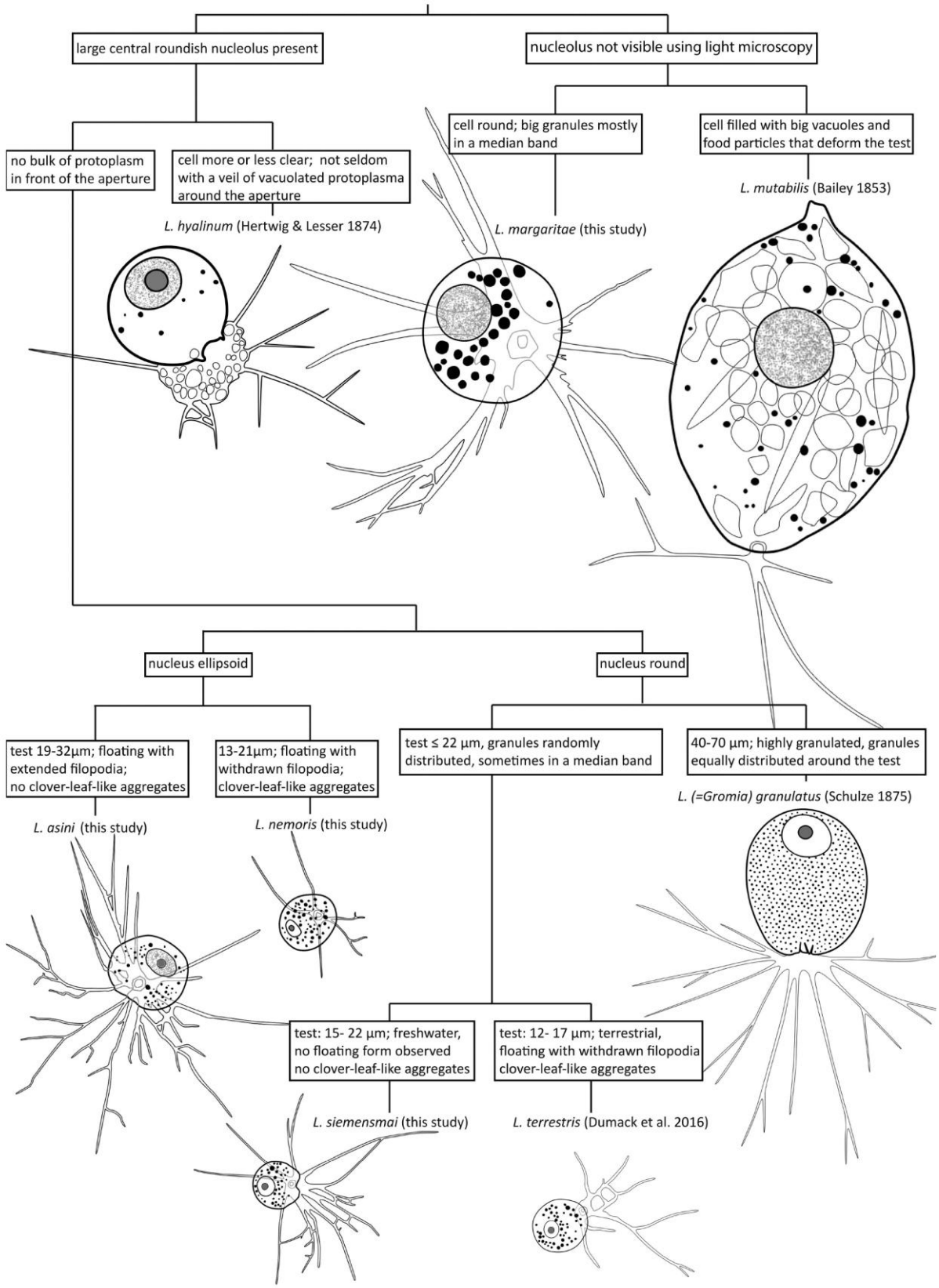


Figure 9. (*Continued*)

assignation, since this name was already in use for a metazoan. Our key enables an easy determination of *Lecythium*-like isolates and links the older references to the according descriptions. However we excluded '*Pamphagus (Lecythium) avidus*' and '*Pamphagus (Lecythium) arcuatus*' described by Leidy (1879) and Penard (1902), respectively. Leidy described '*Pamphagus (Lecythium) avidus*' and compared it in a detailed way to what he believed was *L. mutabilis*. Nevertheless, as stated by Hoogenraad and De Groot (1940), Leidy likely misinterpreted the flattened *Plagiophrys scutiformis* as *L. mutabilis* and when he found a real *L. mutabilis* had to describe it under a new name. Penard (1902) described *Pamphagus arcuatus* based on observations on only one dead and one living individual and due to its flattened appearance, it might as well be identical to *Plagiophrys scutiformis*.

Schulze (1875, not 1874 as often wrongly cited) described '*Gromia (Lecythium) granulatus*'. Due to its filose and not granofilose appearance, it was apparently assigned to the wrong genus and accordingly treated by several authors as a *Lecythium* species (Cash et al. 1915; Penard 1902). Since then, every reported *Lecythium sp.* that contained a "huge amount" of granules was considered *L. granulatus* (e.g. Cash et al. 1915; Penard 1902). However, Schulze described three clear morphological traits beside a "huge amount" of granules; (a) the size ranges from 40–70 μm (b) the nucleolus is dark, big and central to the nucleus, and (c) the rather small granules are equally dispersed in the cell periphery near to the theca (Schulze 1875).

Some of the *Lecythium* species we isolated (*L. margaritae*, *L.* cf. *margaritae* and *L. asini*) share several similar traits with the *L. granulatus* described by Schulze (1875); however all have distinctive morphological differences from each other.

In fact there are several descriptions of *L. granulatus* available that differ in the traits of Schulze's (1875). Penard (1902) observed a *Lecythium* species of 66–100 μm size that did not contain one round nucleolus as described by Schulze, but several refractive spots in the nucleus and rather big granules that were not equally dispersed in the cell periphery, but more often found in a median band or dispersed throughout the cell body. Despite these differences Penard (1902) assigned this specimen to *L. granulatus*. Cash et al. (1915) described *L. granulatus* of 40–140 μm in size with a granular nucleus, whereas a nucleolus was not mentioned. Both of these descriptions fit much better to our description of *L. margaritae* and *L.* cf. *margaritae* than to Schulze's *L. granulatus* due to differences in its nucleolus, cell size, granule size and distribution of granules.

However, of the amoebae we collected, *L. asini* fits best to the original description of *L. granulatus*, regarding the central nucleolus and the small, evenly dispersed granules, but the cell size is by far too small (19–32 μm). We do not consider a "huge amount" of granules as a sufficient trait for species discrimination. This is further confirmed by phylogeny as *L. margaritae*, *L. asini* did not show monophyly within our phylogenetic analyses. Therefore we conclude that we did not find Schulze's *L. granulatus*, but we suppose its phylogenetic position would be close to *L. asini* because of their morphological similarities (Fig. 9). Furthermore, we assume that most of the alleged *L. granulatus* specimens described by various authors were incorrectly assigned to that species.

Lecythium spinosum was described by Penard (1890) as *Trinema spinosum* and later Lauterborn (1901) described probably the same species as *Plagiophrys armatus*. Since *Trinema* is a euglyphid genus with a rigid test built by silica scales *L. spinosum* was wrongly assigned to it. Penard noticed his mistake and changed the name to *Pamphagus armatus* in a later publication (Penard 1902). Nevertheless the description of Penard (1890) clearly shows a radial symmetry and no flattened cell body, therefore it does also not resemble a *Plagiophrys* either as Lauterborn (1901) stated. Cash et al. (1915) assigned it due to morphological similarities (membranous test and radial symmetry) to *Lecythium*. Our key therefore calls it *Lecythium spinosum*.

However, for three species presented in the key (*L. kryptosis, L. minutum* and *L. curvus*; Fig. 9) we found no reported observations since their first description. These species were quite poorly described; sometimes on the basis of observations of a single individual, and their existence and accuracy might be questionable. Chardez (1971) only stated that the pseudopodia of *L. kryptosis* resemble those of Gromiidae (which at the time included more or less all filose testate amoebae). Therefore *L. kryptosis* could as well belong to the Gromiidae, but we nevertheless included it in the key, because the true species identity cannot be confirmed without reexamining it.

Food Selectivity

Lecythium spp. in freshwater samples could be observed directly, and for some we could

determine the ingested material that was most often diatoms, suggesting that the primary food source for limnic *Lecythium* may be diatoms. For terrestrial *Lecythium* spp. a direct observation was not possible and we rely on indirect evidence from food choice experiments.

In general, the major limiting factor for ingestion of diatoms in our experiments appeared to be the size of food particles. On the one hand, the longest (60 μm, *Nitzschia acicularis*) and the broadest diatoms (~22 μm diameter, *Cyclotella meneghiniana*) were hardly ever ingested, even by the largest amoebae (*L. margaritae*), on the other hand, however, the smallest diatom strain (CCAC 5738 B) was also not ingested by all *Lecythium* strains. Maybe the handling time is too long in respect to prey size, or some diatoms produce secondary metabolites against predators.

As all *Lecythium* isolates were surface grazers, we expected the predominantly floating algae with spines, i.e. *Cyclotella meneghiniana* and *Stephanodiscus binderanus* to be ingested less effectively. However that was not the case, since all tested *Lecythium* strains ingested *S. binderanus*. Nevertheless, *C. meneghiniana* despite similar morphology to the former was only ingested by *L. margaritae* and did not support culture growth, leading to the assumption that morphology may not be the only discriminating trait governing the suitability of prey. Since feeding on fungi led to an aggressive grazing and growth of *L. nemoris* and *L. terrestris* (not shown) we suggest fungal cells are the primary food source of these terrestrial species. We are currently performing further experiments to test this hypothesis.

Cysts

We report here the first record of encystment in the genus *Lecythium*. Interestingly, despite the fact that the genera *Chlamydophrys* and *Lecythium* are supposed to be closely related, they (a) divide in different ways (*Chlamydophrys* by 'budding' and *Lecythium* longitudinal, binary, see Dumack et al. 2016b for a discussion) and (b) as our results show, also build cysts in a different way. Cienkowski (1876) described in detailed drawings the formation of cysts in *Chlamydophrys*. This takes place outside of the test, which means that *Chlamydophrys stercorea* exits its own test, becoming for a short time a naked amoeba, and only then builds a cyst wall around the protoplast. *Lecythium asini* in contrast did not exit its own theca but built a cyst within. Molecular data for *Chlamydophrys* are still missing; future studies might reveal *Chlamydophrys* to group somewhere else in Cercozoa (Howe et al. 2011).

The reasons for the sudden encystment of *L. asini* remain unclear. After transferring cells to fresh medium with yeasts (*S. cerevisiae*) and diatoms (*N. communis*) as food source, the cells excysted and showed normal behaviour. A typical factor for encystment of protists is stress, induced by e.g. drought, salt accumulation, food type and density or the temperature (Darby 1929; Corliss and Esser 1974; Griffiths and Hughes 1968; Schönborn 1962). These factors may be excluded as encystment factors in our case as the cultures of *L. asini* were kept under constant environmental conditions, including the humidity, temperature and food, the medium being replaced every week. According to our field observations, seasonality may play a role: our environmental surveys started in early spring and ended at the end of summer, but active *Lecythium* species could only be observed in April and July (Table 1). Accordingly the genus *Lecythium* might comprise some species that show seasonality, most notably in spring, with encystment during summer.

Conclusion

Our results show that with thorough sampling, there are many species of *Lecythium* to be found. Further we give evidence that organisms previously declared as 'unculturable', are in fact, with the right techniques and in this case the right food source, culturable. Our study is an example that still now, in times of mass sequencing, careful morphological descriptions and basic ecological information from culturing studies are a fundamental necessity.

Especially the genus *Lecythium* may generate interesting correlations in next generation sequencing studies, as its species seem to prey exclusively on other eukaryotes. Being microeukaryotic predators at higher trophic levels makes them a valuable target in network analyses.

Methods

Sampling and amoeba identification: We collected 50–100 ml of sediment or detritus rich water samples from rivers, ponds or puddles with small glass or plastic bottles (Table 1). Approximately 10 g of surface soil (upper 2 cm) were collected and 2 cm^2 of moss (*Polytrichum* sp. from withering tree trunks) and were stored in a plastic bottle overnight. The sampling bottle was then filled with approximately 50 ml of Waris-H (McFadden and Melkonian 1986)

and shaken to detach protists from the substrate. Two ml were transferred in each well of a 24-well plate (Sarstedt, Germany). Subsequently the bottom of the sampling bottle was carefully scratched with the pipette tip to detach surface attached amoebae. The multi-well plates were incubated at room temperature for a few hours to allow small organisms to settle to the bottom of the wells. Observation of amoebae was conducted with a light microscope (Nikon Eclipse TS100; Ph1; 40x, 100x, 200x and 400x magnification). All samples were scanned several times up to three weeks after collection. If possible, food organisms in the sample consumed by the filose amoebae were determined by light microscopy and documented (Table 1). Wells with reproducing filose amoebae were sometimes enriched with putative food organisms: a diatom (CCAC culture 1762 B; *Nitzschia communis*), a green alga (*Characium* sp.) or yeast (*Saccharomyces cerevisiae*) (Table 1).

Isolation of *Lecythium* strains: *Lecythium*-like amoebae were carefully transferred to Waris-H medium containing algae or yeast cells as food source. If inside the cells ingested yeast or algae could be observed, the food source was then provided accordingly. Otherwise a mixture of all three food organisms was offered and subsequently amoebae were subcultured with the preferred food source. Subculturings were repeated at intervals of weeks (*L. margaritae, L. asini* and *L. siemensmai*) to a couple of months (*L. nemoris* and *L. terrestris*).

Microscopical observations: Pictures and videos were taken with a Nikon digital sight DS-U2 camera (program: NIS-Elements V4.13.04) with Nikon Eclipse 90i (DIC, up to 600x magnification) and Nikon Eclipse TE2000-E (ph1, up to 400x magnification) microscopes. For measurements and descriptions of all mentioned characteristics at least 20 individuals were used.

Selective feeding experiment: The 10 algal strains were grown at 15 °C; light regime of 14 h light to 10 h dark with a light-intensity of about 5 µmol photons/m^2/sec. *Chlorella vulgaris* was grown in Waris-H, the diatoms in Waris-H+Si (for media composition see http://www.ccac.uni-koeln.de/textfiles/media.htm, last accessed January 6, 2016). The yeast *Saccharomyces cerevisiae* was grown on Potato Glucose Agar (PGA) according to manufacturer's instructions (Sigma-Aldrich, Taufkirchen, Germany).

To test the selectivity of *Lecythium* spp. for algae and fungi, a few cells of each strain were transferred into a 96-well plate (n=4) and co-cultured on a single food source (Table 3). Plates were checked for successful feeding and reproduction every three days for twelve days.

Cysts: Cysts were observed in the *L. asini* cultures after a few months of culturing and rarely some cyst-like structures were observed in cultures of *L. siemensmai*, but due to their very inconsistent appearance the latter could not be further investigated. To prove that the cysts of *L. asini* were viable and able to excyst, 36 wells of 24 well-plates were filled with fresh Waris-H, a mixture of yeast (*Saccharomyces cerevisiae*) and diatoms (*Nitzschia communis*) and one single cyst of *L.* asini in each. The wells were stored without direct light exposure at room temperature and scanned every few days over several weeks for excysted amoebae.

DNA extraction, PCR amplification and sequencing: Single individuals were starved for three days and then with approx. 1 µl of medium transferred into a PCR-tube containing 15 µl ddH_2O. The tube was then frozen at -20 °C for storage. To this sample solution, we added a 35 µl PCR mixture containing 5 µl of 0.1 µM forward and reverse primers (see below), 5 µl 200 µM dNTPs, 5 µl Thermo Scientific Dream Taq Green Buffer, 0.3 µl Dream Taq polymerase (Thermo Fisher Scientific, Germany) and 14.7 µl ddH_2O. The SSU sequences were obtained in two successive steps. First the whole SSU was amplified with the general eukaryotic primers, EukA and EukB (Medlin et al. 1988). Using one µl of the first PCR as template, semi-nested re-amplifications were conducted with primers specifically designed for cercozoans, with the same settings as above and the primers pairs EukA + S963R_Cerco (Fiore-Donno pers. comm.) targeting the 5′ part of the SSU and S616F_Cercomix (Fiore-Donno pers. comm) + EukB for the 3′ part of the gene.

Phylogenetic analyses: The partial sequences were manually checked for sequencing errors and combined into one sequence contig. To create a dataset for phylogenetic analyses they were blasted (blastn 2.3.0) against the NCBI GenBank database (last date of accession: 04.08.2015). Sequences with a similarity of >95% were downloaded and manually aligned in SeaView (V4.5.3, Guoy et al. 2010). Additionally the closely related order Cryomonadida and the class Imbricatea were added with several representative sequences and selected Endomyxa were added as outgroup. An alignment with 41 sequences and 1,767 unambiguously aligned sites of which 60.05% were without polymorphisms was used for phylogenetic analyses. The program jmodeltest (V.2.1.5, Darriba et al. 2012) was used to determine the best fitting model: GTR+I+G, which was selected among 88 models (settings: Substitution Schemes 11; add Base frequencies +I+G rate variation nCat=4, ML optimized NNI as base tree). Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). The support values of the PhyML and the Bayesian analyses are given as follows (ML/BI).

Maximum likelihood phylogenetic analyses were run using PhyML V3.1 (Guindon and Gascuel 2003) with the following settings: GTR model; a proportion of invariable sites and a gamma-shaped distribution of the substitution rates across variable sites (GTR+I+G), with four rate categories; BIONJ distance-based starting tree with all model parameters estimated from the data. The Bayesian analyses were run using MrBayes v.3.2 (Altekar et al. 2004; Ronquist and Huelsenbeck 2003) with the following settings: one million generations, trees samples every 500 generations, convergence of the two runs was estimated every 5,000 generations with a final average of the standard deviation of split frequencies of <0.001 in the end of the run. Of the sampled trees, 25% were discarded as burn-in.

Preparation of the hapantotypes: A fixing solution was obtained by mixing saturated mercury-(II)-chloride solution with 96% ethanol in a ratio of 2:1. Four ml heated to 70 °C were added to one ml of a culture in growing phase. The mixture was washed in 30% ethanol once and further fixed in 1 ml osmium tetroxide for one minute. Subsequently the cells were dehydrated in eight steps consisting of 20 minutes incubation in an ethanol series of increasing concentration (from 30 to 96%). Cells were then preserved on a microscope slide with a drop of glycerin under a cover slide. The slides were then deposited in the Upper Austrian State Museum Invertebrate Collection.

Acknowledgements

We thank the Culture Collection of Algae at the University of Cologne (CCAC) for providing many

algal strains, we thank Anna Maria Fiore-Donno for critical reading of the manuscript. The study was supported by the DFG research group FOR 918 “Carbon flow in belowground food webs assessed by isotope tracers”.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2016.08.001.

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Chapter 5: Shedding light on the polyphyletic genus *Plagiophrys*: The transition of some of its species to *Rhizaspis* (Tectofilosida, Thecofilosea, Cercozoa) and the establishment of *Sacciforma* gen. nov. (Cryomonadida, Thecofilosea, Cercozoa).

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Title

Shedding light on the polyphyletic thecate amoeba genus *Plagiophrys*: Transition of some of its species to *Rhizaspis* (Tectofilosida, Thecofilosea, Cercozoa) and the establishment of *Sacciforma* gen. nov. and Rhogostomidae fam. nov. (Cryomonadida, Thecofilosea, Cercozoa).

Keywords

Rhogostoma, protist, algivorous, testate amoebae, amoeba, phylogeny

Abstract

For over a century testate amoebae have been a favoured group of interest for protistologists, however there is still an endless amount of unanswered questions. The genus *Plagiophrys*, Claparède and Lachmann 1859, is still one of the unresolved mysteries as it comprises species with high morphological diversity of which no molecular data are available. To shed light on the phylogeny and taxonomy of *Plagiophrys* we (a) cultured four isolates of three *Plagiophrys* morphospecies and provided morphological observations (b) obtained three new SSU sequences and conducted phylogenetic analyses of the Thecofilosea and (c) did intensive literature research, showing that *Plagiophrys* is polyphyletic. We partially untangle this polyphyly by combining several of its species with the genus *Rhizaspis*, Skuja 1948. Furthermore, we establish *Sacciforma* gen. nov. to accommodate *P. sacciformis* as it groups within the formerly known Rhizaspididae, which do not

comprise our isolates of *Rhizaspis* (and therefore were renamed as Rhogostomidae) as it groups with maximum support as a sister-group to the Pseudodifflugiidae.

1. Introduction

Testate amoebae have been of high interest for protistologists and ecologists since their discovery. They show the advantage of having clear morphological traits, making them ideal model organisms for protist ecology and evolution. Accordingly the specific ways in which their tests are constructed have been intensively studied and were used for testate amoeba identification and taxonomy. The tests may be siliceous (e.g. Euglyphids; Cavalier-Smith 1998a,b; Wylezich et al. 2002), organic (e.g. Thecofilosea; Dumack et al. 2016a,b; Howe et al. 2011) or composed of agglutinated material, e.g. foreign particles, like sand grains or diatom frustules (e.g. Pseudodifflugiidae; Cavalier-Smith and Chao 2003; Wylezich et al. 2002).

However, taxonomy solely based on morphology lead to contradicting taxonomical concepts (de Saedeleer 1934; Cash, Wailes and Hopkinson 1915). Testate amoeba taxonomy was therefore for a long time confusing and often changed. Phylogenetic approaches finally revealed their polyphyly showing independent lineages in the Amoebozoa (e.g. *Difflugia*, *Arcella*, ..), Stramenopiles (e.g. *Amphitrema*) and Cercozoa (e.g. Euglyphida, amoeboid Thecofilosea) and enabled the unification of a widely accepted consensus (Cavalier-Smith 1998a,b; Kosakyan et al. 2016; Nikolaev et al. 2005).

The highly diverse phylum Cercozoa established by Cavalier-Smith (1998a,b) consists predominantly of naked amoebae, flagellates and amoeboflagellates (Bass et al. 2009a,b; Dumack et al. 2016c; Hess & Melkonian 2013; Hess et al. 2012). Nestling between those, it comprises several polyphyletic testate amoeba lineages: e.g. the order Eugylphida (Cavalier-Smith 1998a,b), the family Rhizaspididae in the order Cryomonadida (Howe et al. 2011) and the families Chlamydophryidae and Pseudodifflugiidae in the order Tectofilosida (Cavalier-Smith & Chao 2003; Howe et al. 2011; Wylezich et al. 2002).

Nevertheless, there are still genera lacking molecular data and therefore a phylogenetic placement. Some of which include species of high morphological diversity which may even be polyphyletic assemblages, like the genus *Plagiophrys*. It was first described by Claparède and Lachmann (1859) to accommodate two species *P. cylindrica* and *P. sphaerica* and was assigned to the Actinophryidae. They described non-‘armoured’ spherical amoebae with a ‘double-contoured’ flexible ‘skin’ and granules in their numerous radiating filopodia.

Later Hertwig and Lesser (1874) pointed out that Claparède and Lachmann distinguished between an 'armour' and 'skin' and came to the conclusion that what they have described as a 'skin' is in fact a theca. Hertwig and Lesser therefore added two new species '*P. scutiformis'* and *'P. sacciformis'* with a 'double-contoured construct' (=theca) to the genus, although they differ in major points to *P. cylindrica* and *P. sphaerica*, as they are lateral compressed and have no granules in their filopodia. Since then the genus *Plagiophrys* caused taxonomic confusion (de Saedeleer 1934; Cash, Wailes and Hopkinson 1915). Cash, Wailes and Hopkinson (1915) pointed already out the presumed polyphyly of the genus but did not create a new one.

Penard (1902) and de Groot (1979) added further species to this genus (*P. parvipunctata*, *P*. *arcuatus* and *P. scutiformis* var*. marginata*) similar to Hertwig and Lessers' description but not to Claparède and Lachmanns'. To make things even more complicated, a phycologist, Skuja (1948) described *Rhizaspis*, a genus highly similar to *Plagiophrys* sensu Hertwig and Lesser, which we herein discuss and combine into one.

Clearly, molecular data are necessary to untangle the confusing taxonomy of *Plagiophrys*. We have conducted intensive literature research, isolated four '*Plagiophrys*' strains from freshwater habitats of which we conducted light microscopy, SSU rDNA sequencing and phylogenetic analyses.

2. Results

2.1. Sampling and culturing

We were able to find and extract four different strains (i.e. KD1015, KD1016, KD1017 and KD1018) of '*Plagiophrys* spp.' out of approximately 30 screened samples. Two of our isolates were morphological similar (strain KD1015 and KD1018) the other two strains of unique morphology. Unfortunately two of the isolates, KD1016 and KD1015, divided two to three times under our culture conditions, but then ceased to grow, leading to the extinction of those cultures. The morphology of KD1015 was well characterized, but the SSU sequence could not be obtained.

2.2. Microscopical observations

2.2.1. *Rhizaspis rugosa* sp. nov.

The cell bodies of strains KD1015 and KD1018 were oval and flattened with a length of 57.51 ± 2.65 µm, a width of 40.79 ± 6.33 µm and a length-width ratio of 1.44 ± 0.22 (Figs 1, 2 and Table 1; n=10).

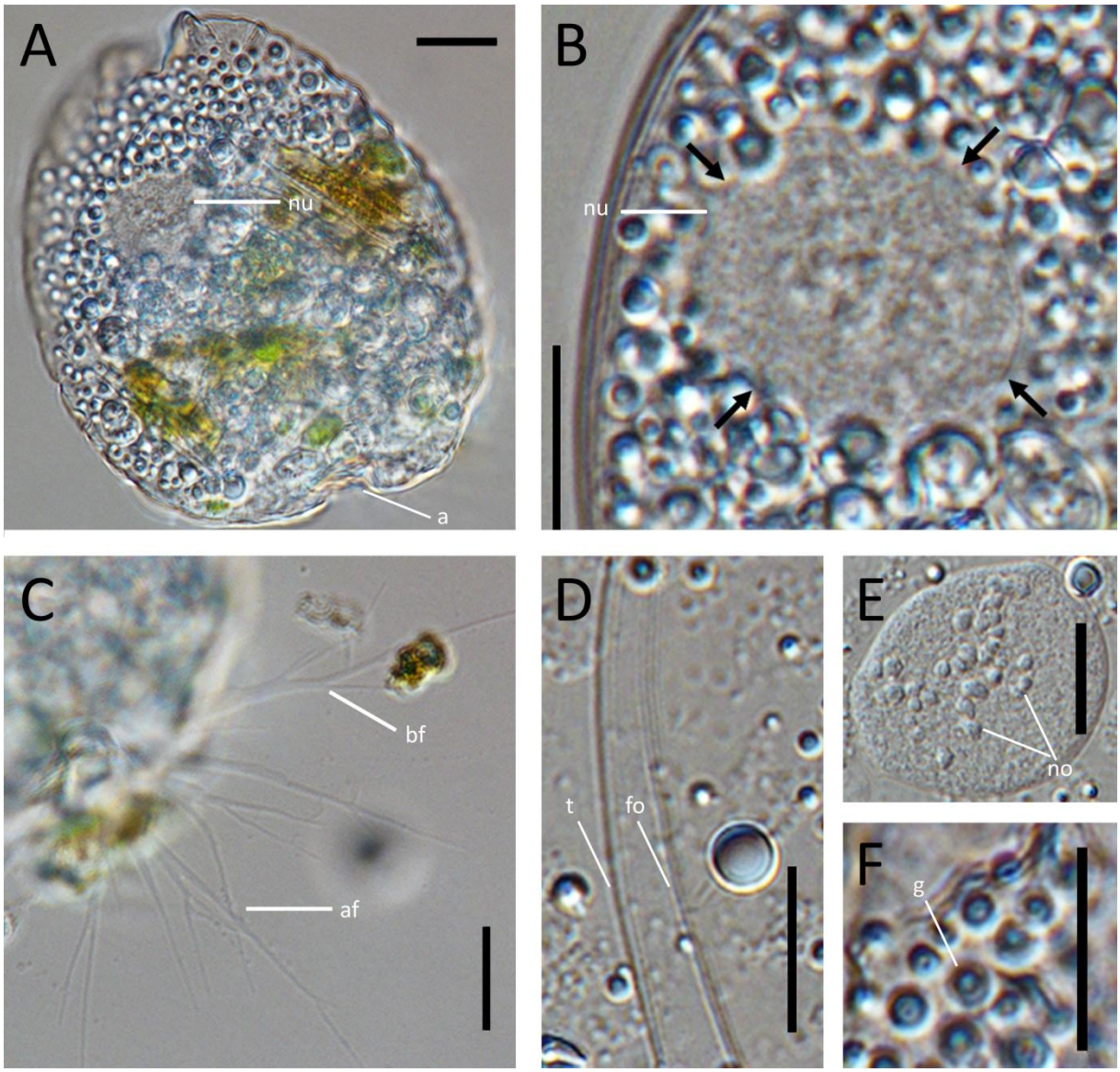

Figure 1: Cellular features of strain KD1015. A: Cell body as an overview. B: Close-up of the nucleus (nu) indicated by arrows. C: Overview of expanding, branched (bf) and anastomosing (af) filopodia. D: Lateral fold (fo) in the margins of a compressed theca (t). E: Nucleolus of a compressed cell: several more or less ovoid nucleoli (no). F: Granules (g). Scale bars indicate 10 µm.

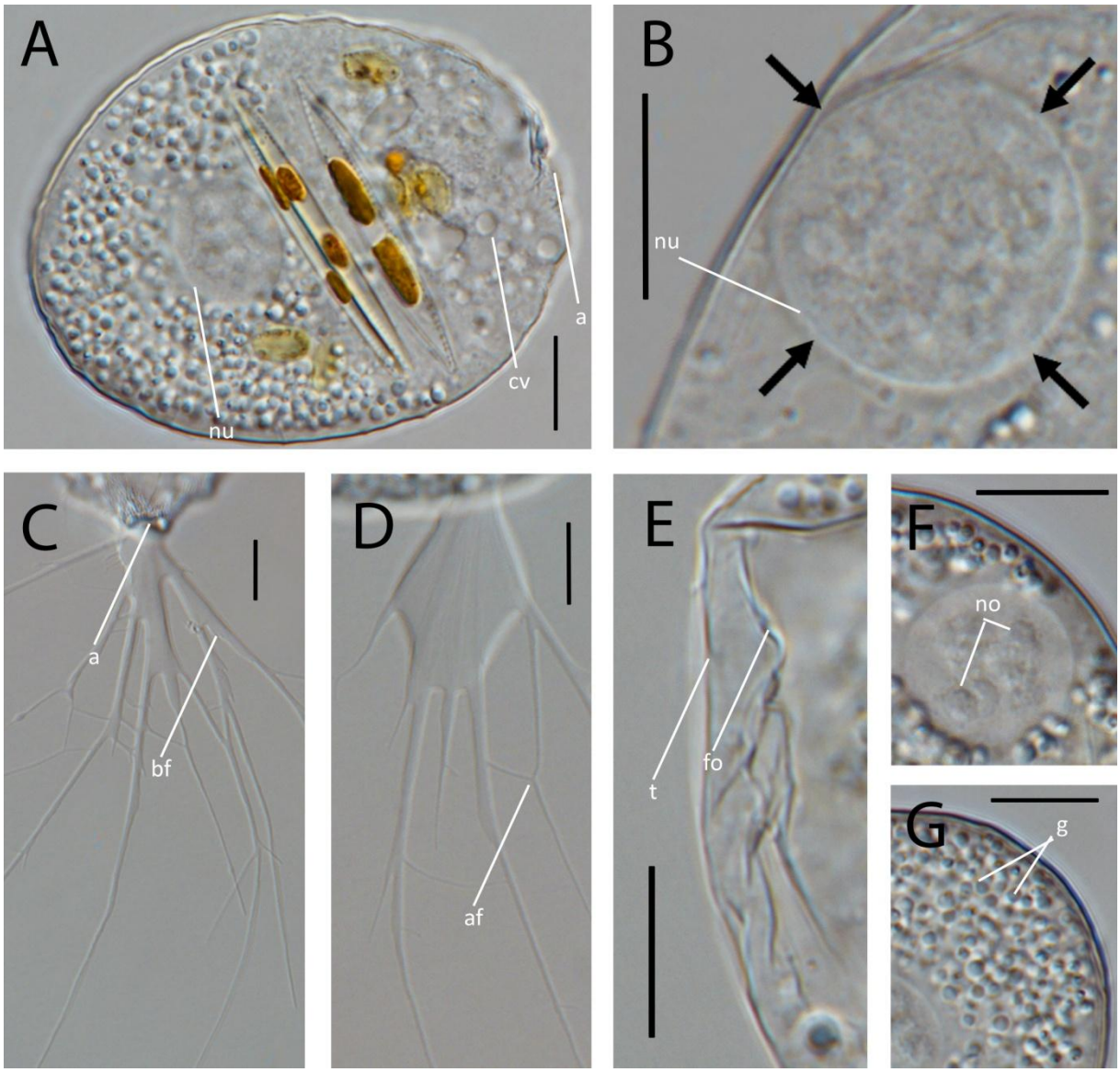

Figure 2: Cellular features of strain KD1018. A: Cell body as an overview, a contractile vacuole (cv) and the aperture (a) highlighted. B: Close-up of the nucleus (nu) indicated by arrows. C: Overview of expanding and branching filopodia (bf). D: Plasma plate of which filopodia emerge with visible cytoskeleton filaments and highlighted anastomosing filopodia (af). E: Lateral fold (fo) in the margins of the theca (t). F: Nucleolus: several more or less ovoid nucleoli (no). G: Granules (g). Scale bars indicate 10 µm.

Table 1: Isolated species and strains with information about, sampling spot and date and corresponding data.

Species	**Strain**	**SSU rDNA accession**	**Sequence length [nt]**	**Country, City**	**Coordinates**	**Isolation date**	**Habitat**
R. rugosa	KD1018	KX580627	1663	Germany, Xanten	51.691441, 6.425774	April 2016	Quarry pond next to river Rhine
	KD1015	-	-	Germany, Much	50.936385, 7.415898	November 2015	Stream Müchelsbach, close to a forest in agricultural land
S. sacciformis	KD1016	KX580629	1685			April 2016	
R. transformis	KD1017	KX580628	1635	Germany, Cologne	50.958675, 7.005476	May 2016	Artificial pond in an urban park

Several zones characterized the cell body (Fig. 3): The nucleus (Figs 1B, 2B; 12.62 ± 1.45 µm; n=10) was located at the apical end of the cell, embedded by numerous granules (1.27 ± 0.41 µm; Figs 1F, 2G; n=30). It contained a varying amount of nucleoli (3.32 ± 1.16 µm; Figs 1E, 2F). A layer of food vacuoles separated this area from several (difficult to detect) contractile vacuoles close to the aperture (Figs 1A, 2A, 3).

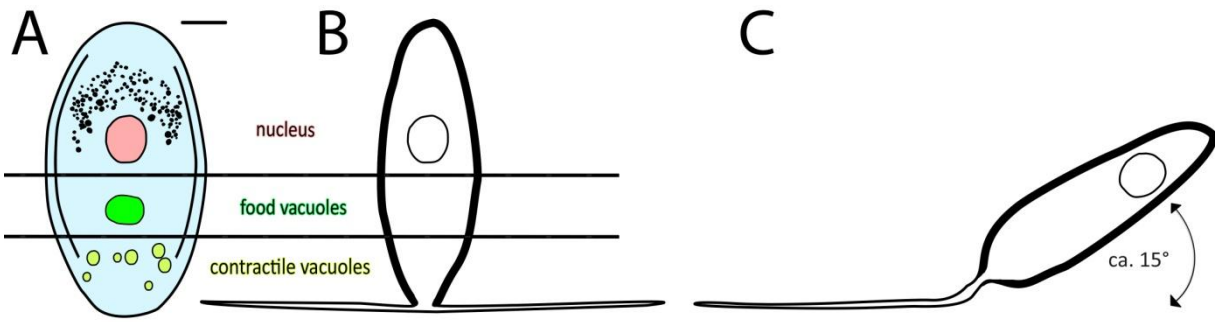

Figure 3: Illustration of the morphological identical strains KD1015 and KD1018. A: Schematic drawing of the cell body B+C: Overview of the lateral compressed side. B: Stationary cell; filopodia expanded in all directions; cell body upright. C: Locomotive cell; filopodia expanded in direction of movement; cell body in dragged position. Scale bar indicates 10 µm.

The cell body was covered by a hyaline, flexible (i.e. stretchable) theca that was most often carried in an upright position sometimes drawn after (Figs 3, 4). It exhibited two lateral folds on each side (Figs 1D, 2E, 3A) that might have only be seen if cells are starving or compressed between glass slides. Additionally, smaller randomly dispersed folds could be observed. The aperture itself usually was slit-like (Figs 4, 5A-C), but showed high flexibility, as it sometimes stretched to different polygon- or almost round shapes. The cells crept by filopodia, which originated from the aperture or at a plate of cell plasma that by itself originated from the aperture (compare Fig. 1C, D and Fig. 4A, B; see Supplementary Video 1). The filopodia branched and anastomosed with a maximum measured length of 110 µm (Fig 4). They lacked granules; instead stripes (cytoskeletal filaments) were prominent (Fig. 2D). Motile cells usually expanded most filopodia in the direction of movement, non-moving cells formed filopodia in all directions (Fig. 3). No floating or flagellated cell stages were observed. Division was longitudinal.

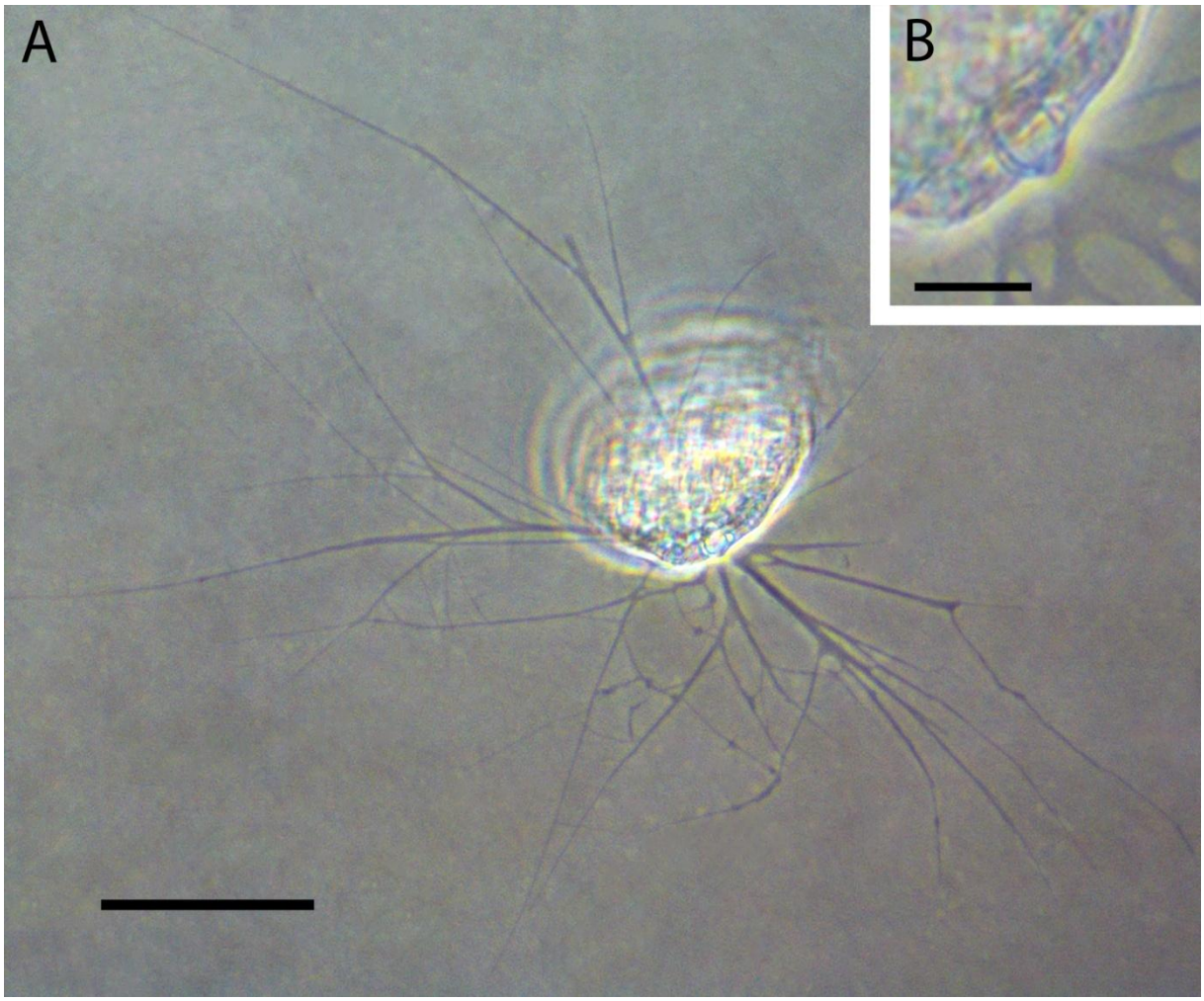

Figure 4: Strain KD1015 taken with an inverse microscope (Ph1). A: Cell with expanded filopodia. B: Close-up of the aperture of the cell. Scale bars indicate 50 µm (A) and 10 µm (B).

2.2.2. *Rhizaspis transformis*

The cell bodies of strain KD1017 were highly metabolic and therefore variable in shape and size with a length of 48.15 ± 16.22 µm, a width of 52.53 ± 16.73 and a length-width ratio of 0.92 ± 0.16 (Table 1; n=10), but were bulky and deformed and never resembled the thin and disc-like shape of *Rhizaspis rugosa* (Fig 5).

The cell body showed no division in zones as described for *Rhizaspis rugosa*. The roundish nucleus (Fig. 6B; 12.36 ± 1.76 µm; n=10) had a varying amount of nucleoli (Fig. 6I) approximately 1-2.5 µm in diameter, which were difficult to observe. Granules quite bulging and various in size (up 0.63 to 3.94

µm; Fig. 6J; n=30), food vacuoles and contractile vacuoles were often randomly dispersed; rarely their arrangement was similar to the zones described for *Rhizaspis rugosa* but not as strict (Fig. 5).

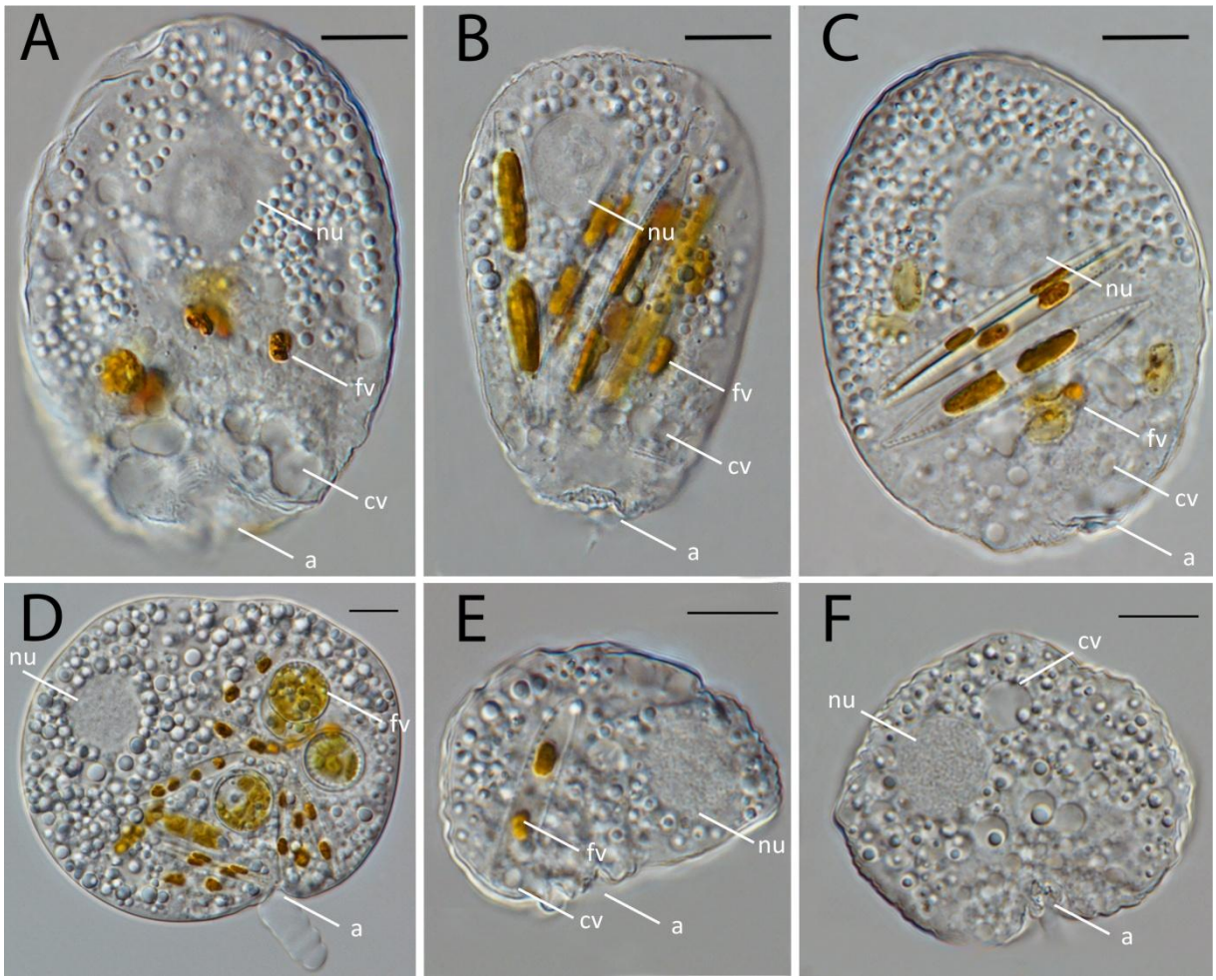

Figure 5: Healthy individuals of growing cultures of strain KD1018 and KD1017, taken with differential interference contrast (DIC). A-C: KD1018 D-F: KD1017. Note that KD1018 always shows an oval cell form with more or less clearly visible zones (granules and nucleus apical, vacuoles basal); in contrast KD1017 varies highly in shape and composition. Scale bar indicates 10 µm. a = aperture; nu = nucleus; fv = food vacuole; cv = contractile vacuole.

The cell body was covered by a hyaline, flexible (i.e. stretchable) theca that was most often carried in an upright position or drawn after (Fig. 7, see also Fig. 3). It exhibited larger lateral folds and smaller horizontally ones (Fig. 6G, H). On the basal end of the theca the aperture was located, usually between folds. The aperture itself was most-often roundish, but showed high flexibility, as it sometimes stretched to different polygon- but mostly oval shapes (Figs 6D, E, 7). The cells crept by filopodia that originated from the aperture or at a plate of cell plasma that by itself originated from the aperture (Fig. 6C, D; see Supplementary Video 1). The filopodia branched and anastomosed (Fig.

6C) with a maximum observed length of 130 µm (Fig. 7). They lacked granules; instead stripes (cytoskeletal filaments) were prominent. Additionally within the filopodia various vesicle-like structures could be observed (Fig. 6D, E). Motile cells usually extended most filopodia in the direction of movement, non-moving cells formed filopodia in all directions (compare Fig. 3).

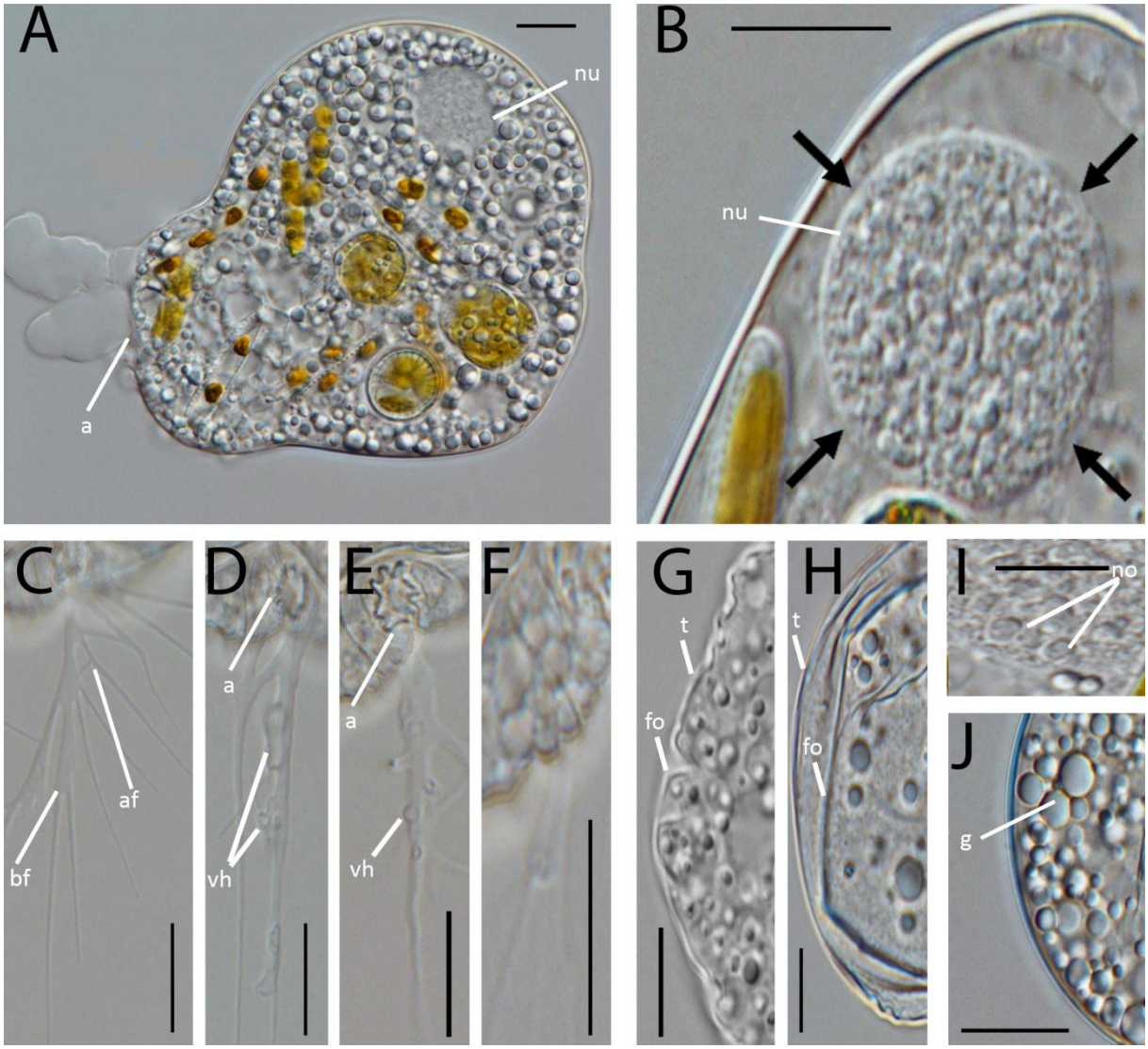

Figure 6: Cellular features of strain KD1017. A: Cell body as an overview, the nucleus (nu) and the aperture (a) highlighted. B: Close-up of the nucleus (nu) indicated by arrows. C: Overview of expanding branched (bf) and anastomosing (af) filopodia. D-F: Close-up to vesicle-like structures (vh) in filopodia. G: Notches (fo) at the lateral surface of the theca (t). H: Lateral fold (fo) in the margins of the theca (t). I: Nucleolus: several more or less ovoid nucleoli (no). J: Granules (g). Scale bars indicate 10 µm.

Cells frequently formed cysts that showed a varying shape and size (38.38 ± 5.39 µm in diameter, n=7; Fig. 8; see Supplementary Video 1): The outermost envelope, the theca, was deformed and comprised irregular notches (Fig. 8C) or formed a smooth 'velum', which might vary in thickness (Fig. 8A, B). In some cases the usual shape was still noticeable (Fig. 8D). Below the theca, a well-defined cyst wall covered the spherical cells; it seemed to be quite constant (i.e. in shape) in all individuals. The content of the cysts had a granular appearance, which was homogenous or contained small round particles. Additionally, most cysts contained a single conspicuous sphere, usually located in the cell center, measuring approximately 12 µm in diameter (Fig. 8D), therefore most likely the nucleus. Transferring cysts to fresh medium containing food organisms did not trigger excystment (n=12).

No floating or flagellated cell stages were observed. Division was longitudinal.

Multinucleate basolaterally merged individuals were spotted in strains KD1015, KD1018 and KD1017 (Fig. 9) occurring most often with unsuitable culture conditions. Unlike described for *Lecythium*, those cells originated by an unfinished fission, no fusion of cells observed.

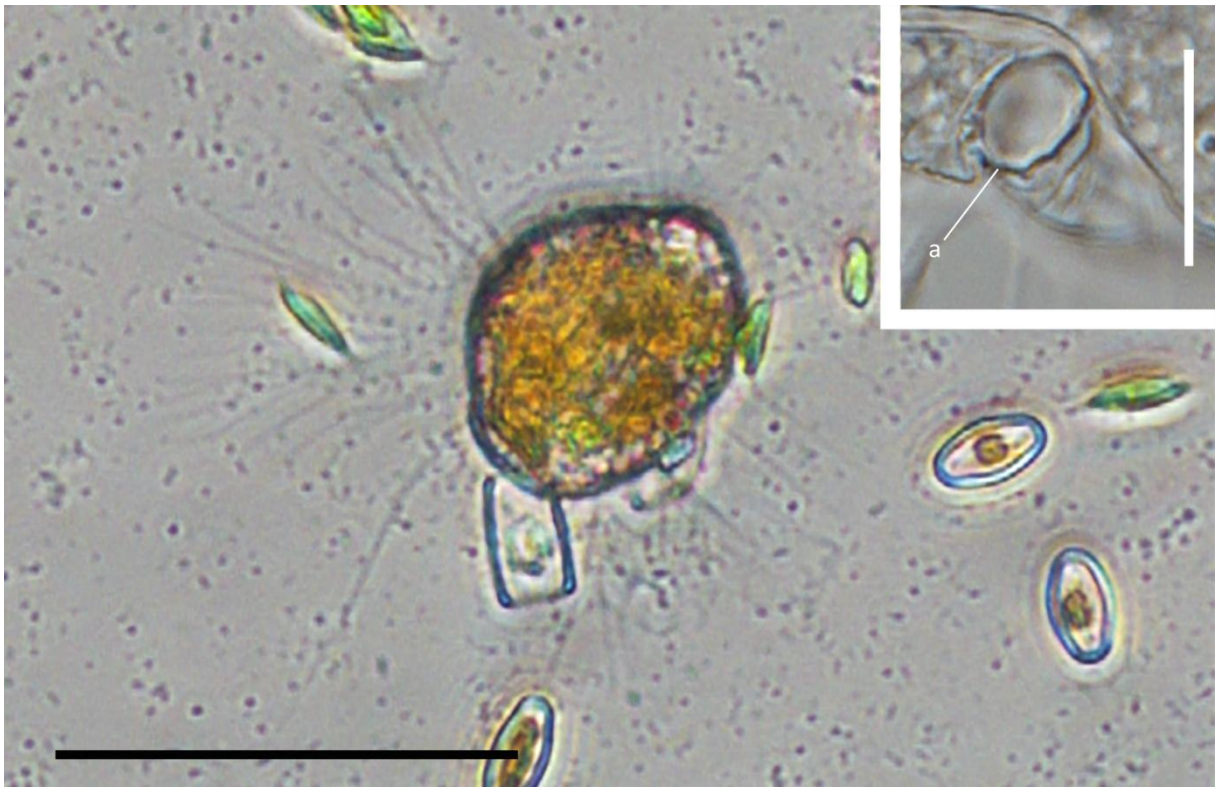

Figure 7: Strain KD1017 taken with an inverse microscope (Ph1). A: Cell with expanded filopodia. B: Close-up of the aperture of the cell. Scale bars indicate 50 µm (A) and 10 µm (B).

2.2.3. *Sacciforma sacciformis* gen. nov.

The cell bodies of strain KD1016 were oval and flattened with a length of approx. 35 µm, a width of approx. 25 µm and a length-width ratio of approx. 1.5 (Table 1, Fig. 10). The nucleus was located at the apical end of the cell. Cells contained numerous granules (2.87 ± 0.67 µm; n=10). Close the aperture several food vacuoles and contractile vacuoles were located.

The cell body was covered by a hyaline, flexible (i.e. stretchable) theca that was carried in an upright position. On the basal end of the theca the slit-like, non-flexible, aperture was located. The cells crept by filopodia which originated of the aperture, cells never showed a large net of filopodia. The filopodia lacked granules and branched. No floating or flagellated cell stages were observed. Division was longitudinal.

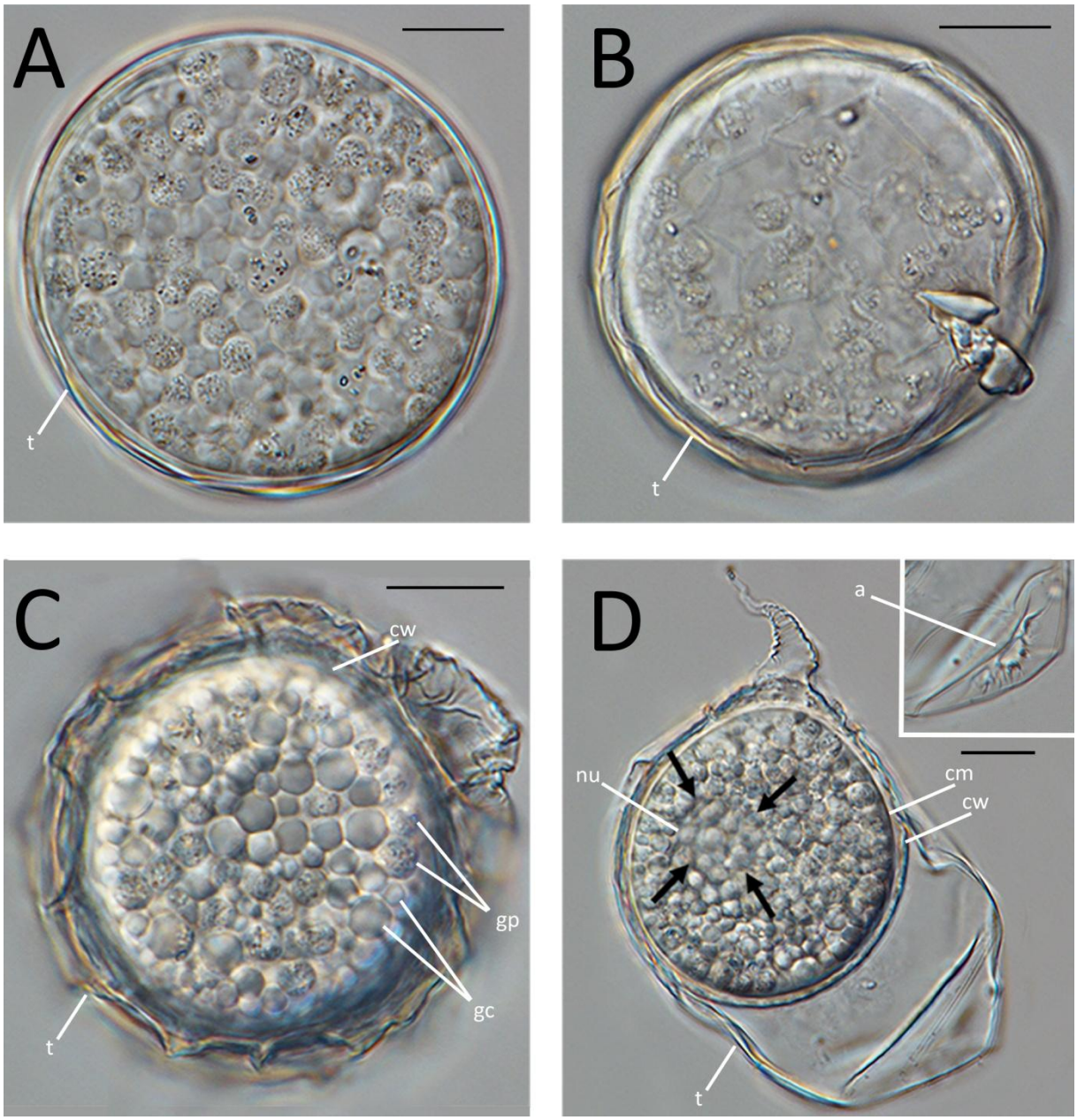

Figure 8: Four different cysts of strain KD1017. Note highly varying shape of the outer envelope (theca). A: Individual with very difficult to detect theca (t). Note hyaline granules and granules with smaller particles. B: Similar cyst in another focus layer. C: Theca clearly visible as it shows many notches and folds D: Individual with less compressed theca, former shape is still distinguishable. Note the clearly separated theca, cyst wall (cw) and cell membrane (cm). Arrows indicate most likely the nucleus (nu). Note close-up to the former aperture. For an animation of cysts, see Supplementary Video 1. Scale bars indicate 10 µm.

2.3. Phylogenetic analyses

Nearly full-length SSU sequences were obtained, ranging from 1,635 to 1,685 nucleotides (Table 2). No introns were found. The maximum likelihood tree (Fig. 11) revealed the cercozoan subphylum Filosa (Cavalier-Smith 1997) and selected sequences of the Endomyxa as out-group. The filosan class Thecofilosea was composed of the Phaeodaria, Cryomonadida and Tectofilosida. Thecate amoeba lineages sequenced in this study and close relatives within the Thecofilosea are highlighted. Similar to previous analyses using SSU rDNA sequence comparisons, many basal branches within the Cercozoa were not supported (Bass and Cavalier-Smith 2004; Bass et al. 2009a,b; Howe et al. 2011).

Sacciforma sacciformis (KD1016), *Rhizaspis rugosa* and *Rhizaspis transformis* (KD1017 and KD1018) were not monophlyletic. Although *Rhizaspis rugosa* and *Rhizaspis transformis* (strain KD1017 and KD1018) formed a maximally supported clade within the Tectofilosida, *Sacciforma sacciformis* (KD1016) grouped in the Cryomonadida. The SSU sequences of *Rhizaspis rugosa* and *Rhizaspis transformis* (KD1017 and KD1018) were to 100% identical and built together with *Pseudodifflugia* cf. *gracilis* (AJ418794) a sistergroup to *Lecythium* with a bootstrap support value of 80 (ML) and Bayesian posterior probability of 1.0 (BI).

Sacciforma sacciformis (KD1016) grouped next to *Rhogostoma* with (96/1.0). The clade has been referred to as the Rhizaspididae by Howe et al. (2011) but was here renamed as Rhogostomidae (see discussion).

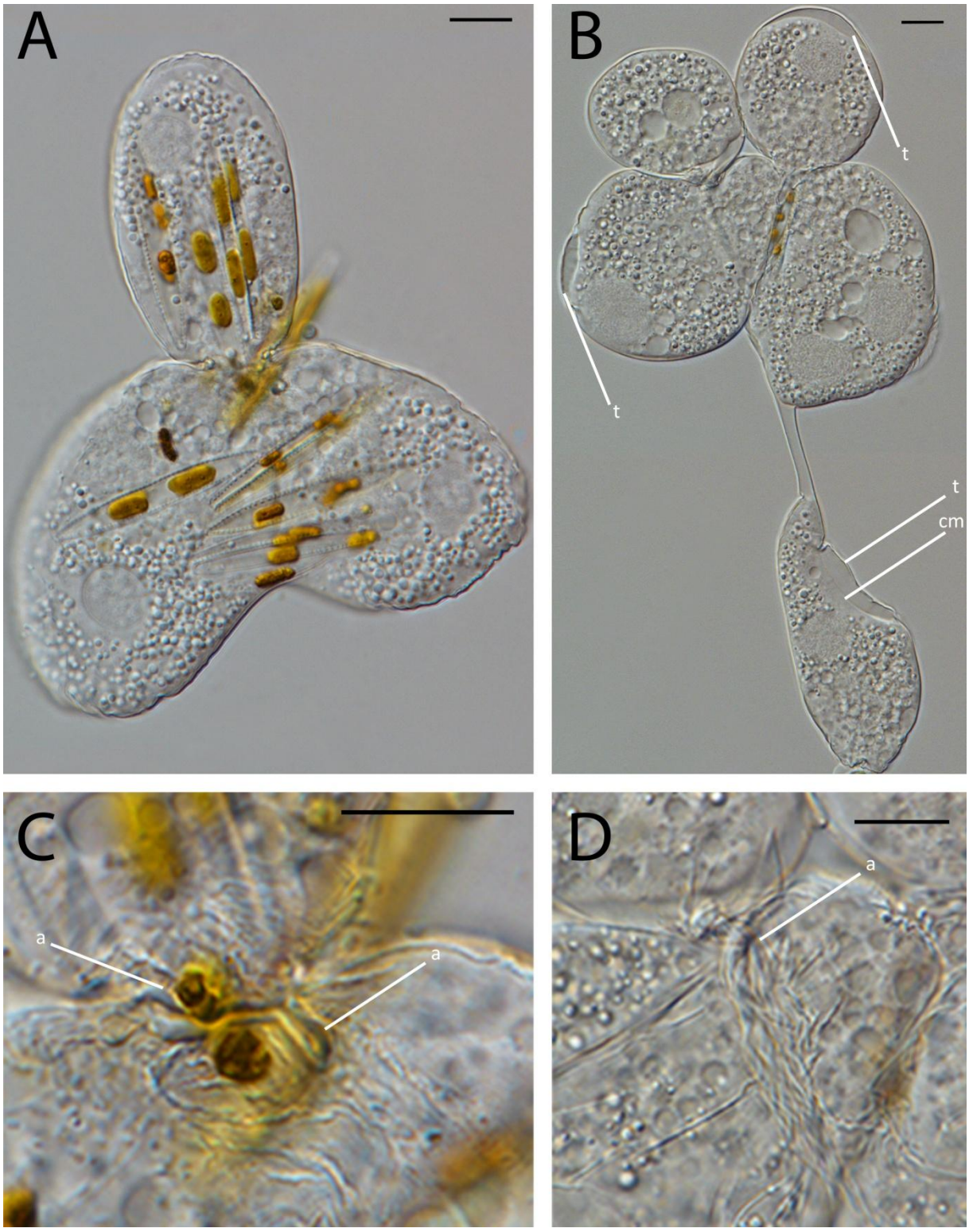

Figure 9: Basolaterally adhering cells of strain KD1017 (A+C) and KD1018 (B+D). A: Five active basolaterally connected cells. Note the highly deformed thecae (t), one compartment does not bear a nucleus, another two. B: Close-up to the center of the joined cells of strain KD1017. Note that thecae only partially are filled with the cell body, therefore revealing the cell membrane (cm). C: Three active basolaterally connected cells, apertures highlighted (a). D: Close-up to the center of the joined cells apertures highlighted. Scale bars indicate 10 µm.

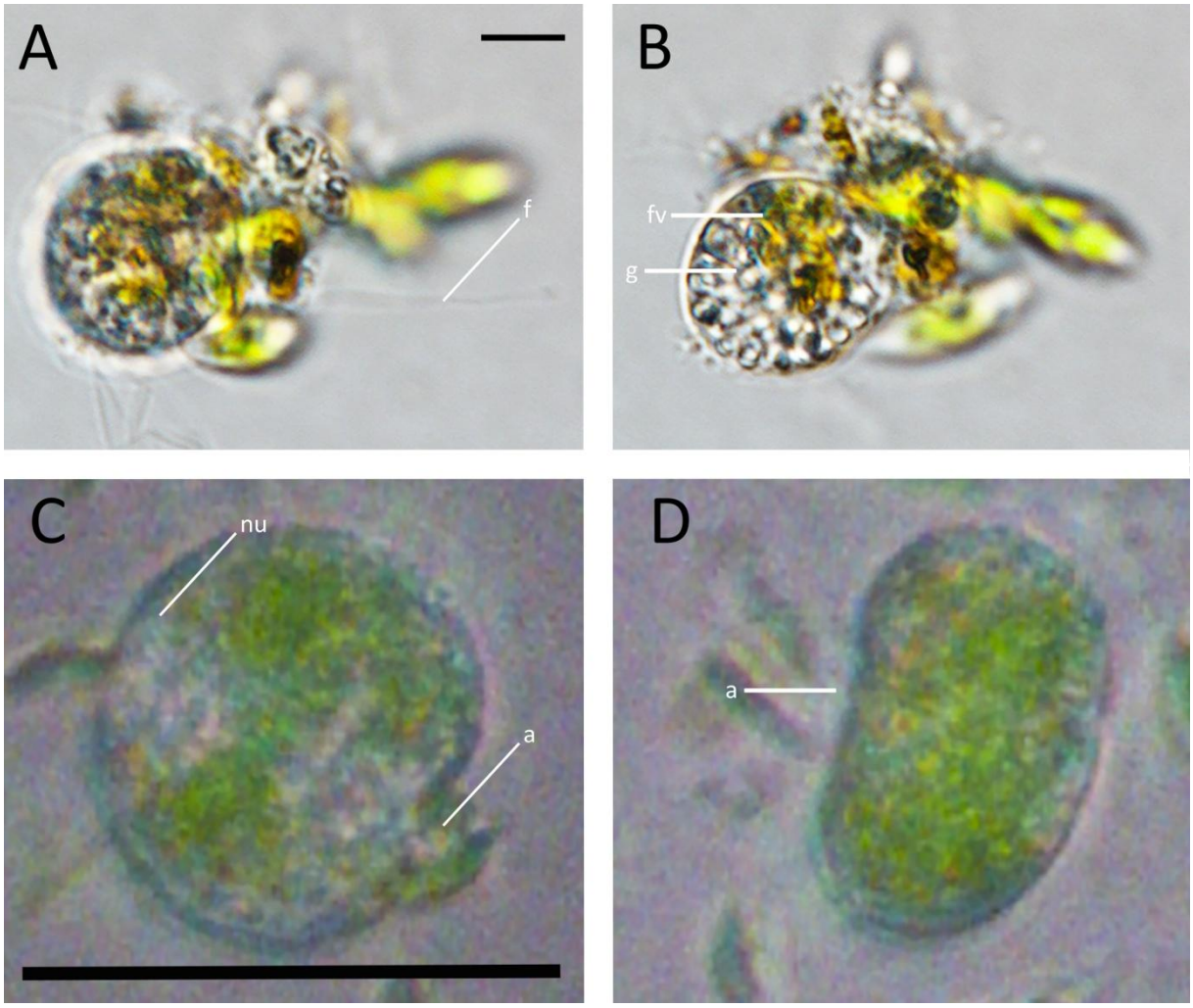

Figure 10: Cells of strain KD1016 as an overview, taken with (A+B) differential interference contrast (DIC) and (C+D) an inverse microscope (ph1). A: Focus on filopodia (f). B: Focus on cell body with granules (g) and food vacuoles (fv). C: Cell body in dragged position, focus on the side of the cell, highlighted are the nucleus (nu) and aperture (a). D: Cell body upright, focus on apical end, note the lateral compressed form. See also Supplementary Video 2. Scale bars indicate 10 µm (A+B) and 50 µm (C+D).

Table 2: Isolated species and strains with information about culture conditions and measurements.

Species	Strain	Cell size [µm]		Length/ Width ratio	Size of granules [µm]	Nucleus size [µm]	Nucleolus [µm]	Food source in culture
		Length	Width					
R. rugosus	KD1018	up to 66	53	-	0.8-2.9	-	1.0-2.0	*Nitzschia communis, Characium* sp., *Saccharomyces cerevisiae*
	KD1015	57.51±3.65	40.79±6.33	1.44±0.22	1.27±0.41	12.62±1.45	3.32±1.16	*Navicula* sp., *Cyclotella meneghiniana, Nitzschia communis*
S. sacciformis	KD1016	~35.6	~23.73	~1.5	2.87±0.67	-	-	*Characium* sp.
R. rugosus var. *transforma*	KD1017	48.15±16.22	52.53±16.73	0.92±0.16	1.86±1.02	12.36±1.76	1.86±0.95	*Nitzschia communis, Nitzschia amphibia, Pinnularia* sp.

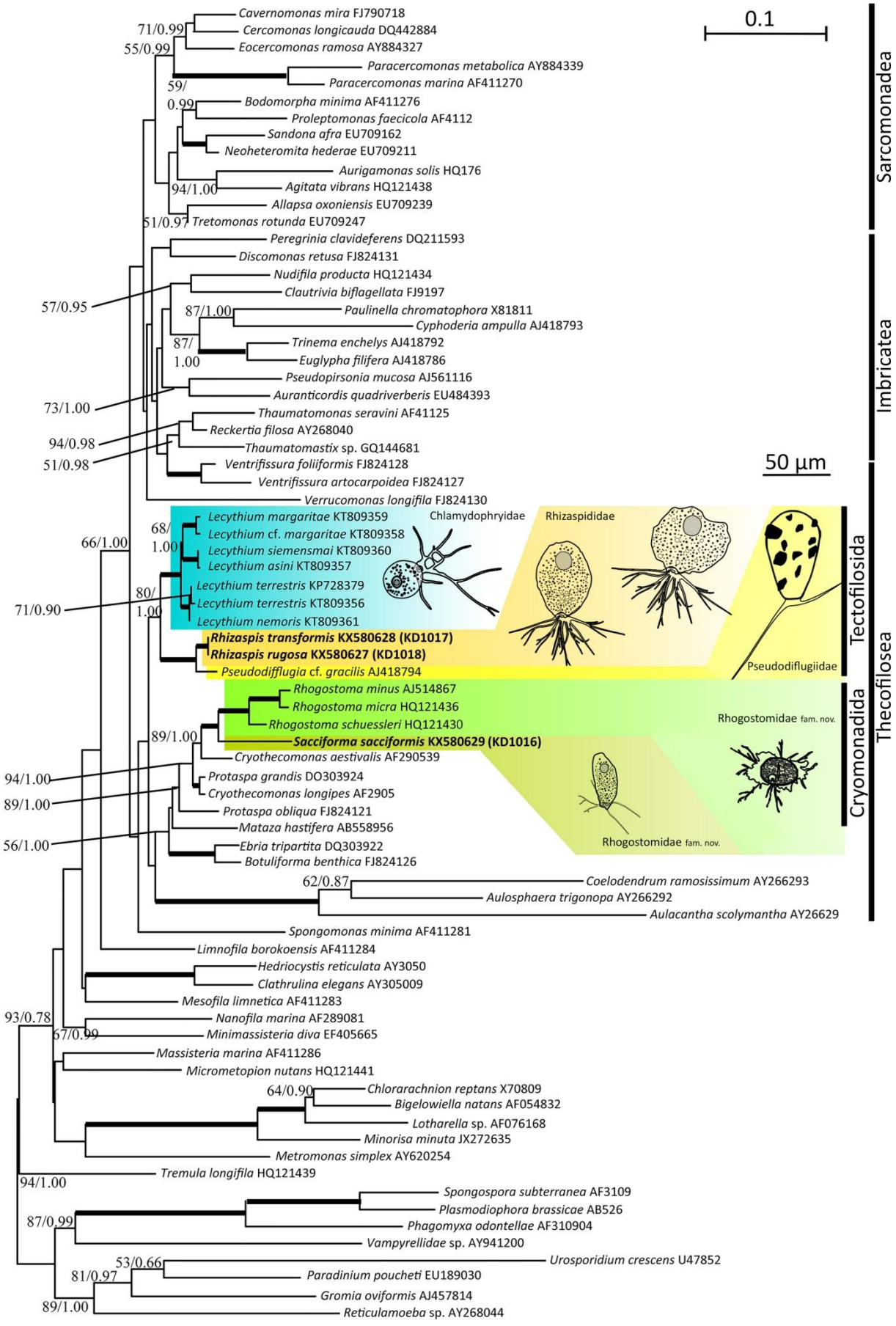
0.1
Cavernomonas mira FJ790718
71/0.99
Cercomonas longicauda DQ442884
55/0.99
Eocercomonas ramosa AY884327
Paracercomonas metabolica AY884339
59/
Paracercomonas marina AF411270
0.99
Bodomorpha minima AF411276
Proleptomonas faecicola AF4112
Sandona afra EU709162
Neoheteromita hederae EU709211
Aurigamonas solis HQ176
94/1.00
Agitata vibrans HQ121438
Allapsa oxoniensis EU709239
51/0.97
Tretomonas rotunda EU709247
Peregrinia clavideferens DQ211593
Discomonas retusa FJ824131
Nudifila producta HQ121434
Clautrivia biflagellata FJ9197
57/0.95
87/1.00
Paulinella chromatophora X81811
Cyphoderia ampulla AJ418793
87/
1.00
Trinema enchelys AJ418792
Euglypha filifera AJ418786
Pseudopirsonia mucosa AJ561116
Auranticordis quadriverberis EU484393
73/1.00
Thaumatomonas seravini AF41125
Reckertia filosa AY268040
94/0.98
Thaumatomastix sp. GQ144681
51/0.98
Ventrifissura foliiformis FJ824128
Ventrifissura artocarpoidea FJ824127
Verrucomonas longifila FJ824130
50 µm
Lecythium margaritae KT809359
Chlamydophryidae
68/
1.00
Lecythium cf. margaritae KT809358
Lecythium siemensmai KT809360
66/1.00
Lecythium asini KT809357
Lecythium terrestris KP728379
80/
1.00
Lecythium terrestris KT809356
71/0.90
Lecythium nemoris KT809361
Rhizaspidae
Rhizaspis transformis KX580628 (KD1017)
Rhizaspis rugosa KX580627 (KD1018)
Pseudodifflugia cf. gracilis AJ418794
Pseudodiflugiidae
Tectofilosida
Rhogostoma minus AJ514867
Rhogostoma micra HQ121436
Rhogostoma schuessleri HQ121430
Rhogostomidae fam. nov.
89/1.00
Sacciforma sacciformis KX580629 (KD1016)
Cryothecomonas aestivalis AF290539
94/1.00
Protaspa grandis DO303924
89/1.00
Cryothecomonas longipes AF2905
Protaspa obliqua FJ824121
Mataza hastifera AB558956
56/1.00
Ebria tripartita DQ303922
Botuliforma benthica FJ824126
Rhogostomidae fam. nov.
Cryomonadida
Thecofilosea
62/0.87
Coelodendrum ramosissimum AY266293
Aulosphaera trigonopa AY266292
Aulacantha scolymantha AY26629
Spongomonas minima AF411281
Limnofila borokoensis AF411284
Hedriocystis reticulata AY3050
Clathrulina elegans AY305009
Mesofila limnetica AF411283
93/0.78
Nanofila marina AF289081
67/0.99
Minimassisteria diva EF405665
Massisteria marina AF411286
Micrometopion nutans HQ121441
64/0.90
Chlorarachnion reptans X70809
Bigelowiella natans AF054832
Lotharella sp. AF076168
Minorisa minuta JX272635
Metromonas simplex AY620254
Tremula longifila HQ121439
94/1.00
Spongospora subterranea AF3109
Plasmodiophora brassicae AB526
87/0.99
Phagomyxa odontellae AF310904
Vampyrellidae sp. AY941200
53/0.66
Urosporidium crescens U47852
81/0.97
Paradinium poucheti EU189030
Gromia oviformis AJ457814
89/1.00
Reticulamoeba sp. AY268044
Sarcomonadea
Imbricatea

Figure 11: Reconstructed phylogenetic tree of the Cercozoa. Shown is the maximum likelihood tree obtained by the PhyML GTR+I+G analyses including 76 sequences and 1587 unambiguously aligned sites. On the respective branches are the support values of the PhyML and the Bayesian analysis shown (ML/BI), if higher than 50. Bold lines indicate a support value above or equal 95/0.95. Highlighted are the Tectofilosida (blue, orange and yellow) and Rhogostomidae in the Cryomonadida (green-colours).

3. Discussion

3.1. Untangling *Plagiophrys* on morphological basis

Skuja (1948) described a species, highly similar to '*Plagiophrys scutiformis*', only differing in the nucleus structure. He described *Rhizaspis granulata* as an oval and flattened cell with longitudinal folds and filopodia, which are used for locomotion by creeping over the surface and may be used for floating stages. He even mentioned the zoning of vacuoles and granules. Since he was a phycologist and was not familiar with filose amoebae, he saw resemblance in the petalomonads and suggested that his isolates completely lost their flagella and evolved therefore filopodia *de novo*.

This is highly unlikely and since his description of *R. granulata* was in all major points (e.g. filopodia without granules, aperture being slit-like, cell shape and nourishment) in common with Hertwig and Lessers', we conclude that Skuja unknowingly redescribed '*Plagiophrys*' sensu Hertwig and Lesser (1874).

We therefore adopt the name *Rhizaspis* for '*Plagiophrys*' sensu Hertwig and Lesser (1874), therefore all to us known *Plagiophrys* species except *Plagiophrys* sensu Claparède and Lachmann (i.e. *Plagiophrys cylindrica* and *Plagiophrys sphaerica*) and transfer following species to *Rhizaspis*: *P. scutiformis*, *P. parvipunctata*, *P. arcuatus* and *P. scutiformis* var. *marginata*.

Since pseudopodia structure (i.e. being lobose or filose and granule content) is a morphological character of high specificity (Bass et al. 2009a, Dumack et al. 2016a, Nikoleav 2005), it is highly unlikely, that these are monophyletic with *Plagiophrys* sensu Claparède and Lachmann.

The species described by Claparède and Lachmann (i.e. *Plagiophrys cylindrica* and *Plagiophrys sphaerica*) show closer morphological affinity to *Allogromia*, *Microgromia* or other amoebae with prominent granules in their filopodia and therefore might group in the Foraminifera or elsewhere in

the Cercozoa (for instance in the Granofilosea), whereas the species described by Hertwig and Lesser are presumed to group in the Thecofilosea.

3.2. Morphological analyses of our isolates

All our isolates show high similarities with Hertwig and Lessers' '*Plagiophrys*'. Hertwig and Lesser (1874) reported '*Plagiophrys sacciformis'* as irregular cylindrical (35 µm in length and 25 µm in width), just like our measurements of KD1016. We conclude that KD1016 resembled Hertwig and Lessers' *Plagiophrys sacciformis*.

Further they describe *P. scutiformis* as oval, 60 µm in length and 40 µm in width, with an often-wrinkled surface. They did not describe contractile vacuoles, which are difficult to observe within thecate amoebae as (a) thecate amoebae often are thicker than most naked amoebae and other cell contents shade the hyaline vacuoles and (b) the vacuoles usually 'beat' very slowly compared to most naked amoebae. However, Skuja (1948) described them. Both described the withdrawn slit-like aperture and clear zones by pointing out that food vacuoles aggregate and granules, in the center of the cell, embed the nucleus. These descriptions fit to our observations of strains KD1015 and KD1018.

However, there is one significant difference between their and our descriptions. Opposing to our observation Hertwig and Lesser (1874) described the nucleus to be homogeneous, even after treatment with acetic acid, no nucleolus could be observed. Skuja (1948) however described his *R. granulata* with one large central nucleolus. In contrast, we could define a varying amount of nucleoli. Since nucleus structure is a morphological characteristic of high specificity and importance, we conclude therefore, that we need to establish a new species name for our isolates KD1018 and KD1015, *Rhizaspis rugosa*.

KD1017 resembled in many points *Rhizaspis rugosa*. But up to now, the highly different and varying cell shape of strain KD1017 was never described to this extend for any other '*Plagiophrys*' or *Rhizaspis* species and never resembled the typical shield-like form of the previously mentioned species.

3.3. Untangling *Plagiophrys* on phylogenetic basis

Surprisingly strain KD1016, *Rhizaspis rugosa* and *Rhizaspis transformis* are not monophyletic within our analyses. Although both group within the Thecofilosea, KD1016 groups close to *Rhogostoma*, *Rhizaspis rugosa* and *Rhizaspis transformis* instead as a sistergroup to the Pseudodifflugiidae. We therefore cannot adopt the name *Rhizaspis* for Hertwig and Lessers' '*Plagiophrys sacciformis*' and therefore establish the new genus *Sacciforma* gen. nov., comprising exclusively *Sacciforma sacciformis*, yet.

Howe et al. (2011) established the Rhizaspididae although no *Rhizaspis* has been sequenced back then. They made this assumption based on morphological similarity between *Rhogostoma, Capsellina* and *Rhizaspis* (i.e. the slit-like aperture, which is, as we could show, highly flexible in *Rhizaspis*). *Rhizaspis* does not group close to *Rhogostoma*, although *Sacciforma* does. Those genera can therefore not build a family which bears the name Rhizaspididae. We therefore have to switch *Rhogostoma* and the presumed closely related *Capsellina* (see Howe et al. 2011) to a new family, which includes *Sacciforma* and should be called Rhogostomidae.

'*Plagiophrys*' sensu Hertwig and Lesser (=*Rhizaspis*) was early believed to be closely-related to *Lecythium* (de Saedeleer 1934). We could show that this is the case as *R. rugosa* and *R. transformis* group within the Tectofilosida. However, they build no direct sistergroup to *Lecythium* but show a closer relationship with the agglutinating testate amoeba *Pseudodifflugia*.

Although the morphology of the here described *Rhizaspis* species clearly differs as they could never be confused with each other, surprisingly, their SSU sequences were identical. Several studies already highlighted that the SSU gene is a limited marker for diversity of protists. More variable genes probably will show genetic differences between *Rhizaspis rugosa* and *Rhizaspis transformis*. Since the cultures are deposited in a culture collection and are therefore made available to the public, genes such as ITS or LSU may be sequenced by us or others in future studies.

The other described '*Plagiophrys*' species (*P. scutiformis* by Hertwig and Lesser 1874, *P. parvipunctata* and *P. arcuatus* by Penard 1902 and *Plagiophrys scutiformis* var. *marginata* by de Groot 1979) of which molecular data are still lacking, may also group with Tectofilosida, because of their similarity to *Rhizaspis rugosa* and *Rhizaspis transformis.* However, this study confirmed that assumptions about taxonomy solely based on few morphological characteristics may be incorrect and are often confusing. Molecular data are therefore urgently required to shed more light on the phylogeny of the highly interesting group of filose thecate amoebae.

3.4. Culture conditions

Although we provide stable long-time maintainable cultures, we were not able to provide cultures of two of our four strains (*Rhizaspis rugosa* (strain KD1015) and *Sacciforma sacciformis*), as they ceased to grow after a couple divisions. In case of *R. rugosa*, we were not able to culture strain KD1015 with a mixture of food sources including yeasts and green algae. We optimized culture conditions by adding a variation of different diatoms and excluding yeasts and green algae, making KD1017 and KD1018 well culturable (Table 1).

Nevertheless, strain KD1017 constantly formed cysts (in contrast to KD1018 and many other Thecofilosea cultures) which we interpret as a stress response to our culture conditions. *Sacciforma sacciformis* grouped in direct relation to *Rhogostoma*, an omnivorous amoeba, feeding on eukaryotic cells like algae but not culturable without sufficient supply of bacteria as food source (own observations, unpublished). We therefore conclude that *Sacciforma* needs bacteria as food source and ceases to grow under our low bacteria conditions, similar to *Rhogostoma*.

However, as this study and many other recent studies show (Dumack et al. 2016 a,b; Hess & Melkonian 2013; Hess et al. 2012), previously as 'unculturable' assigned protists, are culturable if cultured under the right conditions.

4. Taxonomic acts

4.1. *Rhizaspis* (Skuja 1948)

Revised diagnosis: Thecate amoebae with ventral slit-like, but flexible, cleft that emits filopodia; theca thin, flexible, in active cells adherent throughout to cell surface, with exosomes (*R. parvipunctata*) or without (all other species), sometimes deformed by ingested food, often wrinkled or folded, consisting of single smooth dense layer outside and scarcely thicker than the plasma membrane; thus with bilateral symmetry (ventral flattened). Round nucleus; nucleoli may be visible by light microscopy, often one, sometimes several. Cells may be divided in zones (basal to apical): several contractile vacuoles, food vacuoles, nucleus embedded by a large number of in size varying

granules. Inhabiting freshwaters. Eukaryvorous, mainly feeding on algae, especially diatoms. Division longitudinal, binary. Sexual reproduction unknown.

Type species: *R. granulata* Skuja 1948

Other species: *R. simplex* by Skuja 1948, *R. scutiformis* comb. nov. by Hertwig and Lesser 1874, *R. parvipunctata* comb. nov. and *R. arcuatus* comb. nov. by Penard 1902 and *R. scutiformis* var. *marginata* comb. nov. by de Groot 1979

4.2. *Rhizaspis rugosa* sp. nov.

Diagnosis: The theca comprises lateral and basal folds. Round nucleus, several roundish nucleoli. Cell divided into zones as described for the genus. No floating stages observed. Cysts not observed. In cultures cells may occur basolaterally connected in a ring, built through unfinished fission, not fusion. Easy to confuse with *Rhizaspis scutiformis* or *Rhizaspis granulata* which have different nucleoli.

Deposited preserved material: A glass slide containing several fixed individuals is deposited in the Upper Austrian State Museum Invertebrate Collection (2016/119).

Type generating strain: KD1018; deposited in the Culture Collection of Algae and Protozoa under the accession number CCAP 1267/1

SSU Sequence of type generating strain: KX580627

Illustrations of type generating strain: Figs 1, 3, 10, Supplementary Video 1; this material constitutes the name-bearing type of this species.

Type locality: quarry pond next to river Rhine, in Xanten, Germany (51.691441, 6.425774)

Etymology: rugosa {adj} [Latin] = 1) wrinkled, wrinkly. Referring to the numerous folds in the theca.

4.3. *Rhizaspis transformis* sp. nov.

Diagnosis: Similar to *Rhizaspis rugosa,* with following differences*:* Highly metabolic and therefore varying in shape and size, usually bulky and deformed; never resembled the thin and disc-like shape of *Rhizaspis rugosa*; no clear separation in zones; cysts vary in form: theca builds an envelope around the spherical cyst, compressed with notches and protuberances; filopodia with numerous non-contractile vacuoles.

Deposited preserved material: A glass slide containing several fixed individuals is deposited in the Upper Austrian State Museum Invertebrate Collection (2016/120).

Type generating strain: KD1017; deposited in the Culture Collection of Algae and Protozoa under the accession number CCAP 1267/2

SSU Sequence of type generating strain: KX580628

Illustrations of type generating strain: Figs 6, 7, 8, 9, 10; Supplementary Video 1; this material constitutes the name-bearing type of this species.

Type locality: pond in urban park, in Cologne near Mülheim, Germany (50.958675, 7.005476)

Etymology: transforma –re {verb} [Latin] = 1) (to) transform, (to) reform. Referring to the highly variable cell shape.

4.4. Rhogostomidae fam. nov.

Diagnosis: Thecate amoebae with ventral slit-like and not flexible cleft that emits filopodia; theca thin, flexible, in active cells adherent throughout to cell surface, consisting of single smooth dense layer outside and scarcely thicker than the plasma membrane; thus with bilateral symmetry. Theca with exosomes (*Capsellina*) or without (*Rhogostoma*, *Sacciforma*). Omnivorous (mainly bacteria, also yeasts, algae). Electron microscopy of *Capsellina* and *Rhogostoma* by Simitzis and Le Goff (1981). Division longitudinal, binary. Sexual reproduction unknown.

Type genus: *Rhogostoma* Belar, 1921

other genera: *Capsellina* (Penard 1909; see Howe et al. 2011) *Sacciforma* (this study)

4.5. *Sacciforma* gen. nov.

Diagnosis: Cell body in contrast to the apical-basal compressed *Rhogostoma* and *Capsellina,* to which it is closely related, elongated (length-width ratio approx. 1.5) and ventral flattened. Freshwater. Cysts, floating- or flagellate stages unknown.

Type species: *Sacciforma sacciformis* comb. nov., Hertwig and Lesser 1874

Etymology: saccus {noun} [Latin] = bag; formis {noun} [Latin] = shape; refers to the bag-like shape of the cell body, derived from the species name of the type species *Sacciforma sacciformis*, described as *Plagiophrys sacciformis* by Hertwig and Lesser, 1874.

5.Materials and methods

5.1.Sampling and isolation

We collected 100-200 ml of sediment or detritus rich water of freshwaters, like ponds and rivers (Table 1). Most samples were stored at room temperature over night; few samples were stored at 10 °C incubation over two to three days before investigation.

The samples were then shaken to detach surface attached protists and 1 ml was given in each well of a 24-well plate (Sarstedt, Germany). The plate was incubated for approx. 20 min to allow small organisms to settle. Observation of amoebae was conducted with a light microscope (Nikon Eclipse TS100; Ph1; 40x, 100x, 200x and 400x magnification). All samples were scanned for *Plagiophrys*-like cells several times up to three weeks after collection. Promising cells were transferred to Waris-H (McFadden & Melkonian 1986) and enriched with a mixture of putative food organisms: *Nitzschia communis* (CCAC 5737 B), *Nitzschia amphibia* (CCAC 5733 B), *Navicula* sp. (CCAC 1772 B), *Pinnularia* sp. (CCAC 0222 B), *Cyclotella meneghiniana* (CCAC 5735 B), *Characium* sp. and *Saccharomyces cerevisiae* (Table 2). Cells were subcultured every week.

5.2.Microscopical observations

Pictures and videos were taken with a Nikon Eclipse TE2000-E (Ph1, up to 400x magnification) Nikon Eclipse 90i (DIC, up to 600x magnification), both with the mounted camera Nikon digital sight DS-U2 (program: NIS-Elements V4.13.04).

5.3. SSU sequencing

Single cells were starved for two to three days and transferred with approx. 1 µl of medium into 15 µm ddH_2O into PCR-tubes. To this, 5 µl Green Taq Dream Buffer, 5 µl of 2 µM dNTP's, 5 µl of 1 µM forward and reverse primer each and 0.3 µl DreamTaq DNA-polymerase (Thermo Fisher Scientific, Dreieich, Germany) were added with additional 14.7 µl ddH_2O.

The SSU sequences were obtained in two successive steps. First the whole SSU was amplified with the general eukaryotic primers, RibA and RibB (Cavalier-Smith and Chao 1995; Pawlowski 2000). Using one µl of the first PCR as template, a semi-nested re-amplification was conducted with the primers RibA+1300R targeting the 5' part and 590F+RibB targeting the 3' part of the gene (Quintela-Alonso et al. 2011).

PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles (denaturation at 96 °C for 32 s, annealing at 50 °C for 36 s, elongation at 72 °C for 2 min), terminal extension at 72 °C for 7 min, and hold at 4 °C.

For sequencing the Big dye Terminator Cycle sequencing Kit and an ABI PRISM automatic sequencer were used.

5.4.Phylogenetic analyses

The partial sequences were manually checked for sequencing errors and combined into one sequence contig. To create a dataset for phylogenetic analyses they were blasted (blastn 2.3.0) against the NCBI GenBank database (last date of accession: 23.06.2016). Sequences with a similarity of ≥ 96% were downloaded and manually aligned in SeaView (V4.5.3, Gouy et al. 2010). Additionally

representative sequences of the major cercozoan subphyla were added, Endomyxa sequences were used as outgroup. An alignment with 76 sequences and 1587 unambiguously aligned sites of which 46.82% were invariant was used for phylogenetic analyses. The program jmodeltest (V.2.1.5, Darriba et al. 2012) was used to determine the best fitting model: GTR+I+G, which was selected among 88 models (settings: Substitution schemes 11; add Base frequencies +I+G rate variation nCat=4, ML optimized NNI as base tree).

Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood phylogenetic analyses were run using PhyML V3.1 (Guindon & Gascuel 2003) with the following settings: GTR model; a proportion of invariable sites and a gamma-shaped distribution of the substitution rates across variable sites (GTR+I+G), with four rate categories; BIONJ distance-based starting tree with all model parameters estimated from the data. The Bayesian analyses were run using MrBayes V3.2 (Altekar et al. 2004; Ronquist & Huelsenbeck 2003) with the following settings: 5 million generations, trees were sampled every 100 generations, convergence of the two runs was estimated every 500 generations. Of the sampled trees, 25% were discarded as burn-in.

5.5.Preparation of cells for deposition

Saturated mercury-(II)-chloride solution and 96% ethanol were mixed (ratio 2:1) to obtain a fixing solution. It was heated to 70 °C and 30 ml were added to 1.5 ml of a culture in growing phase. The mixture was washed in 30 % ethanol once and further fixed in 1 ml osmium tetroxide for one minute. Subsequently the cells were dehydrated in eight steps consisting of 20 min incubation in an ethanol series of increasing concentration (from 30 to 96%). Cells were then preserved on a microscope slide with a drop of glycerine under a cover slide. The slides were deposited in the Upper Austrian State Museum; accession numbers pending.

6.Acknowledgments

The study was supported by the DFG research group FOR 918. We thank the Culture Collection of Algae at the University of Cologne (CCAC) for their service and the provided algal strains.

7.References

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Chapter 6: Polyphyly in the thecate amoeba genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa), redescription of its type species *L. hyalinum*, description of *L. jennyae* sp. nov. and the establishment of *Fisculla* gen. nov. and Fiscullidae fam. nov.

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Title

Polyphyly in the thecate amoeba genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa), redescription of its type species *L. hyalinum*, description of *L. jennyae* sp. nov. and the establishment of *Fisculla* gen. nov. and Fiscullidae fam. nov.

Keywords

Chlamydophyrs, protist, algivorous, testate amoebae, ectocytobiotic bacteria

Abstract

Testate amoebae have been studied and classified for more than 150 years. There are however some groups of which little is known, such as the genus *Lecythium* in the family Chlamydophryidae. Recently, five monophyletic species of thecate amoebae within Cercozoa were described, and grouped into the genus *Lecythium* on morphological basis. Since sequences of the type species, *Lecythium hyalinum* Hertwig and Lesser 1874, were lacking these species descriptions were based on morphology. To clarify the taxonomy and phylogeny of *Lecythium*, we screened for *L. hyalinum* in freshwater samples of Germany and the Netherlands. Four different isolates of *L. hyalinum* and one novel species were cultured. We provide (a) light microscopy data, (b) five SSU sequences of the genus *Lecythium* and (c) an updated phylogeny of *Lecythium* and the Thecofilosea. The data show that the genus *Lecythium* is polyphyletic with the type species *L. hyalinum* grouping within the 'Novel Clade 4', which was predominantly composed of environmental sequences. We therefore split this genus into *Lecythium* and *Fisculla* gen. nov. and establish the Fiscullidae fam. nov.

Introduction

Testate amoebae have been of high interest for protistologists and ecologists since their discovery. Since they are one of the few protist groups with clear and easily identifiable morphological traits, they represent ideal model organisms for protist ecology and evolution. The specific ways in which their tests are constructed have been intensively studied and were used for testate amoeba identification and taxonomy. Research solely based on morphology however led to complex taxonomical concepts that were often contradicting and thus frequently changed (Cash et al. 1915; de Saedeleer 1934). With phylogenetic approaches a widely accepted consensus has been achieved. It has been shown that testate amoebae are polyphyletic and belong to different phyla, like Amoebozoa and Cercozoa (Cavalier-Smith 1998a,b; Kosakyan et al. 2016; Nikolaev et al. 2005). The phylum Cercozoa, established by Cavalier-Smith (1998a,b) is highly diverse in morphology and ecology. The Cercozoa consist predominantly of naked amoebae, flagellates and amoeboflagellates (Bass 2009; Dumack et al. 2016a; Hess and Melkonian 2013; Howe et al. 2011). Nestling between those, it comprises several polyphyletic testate amoeba lineages: e.g. the order Euglyphida with tests made out of siliceous plates (Cavalier-Smith 1998a,b; Wylezich et al. 2002) and the classes Cryomonadida and Tectofilosida, both in the order Thecofilosea, comprising amoebae with organic and agglutinated tests (Dumack et al. 2016b,c; Dumack et al. (in review); Howe et al. 2011; Wylezich et al. 2002).

The Tectofilosida, established by Cavalier-Smith and Chao (2003), contain up to now the testate amoeba families Rhizaspididae, Pseudodifflugiidae and Chlamydophryidae (Wylezich et al. 2002; Dumack et al. 2016b,c; Dumack et al. 2016d).

Knowledge about the Chlamydophryidae is scarce. De Saedeleer (1934) established the family Chlamydophryidae as a subfamily of the Gromiidae, to accommodate genera such as *Lecythium* and *Chlamydophrys*, thus containing amoeba species with thin hyaline tests, but until recently no molecular data was available. Dumack et al. (2016b,c) shed some light on the Chlamydophryidae by culturing and describing several strains of amoebae with spherical organic tests and longitudinal division. They showed that these isolates grouped as a sister clade to the Pseudodifflugiidae.

Nevertheless the type species described by Hertwig and Lesser (1874), could not yet be sequenced, leaving the possibility open that the genus *Lecythium* might be polyphyletic. We therefore decided to screen samples for *L. hyalinum*, isolate it and perform genetic and morphological analyses to clarify the existing taxonomy.

We isolated four strains of *Lecythium hyalinum* from two central European countries, sequenced their SSU sequence and conducted phylogenetic analyses. We show its phylogenetic placement in

the Cercozoa and discuss the polyphyly of the genus *Lecythium*. Additionally, we isolated another *Lecythium*-like amoeba. After morphological observations and intensive literature research, we are convinced that this species is new to science, which we herein describe.

Results

Footnotes:

[1] List of abbreviations: a = aperture; nu = nucleus; no = nucleolus; g = granules; bf = branched filopodia; af = anastomosing filopodia; fv = food vacuole; cv = contractile vacuole; fo = folds; vg = vacuole with granule; hv = hyaline vacuole; lg = large granule; sg = small granule; c = cyst wall; t = theca

Sampling and culturing

We isolated four different strains of *Lecythium*-like amoebae from Germany and the Netherlands (Table 1). Three cultures of *L. hyalinum* were not stable and therefore lost during the study. Strains of *Lecythium* reacted highly sensitive on the composition of culture medium and food organisms: Strains of *L. hyalinum* were only viable in WC-Medium (Guillard and Lorenzen 1972), whereas strain KD1014 was only cultivable in Waris-H (McFadden and Melkonian 1986). *L. hyalinum* cultures fed only with the green alga *Characium* sp. (or with a combination of *Nitzschia communis* and *Characium* sp.) as food source suddenly collapsed after few weeks of culturing. Only the last isolated *L. hyalinum* strain KD1013 which was cultured with a combination of *Nitzschia communis* (CCAC 5737B), *Characium* sp., *Nitzschia amphibia* (CCAC 5733B) and *Pinnularia* sp. (CCAC 0222B) was culturable over the whole time of our analyses (~ 5 months). Strain KD1014 was cultured with *Nitzschia amphibia* (CCAC 5733B), *Characium* sp. and *Pinnularia* sp. (CCAC 0222B).

Table 1: Isolated species and strains with information about phylogenetic analyses, sampling spot and date, and culture conditions

Species	Strain	SSU rDNA accession	Sequence length	Sampling spots	Coordinates	Isolation date	Habitat	Observed food source in sample	Food source in culture
Lecythium hyalinum	KD1010	KX580626	1427	Germany, Xanten	51.691441, 6.425774	April 2016	Quarry pond next to the river Rhine	Diatoms	*Nitzschia communis* (CCAC 5737B), *Characium* sp.
	KD1011	KX580624	1707	Germany, Cologne	50.926161, 6.928794	April 2016	Small urban artificial pond	Diatoms	*Characium* sp.
	KD1012	KX580623	1689	Germany, Cologne	50.922577, 6.946049	April 2016	Big urban artificial pond in the Volksgarten-Park	Diatoms	*Nitzschia communis* (CCAC 5737B), *Characium* sp.
	KD1013	KX580625	1707	Netherlands, s'Hertogenbosch	51.715207, 5.312476	April 2016	Channel-like urban artificial pond in a park	Diatoms	*Nitzschia communis* (CCAC 5737B), *Characium* sp., *Nitzschia amphibia* (CCAC 5733B), *Pinnularia* sp. (CCAC 0222B)
Lecythium jennyae	KD1014	KX580622	1710	Germany, Cologne	50.958675, 7.005476	May 2016	Urban artifical pond in a park	Diatoms	*Nitzschia amphibia* (CCAC 5733B), *Pinnularia* sp. (CCAC 0222B), *Characium* sp.

Morphological observations

Lecythium hyalinum

All isolates of *L. hyalinum* (strains KD1010, KD1011, KD1012 and KD1013) were of similar morphology. The amoebae bear a thin and hyaline theca (18-50 µm in length and 24-61 µm in width; n=37) of roundish or slightly curved shape and radial symmetry. Around the aperture of the cells, small wrinkles and folds may be seen, occurring most often in starving cells (Figs 1E, 2E for individuals without wrinkles or folds see: Figs 2F, 3D). The aperture might be retracted into an invagination of the test (Fig. 1F). No obvious deformation of the test in consequence of ingested food was detected.

The nucleus (11.5-21 µm; n= 36; Figs 1B, 2B, 3B) is located at the apical end of the cell (opposite to the aperture), most often ellipsoid, sometimes spherical, and a fine marbled structure. The nucleolus (4-8 µm; n=36), rarely two nucleoli (Fig 4, Supplementary Video 2), are spherical and exhibit a more rough structure, with indentations (Figs 1D, 2D, 3B). The test is carried in an upright position and four different zones within the cell body are separated (Fig. 4). From the apical to the basal end: The first zone (I) contains the nucleus embedded in hyaline cytoplasm and usually lacks other structures (e.g. granules, vacuoles, etc.), the second zone (II) contains granules (0.92-2 µm, n=36). The third zone (III) is dominated by food vacuoles and the fourth zone (IV), which lies in front of the aperture and therefore outside of the theca, is defined by a highly vacuolated cytoplasm and emerging filopodia. The vacuoles are contractile (Supplementary Videos 1), within the theca no contractile vacuoles were observed. Thin, often branched and sometimes anastomosing filopodia arise from the vacuolated plasma (IV) with a length of up to 100 µm (Figs 1C, 2C, 3C, 5). The filopodia may contain small granules (Fig. 1G) which are difficult to observe, but seem to be connected to the cytoskeletal filaments, as they move together in the same direction with similar velocity (Supplementary Video 1). All isolates harbored in close connection to the cytoplasm at the base of the filopodia 'dancing particles', possibly ectocytobiotic bacteria (Fig. 5, Supplementary Video 1). Those particles have been present in all *L. hyalinum* isolates and individuals although in variable amounts.

When transferred to new culture medium with a high food density, most amoebae covered themselves by an accumulation of algae, so that only the filopodia could be seen. Swimming stages with slowly moving, non-branching and non-anastomosing filopodia were observed with a decline of food density or when cultures were older than five days. In one strain (KD1013) clover leaf-shaped swimming aggregates (Fig. 2G) as well as large aggregates of individuals forming a feeding community were observed (Fig. 2H). Those aggregates may fuse partially in the areas of the vacuolated cytoplasm in region IV. No cysts or flagellated cell stages were observed. Division longitudinal.

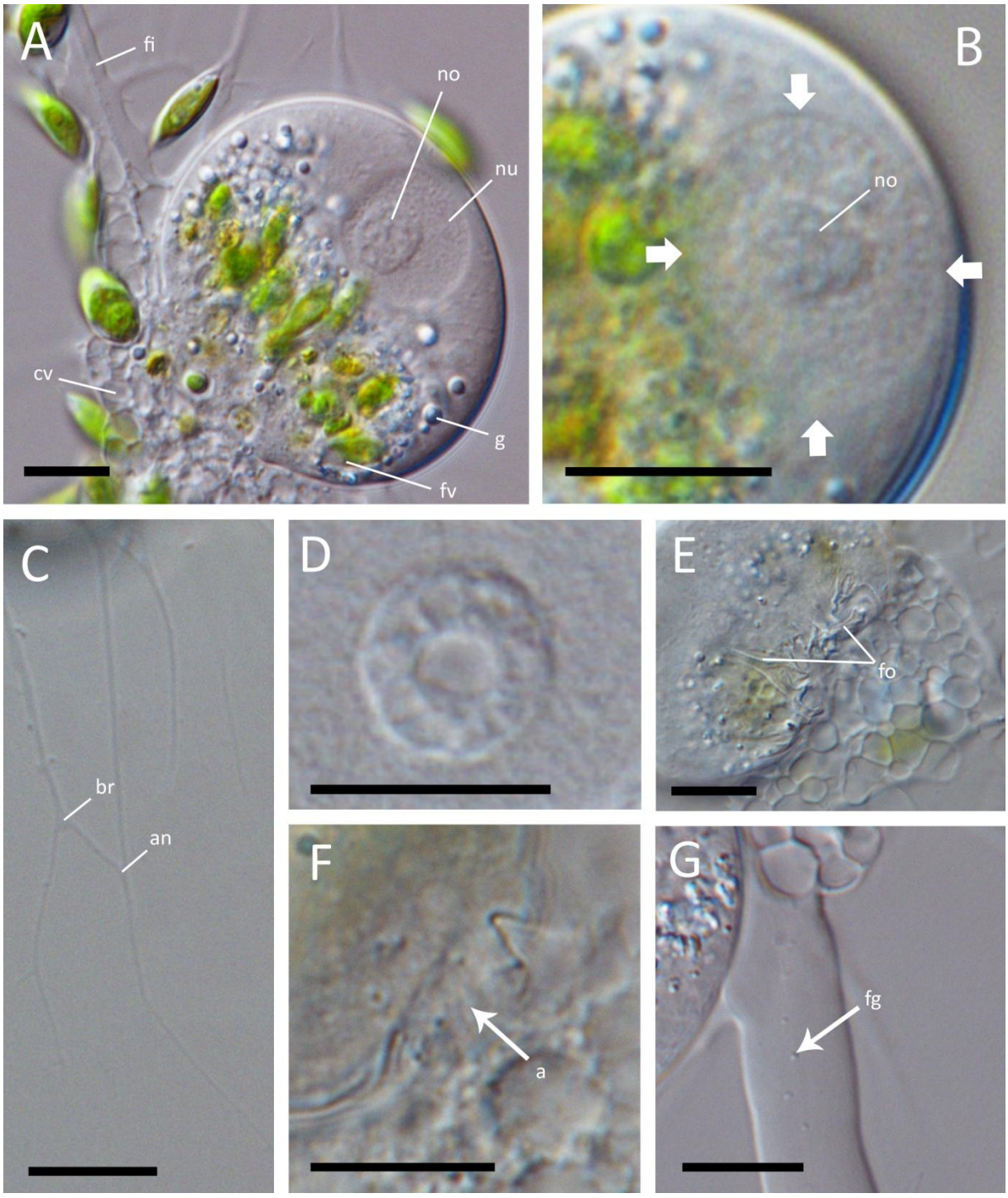

Figure 1: Cellular features of *Lecythium hyalinum* strain KD1010. Scale bars indicate 10 µm, pictures were taken with DIC. A: Overview of the cell body. B: Close-up of the nucleus. C: Branching and anastomosing filopodia. D: The nucleolus and its structure. E: Close up of the folds surrounding the aperture and the vacuolated cytoplasm. F: The aperture seen from the side. G: Small granules within the filopodia.

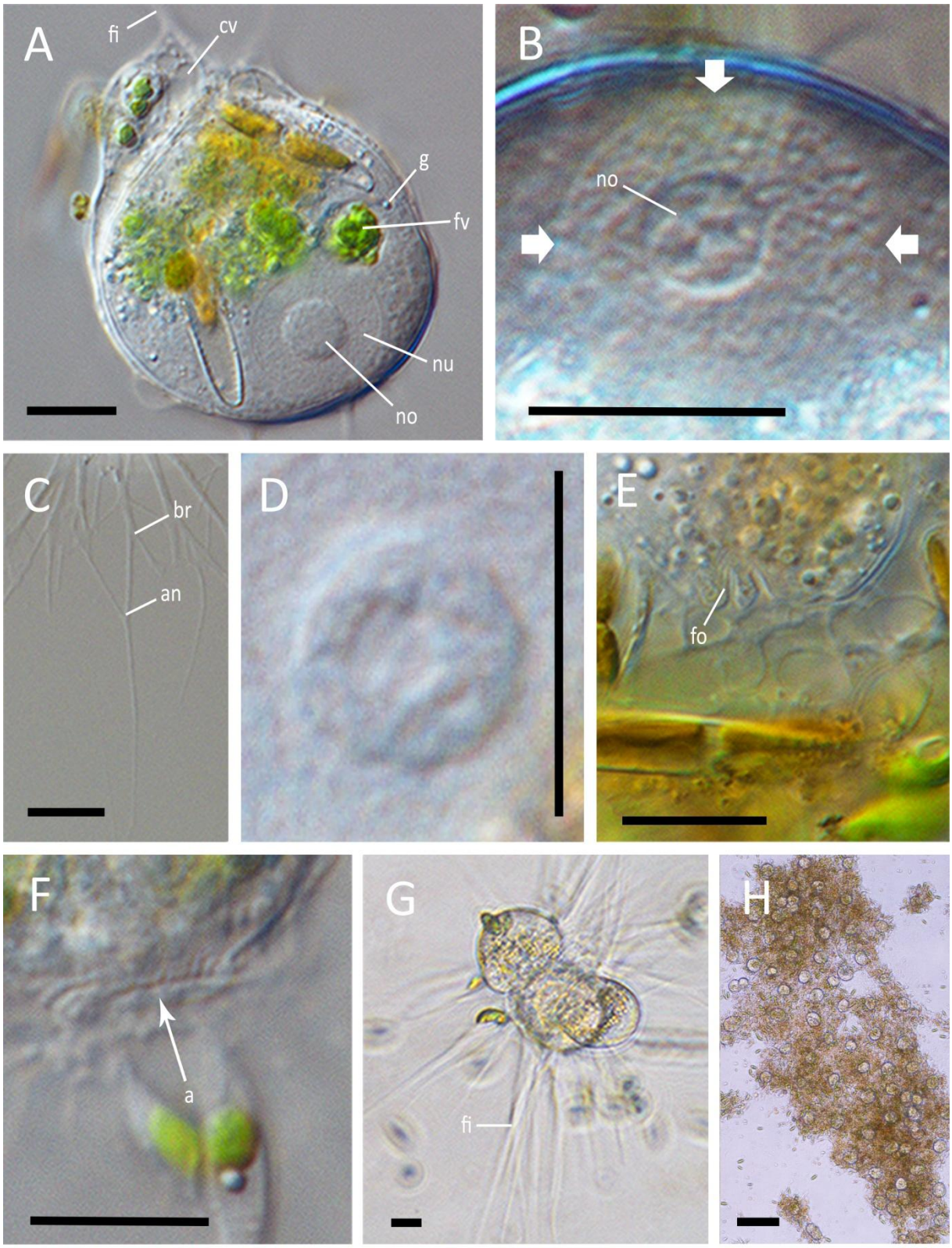

Figure 2: Cellular features of *Lecythium hyalinum* strain KD1013. A-F: Scale bars indicate 10 µm, pictures were taken with DIC. G+H: Scale bars indicate 100 µm, pictures were taken with ph1. A: Overview of the cell body. B: Close-up of the nucleus. C: Branching and anastomosing filopodia. D: The nucleolus and its structure. E: Close up of the folds surrounding the aperture and the vacuolated cytoplasm. F: The aperture seen from the side. G: Three swimming, adhering cells (aggregate) with non-branched and non-anastomosing filopodia; H: Feeding cells in a culture.

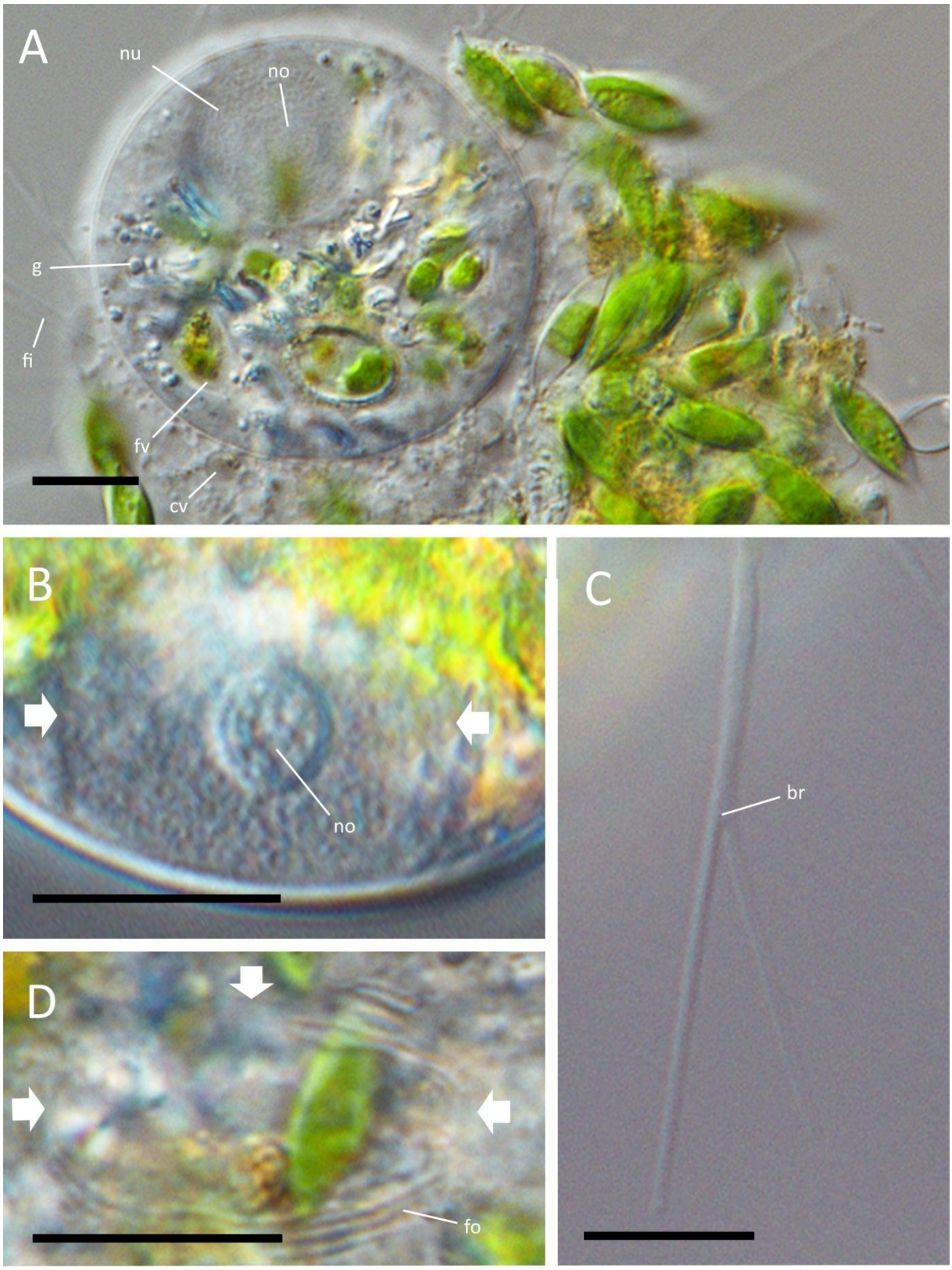

Figure 3: Cellular features of *Lecythium hyalinum* strain KD1012. Scale bars indicate 10 µm, pictures were taken with DIC. A: Overview of the cell body. B: Close-up of the nucleus with nucleolus. C: Branching filopodia. D: The aperture seen from below.

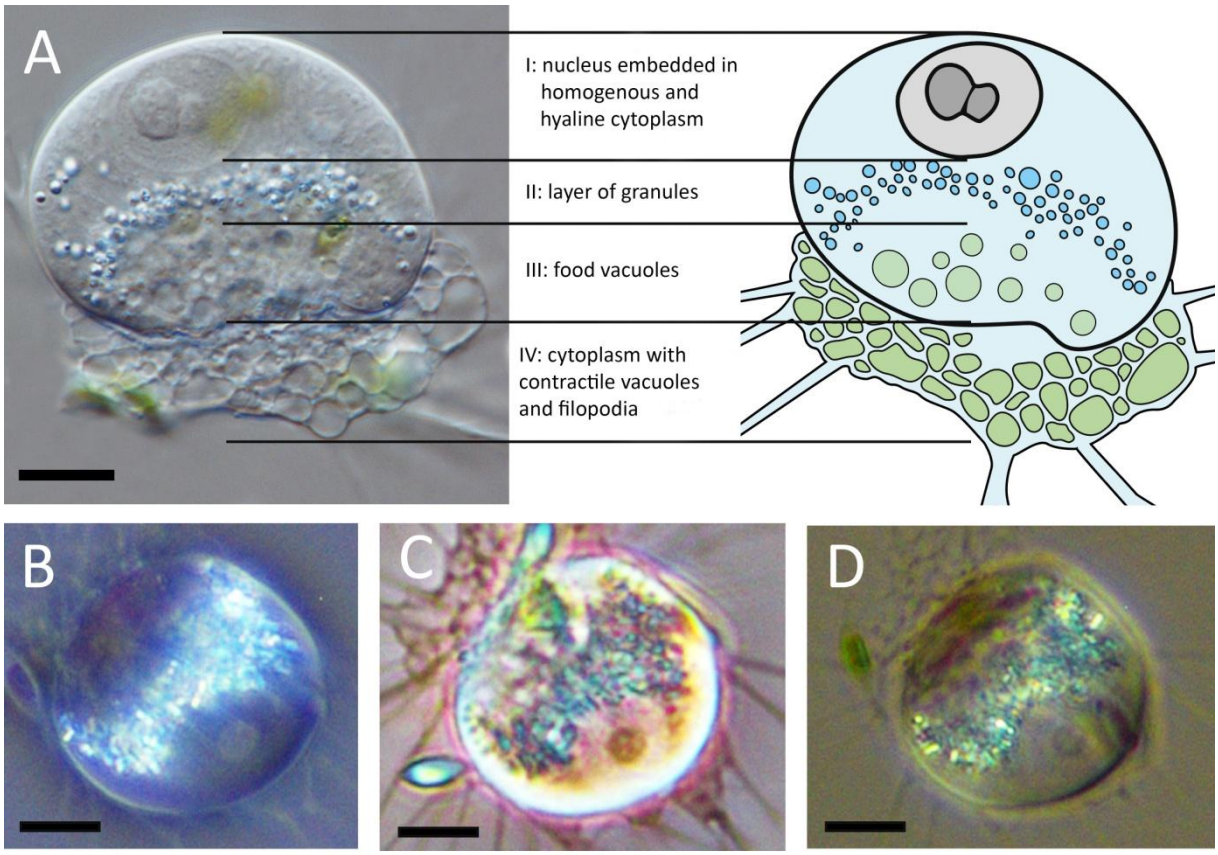

Figure 4: Schematic drawing of the cell composition of *Lecythium hyalinum*. Scale bars indicate 10 µm. Pictures taken with DIC (A), ph2 (B), ph1 (C) and ph3 (D).

Figure 5: Full overview of *Lecythium hyalinum* exemplified on strain KD1013 with expanded filopodia in a typical and undisturbed shape. Scale bar indicates 50 µm.

***Lecythium jennyae* sp. nov.**

The amoebae bear a thin and hyaline test, carried in an upright position. The theca is roundish or slightly curved with 24-42 µm in length and 29-48 µm in width (Fig. 6A, n=16). The theca usually has many folds all over the surface (Fig. 6B,D,F; Supplementary Video 3), and they are concentrated around the aperture (Fig. 6B,F). The surface can be also smooth (Fig. 6A; Supplementary Video 3), usually as a result of large ingested food organisms.

The nucleus (12.8-19.5 µm; n=12; Fig. 6C) is located at the apical end of the cell (opposite to the aperture) and most often ellipsoid, sometimes spherical, with a fine marbled structure. No nucleolus was detected. Granules with a size of 0.96-2.9 µm (n=16), vacuoles and contractile vacuoles are dispersed all over the cell body, whereas food vacuoles are restricted to the basal region of the cell (Fig. 6A, J, Supplementary Video 3).

The amoebae move with thin often branched and anastomosing filopodia with a measured length of maximal 60 µm (Fig. 6B, Supplementary Video 3). No floating or swimming stages were observed. Cell division longitudinal.

In cultures often aggregates occurred of usually two, rarely up to six, amoebae (Fig. 6G, 6F; Supplementary Video 3). The amoebae adhered at the apertures, but no fusion of the cell bodies was detected. After approximately four months of culturing the first cysts appeared in our cultures. They were built within the theca that remained as a loose envelope around the cyst wall (Fig. 7). Cysts contained granules with smaller particles and hyaline granules (Supplementary Video 3), in their center a transparent sphere was located, most probably the nucleus (Fig. 7B).

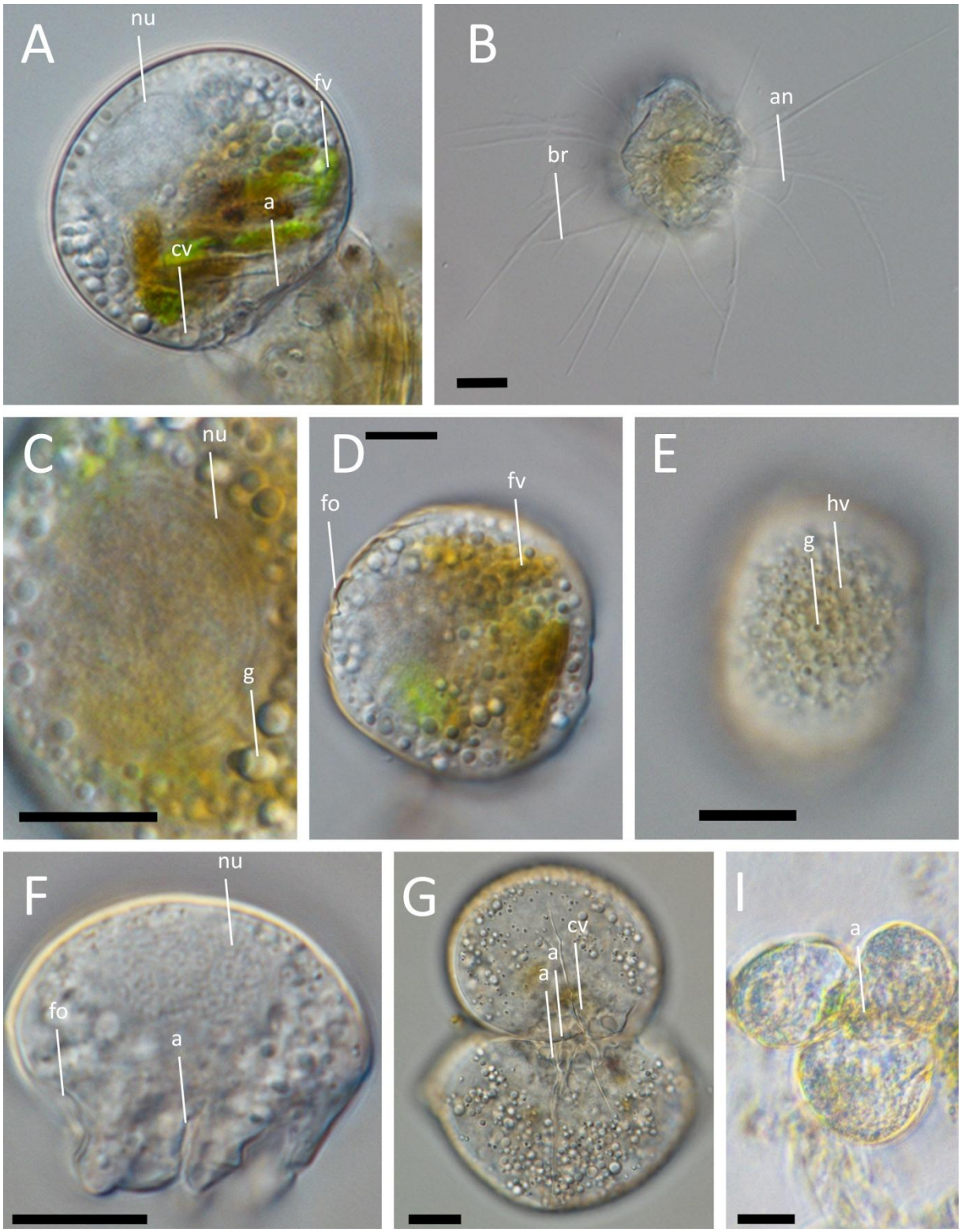

Figure 6: Cellular features of *Lecythium jennyae* (KD1014). Scale bars indicate 10 µm. A-G: pictures were taken with DIC; H: picture was taken with phase contrast. A: Overview of the cell body. B: Branching and anastomosing filopodia and many folds around the aperture. C: Close-up of the nucleus. D: Folds in the theca on the lateral side of the cell. E: Surface of the apical end of the cell. F: Starving cell seen from the side with folds around the aperture. G: Two adhering cells (aggregate). H: Three swimming and adhering (aggregate) cells.

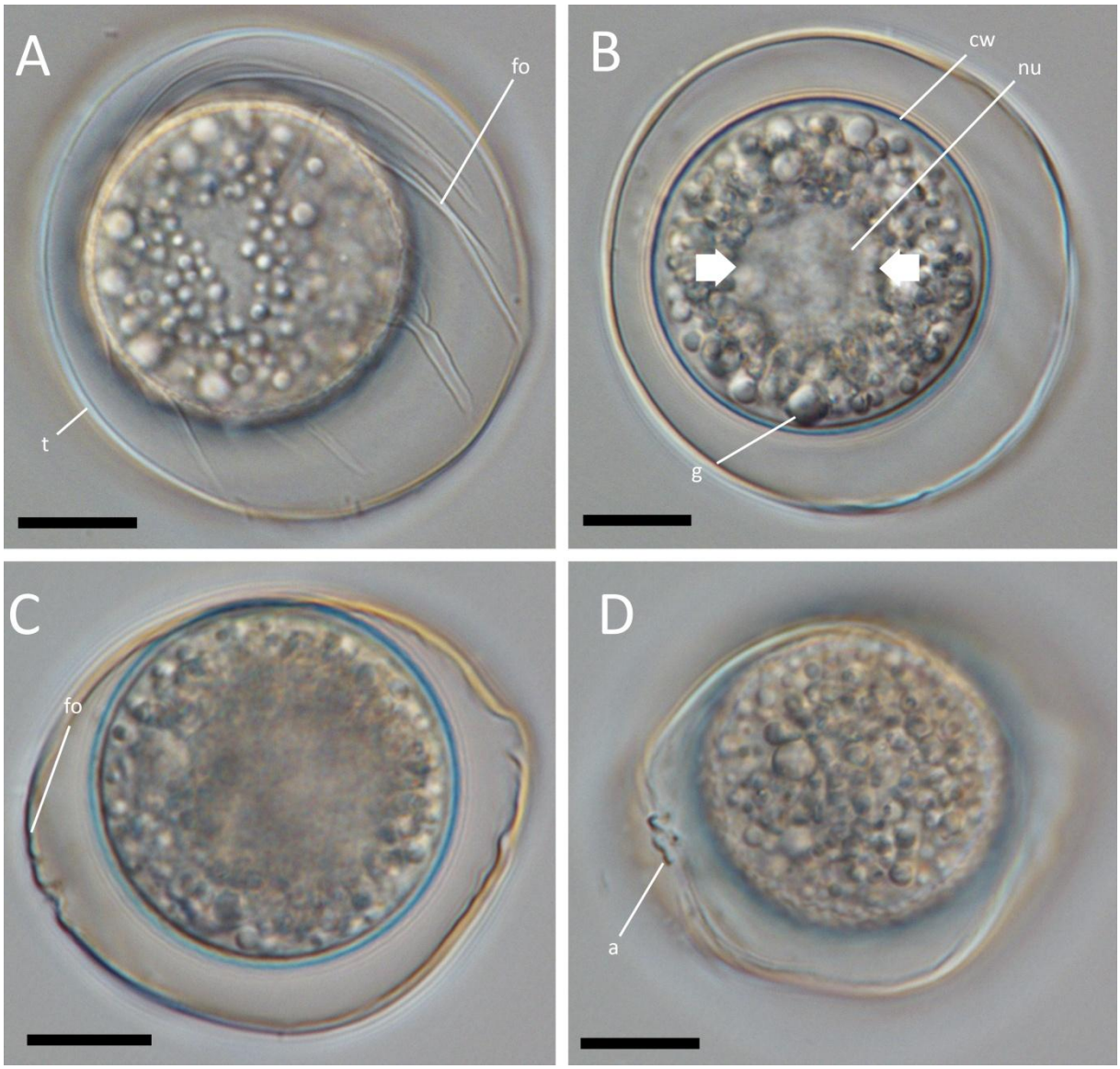

Figure 7: Cellular features of *Lecythium jennyae* in its dormant stage (cyst) provided on two different individuals (A+B; C+D) by light micrographs (DIC). Scale bars indicate 10 µm. For an animation of the cysts see Supplementary Video 3.

Sequencing of cultured amoebae and phylogeny

Near full length SSU gene sequences were obtained. The sequenced parts ranged from 1427 to 1710 nucleotides (Table 1). No introns were found. The sequences of all *L. hyalinum* strains were identical. The best maximum likelihood tree (Fig. 8) reveald the cercozoan subphylum Filosa (consisting of the Thecofilosea, Sarcomonadea and Imbricatea) and selected sequences of the Endomyxa as outgroup. As in previous analyses using SSU rDNA sequence comparisons, some basal branches within the Cercozoa were not supported (Bass et al. 2009; Howe et al. 2011). The Thecofilosea are composed of the Phaeodaria, Cryomonadida, Tectofilosida and with moderate support of 51/0.97 the Novel Clade 4 (Bass and Cavalier-Smith 2004). The Novel Clade 4 was highly supported (100/1.00) and composed of the four strains of *L. hyalinum* (KX580623, KX580624, KX580625, KX580626), *Lecythium jennyae* sp. nov. (KX580622), the recently described *Trachyrhizium urniformis* (Shiratori and Ishida 2016) and two marine environmental sequences.

Diagnoses

Genus ***Lecythium*** HERTWIG ET LESSER 1874 emend.

Emended diagnosis: Filose, radially symmetric amoebae. Cells bear a colourless, thin and organic test (theca), carried in an upright position, with one basal opening from which branching and anastomosing filopodia arise. Filopodia may show small granules. Nucleus at the apical end of the cell. Cells with numerous contractile vacuoles and granules. Might form floating or swimming stages (single cells and aggregates) with straight, non-branched and non-anastomosing filopodia. Aggregates with fused filopodia or fused vacuolated cytoplasm occur. Cysts are built within the theca that then remains as a loose envelope around the cyst wall. No flagellated cells observed. Species feed on eukaryotic prey (i.e. algae); ingestion of bacteria not observed. Division: longitudinal, binary. Sexual reproduction unknown.

Etymology: Lecythium [Greek] = flask, jug; referring to the vitreous test

Type species: *L. hyalinum*

Lecythium hyalinum HERTWIG ET LESSER 1874 emend.

Emended diagnosis: *Lecythium* as above. Test: round or spherical in shape, 18-50 µm (length), 24-61 µm (width), ratio 0.7-1 µm (length/width). Nucleus: ellipsoid or spherical, about 11.5-21 µm in width. Nucleolus:one, rarely two adhering, round, 4-8 µm in diameter; central to nucleus. Aperture: roundish, vacuolated cytoplasm arises from the aperture. Cells show a clear zonation (apical to basal): (I) zone of nucleus (no granules or vacuoles), (II) zone of granules (III) zone of food vacuoles (IV) highly vacuolated cytoplasm in front of the aperture (contractile vacuoles) of which branching and anastomosing filopodia arise. Locomotion: (i) actively creeping, filopodia rarely extend longer than 70 µm; (ii) swimming with extended, non-branched and non-anastomosed and slowly moving filopodia. Filopodia with small granules attached to cytoskeletal filaments occur. Prey: unicellular algae, no ingestion of bacteria observed. Cell division: longitudinal, binary.

Etymology: hyalinum [Latin], due to the hyaline test.

Deposited culture: deposited in the Culture Collection of Algae and Protozoa under the accession number CCAP 1943/6

Lecythium jennyae MAUSBACH DUMACK ET BONKOWSKI, sp. nov.

Diagnosis: *Lecythium* as above. Test: round or spherical in shape, 24-42 µm (length), 29-48 µm (width), ratio 0.7-1 (length/width), often deformed due to folds. Nucleus: ellipsoid or spherical, marbled structure, about 12.8-19.5 µm, located at the apical end of the cell; nucleolus not detectable. Granules: about 0.96-2.9 µm, round, dispersed all over the cell. Vacuoles: contractile vacuoles dispersed all over the cell, food vacuoles restricted to the basal end. Aperture: roundish, filopodia arise directly from the aperture and not from a vacuolated cytoplasm, like in *L. hyalinum*. Locomotion: actively creeping, filopodia rarely extend longer than 60 µm; Prey: unicellular algae, no ingestion of bacteria observed. Cell division: longitudinal, binary.

Type material (hapantotype): A glass slide containing several fixed individuals is deposited in the Upper Austrian State Museum Invertebrate Collection as Inv. Nr. (2016/121); this material constitutes the name-bearing type of this species.

Culture of type generating strain: deposited in the Culture Collection of Algae and Protozoa under the accession number CCAP 1943/7

Sequence of type generating strain (SSU rDNA): KX580622

Type locality: urban pond in a park at Cologne-Mühlheim, Germany; 50.958675, 7,005476

Etymology: jenny- ae, this species is dedicated to Jennifer Schmitz.

Family **Fiscullidae** DUMACK MAUSBACH ET BONKOWSKI, fam. nov.

Limnic or terrestrial filose testate amoebae. Test: flexible, organic, round, pyriform or spherical, with one basal aperture of which filopodia arise. No flagella or cilia. Surface grazer. Trophic stage: incessant creeping with branched and anastomosed, tapering filopodia. Preying on eukaryotes with filopodia. Food ingested through aperture, digested in the test. Nucleus round or ellipsoid. Granules and vacuoles occur (contractile vacuoles hard to detect). May form cysts or floating stages. No sexual reproduction observed. In contrast to *Lecythium*: no granules in filopodia, cells form aggregates by fusion, often between the apical end and the nucleus an additional layer of granules which could not be observed in *L. hyalinum*.

Etymology: Name derived from type genus.

Type genus: *Fisculla*

Genus ***Fisculla*** DUMACK MAUSBACH ET BONKOWSKI, gen. nov.

Filose radially symmetric amoebae. Theca often deformed by ingested food, sometimes wrinkled or folded. Apical ellipsoid or round nucleus, clear or grainy; round nucleolus sometimes visible by light microscopy. One or several contractile vacuoles (hard to see) and food vacuoles, usually basal. Granules vary in amount, sometimes in layers, sometimes spread evenly. Cells creep by filopodia, which may branch or anastomose but lack granules; cell body carried upright. May form floating stages with retracted or expanded filopodia. Cysts formed within the theca, the theca forms a loose envelope around the cyst wall. Cells may aggregate basolaterally in a ring, each retaining its nucleus and individual shape, or apparently partially fuse basally within one multinucleate lobed theca. Eukaryotic prey (yeasts, algae) caught by filopodia; dragged to thecal opening for ingestion. No bacterial ingestion observed. At least *F. terrestris* can form a tubular feeding siphon. Freshwater or terrestrial. Division longitudinal, binary. Sexual reproduction unknown.

In contrast to *Lecythium*: no granules in filopodia, cells form aggregates by fusion, often between the apical end and the nucleus an additional layer of granules which could not be observed in *L. hyalinum or L. jennyae*.

Etymology: Fisculla [Latin] = Belittlement of the noun fiscus (= money bag); referring to the bag-like shape of the test.

Type species: *F. terrestris*

Discussion

Morphological analysis

Lecythium hyalinum was first described by Hertwig and Lesser (1874) as a filose testate amoeba with a non-flexible, hyaline and almost round test. Penard (1902), Belar (1921) and de Saedeleer (1934) provided redescriptions of *L. hyalinum* which were in accordance with the original description of Hertwig and Lesser (1874), but provided further details. They described the length of the test with a range of 20-40 µm, which fits in our measurements (18-50 µm). Hertwig and Lesser (1874), Penard (1902) and Belar (1921) did not describe any folds in the theca, but de Saedeleer (1934) did and described them as restricted to the surroundings of the aperture, as they occurred often in our isolates. Since not all our individuals carried folds, these folds might have also not been observed by Hertwig and Lesser (1874).

We observed that the aperture showed a specific form, a neck-like structure reaching out of a notch, which was described by Hertwig and Lesser (1874), Penard (1902) and de Saedeleer (1934). Further and maybe most important, we could observe a protuberant, vacuolated cytoplasm in front of the aperture, from which the filopodia arise. Up to now, this vacuolated cytoplasm was never described for any other *Lecythium* species (Dumack et al. 2016b,c) and is therefore considered by us as the most important discrimination factor of *L. hyalinum* to other species. In accordance to our observations, Hertwig and Lesser (1874) also observed a well detectable nucleus with a visible nucleolus in a homogenous area of cytoplasm at the apical end of the cell. In this area neither granules nor vacuoles appear, which was clearly described by Penard (1902) and de Saedeleer (1934).

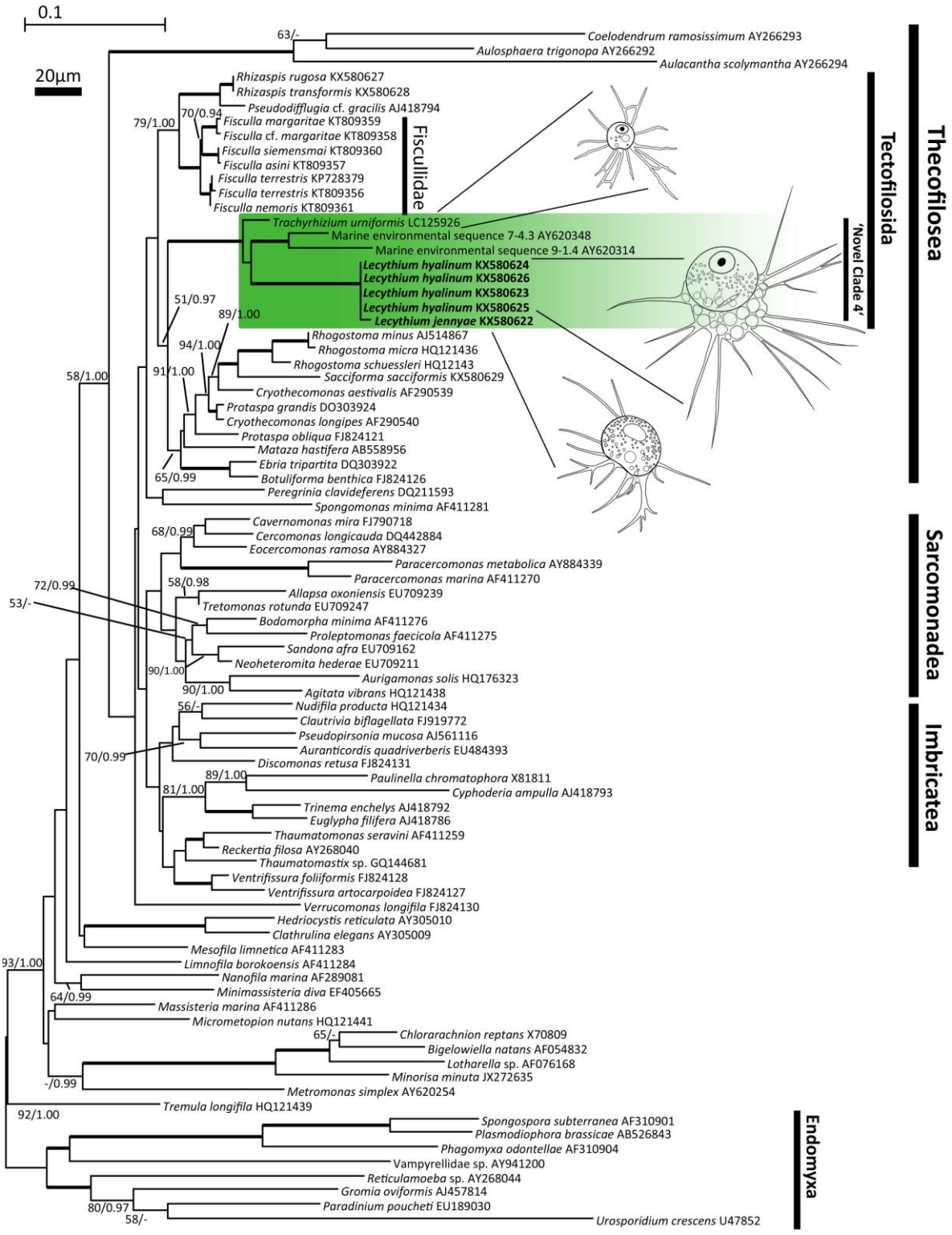

Figure 8: SSU rDNA phylogeny of Filosa with selected Endomyxa as outgroup with focus on the Thecofilosea. The Thecofilosea Shown is the maximum likelihood tree obtained by the PhyML GTR+I+G analyses including 84 sequences and using 1587 aligned sites. The support levels of the PhyML and the Bayesian analysis are shown on the respective branches (ML/BI) in support was over 50/0.95. Bold lines: Support above 95/0.95. Support under 50% or 0.95 are omitted. In this study sequenced amoebae are highlighted.

Unfortunately, Hertwig and Lesser (1874), did not give any descriptions of the nucleolus structure but Belar (1921) described it either as homogenous or as vacuolated, as we could observe in our isolates. In stable cultures, he observed large aggregates moving as a planar, outspread community, what we call a feeding community. Similar to our redescription, Belar (1921) observed swimming stages either as single cell or as an aggregated, roundish group of amoebae, when, as he states, 'products of metabolism accumulated in cultures'. These swimming groups aggregate basolaterally in a ring or sphere. As observed in our isolates, filopodia of the swimming stages were described by Belar (1921) as being straight, which rarely branch and never anastomose. We decided to use the word 'swimming' as the filopodia most often show slow but active movement in contrast to many floating stages that seem immotile, being inactively carried by the current.

Accordingly, our morphological analyses confirm that our isolates (KD1010-KD1013) are strains of *Lecythium hyalinum*, especially the highly vacuolated cytoplasm in front of the aperture, the presence of the nucleus at the apical end with a vacuolated nucleolus, and the zonation of the cells are considered as crucial morphological traits.

The isolate KD1014 shows differences in several characteristics to *L. hyalinum*. KD1014 never showed a zonation of the cell body as in *L. hyalinum*. It contained neither an apical zone with the nucleus embedded in homogenous cytoplasm, nor any zonation in three areas as described above. Granules and vacuoles were dispersed all over the cell body. We never observed of the mentioned vacuolated cytoplasm around the aperture. Also a nucleolus was never observed. The test often features numerous folds to an extent as it was never seen in *L. hyalinum*. We therefore conclude that strain KD1014 does not resemble *L. hyalinum* but must be a closely related species.

There are six other described *Lecythium* species of which molecular data is lacking and we therefore have to compare their morphology with strain KD1014. The species are: *L. granulatus* (Schulze, 1875), *L. mutabilis* (Bailey, 1853), *L. spinosum* (Penard, 1890), *L. curvus* (Leidy, 1879), *L. kryptosis* (Chardez, 1972) and *L. minutum* (de Saedeleer, 1934). The morphology of these species is quite different to our isolate (see also identification key provided by Dumack et al. 2016c). *L. minutum* is marine and by far too small to be the same species as our isolate. Due to the almost round test our isolate cannot be identified as *L. kryptosis*, *L. curvus*, *L. spinosum* or *L. mutabilis.* Some similarities are detectable between our isolate and *L. granulatus* (most prominently the occurrence of a 'large amount of granules', for a discussion of this morphological character see Dumack et al. 2016c), but the size difference and perhaps most importantly the absence of a clearly visible nucleolus in our isolate are an obvious difference. Finally, we conclude our isolate is a novel species of *Lecythium* that we call from now on *L. jennyae*.

***Lecythium* phylogeny and taxonomy**

Morphological analysis of the thecofilosean thecate amoebae is a difficult task. Dumack et al. (2016b,c) grouped novel species into the genus *Lecythium*, which we show here was incorrect. As discussed in Dumack et al. (2016c), Penard (1902) probably wrongly described *L. margaritae* as *L. granulatus*, this shows that morphological misidentification of even distantly related species occurred even to well trained protistologists. With molecular analyses this problem can be resolved. Surprisingly, *L. hyalinum* and *L. jennyae* cluster within the Novel Clade 4 which groups as a novel lineage with moderate support in the Thecofilosea (Bass and Cavalier-Smith, 2004), and not as expected among the recently described '*Lecythium'* species (Dumack et al. 2016b,c). The Novel Clade 4, was established by Howe et al. (2011) and until recently exclusively composed of environmental sequences. Cavalier-Smith and Chao (2003) established the order Tectofilosida to accommodate next to others the Chlamydophryidae. Since *Lecythium* currently is assigned to the Chlamydophryidae we suggest to include the Novel Clade 4 into the order Tectofilosida although SSU phylogenetic support in our analyses is only moderate.

Due to the fact that *L. hyalinum* is the type species of the genus, we have to provide new taxonomic descriptions for the former genus, which we renamed *Fisculla*. In addition, we had to establish a new family, the Fiscullidae. Accordingly, the other described *Lecythium* species (*L. kryptosis*, *L. curvus*, *L. spinosum*, *L. mutabilis*, *L. minutum*, *L. granulatus*) of which molecular data is still lacking, might also not group with the morphologically diverse genus *Lecythium*, but could instead group in the Fiscullidae or Rhizaspididae. Until molecular data are provided, their taxonomy cannot be clarified.

Recently Shiratori and Ishida (2016) described *Trachyrhizium urniformis*, a small marine thecate amoeba that groups within Novel Clade 4. They excluded the possibility, that their isolate resembled *L. minutum* (the only known eukaryvorous marine *Lecythium* species) on morphological basis, since it had granules in the filopodia and a test size of 12.0 µm on average (range: 7.5-17.6 µm). Small granules could also be observed in the filopodia of our *L. hyalinum* isolates despite this character had not been described before, possibly because these granules are very small, rare, and therefore hard to detect. De Saedeleer (1934) described *L. minutum* from a single observation of two cells which were 9.3-11 µm in size and as he wrote: 'we may not have been able to get a clear image of the whole filopodia network due to the fast movement of the cells'. Although Shiratori and Ishida (2016) stated a size difference of *T. urniformis* to *L. minutum*, these values lie within their measured variation and therefore fit in the size range of *T. urniformis.* We therefore suggest that *T. urniformis* might resemble *L. minutum* or a close relative of it.

The 'dancing particles' of *L. hyalinum*

The 'dancing particles' on the cell surface of the vacuolated cytoplasm of *L. hyalinum* might resemble ectocytobiotic bacteria. Protists with ectocytobiotic 'symbionts' are already described for few protists, predominantly studied in the genus *Nuclearia* (Dirren et al. 2014; Dirren and Posch 2016). Further studies will be conducted investigating this possible eukaryotic-prokaryotic 'symbiosis'.

Material and methods

Sampling and identification

Freshwater samples were collected from lakes, ponds, creeks and rivers in Germany and the Netherlands. Plastic bottles were scratched over the bottom to detach detritus, sediment and the surface grazing amoebae. Two ml of the sampled 100- 200 ml were given in each well of a 24-well plate (Sarstedt, Germany). The samples were screened for *L. hyalinum*-like cells with an inverse microscope (Nikon Eclipse TS100; Ph1; 40x, 100x, 200x and 400x magnification).

Isolation and cultivation

Cells were picked with a glass micro pipette and transferred into a new well, containing WC-Medium or Waris-H. Additionally, diatoms (*Nitzschia communis*, CCAC 1762 B; *Nitzschia amphibia*, CCAC 5733 B; *Pinnularia* sp. CCAC 0222B) or green algae (*Characium* sp.) were provided as food source (Tab 1). Cells were sub-cultured approximately every three to ten days.

Microscopial observations

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

Sequencing of cultured amoebae

For sequencing, clonal individuals were starved for up to three days in fresh culture medium and with approximately 1 µl of medium a single individual was transferred into PCR-tubes, containing 15 µl ddH_2O. The tubes were frozen at -20 °C for storage. Then 35 µl PCR mixture was added. The mixture included 5 µl 0.1 µM forward and 5 µl 0.1 µM reverse primer, 5 µl 200 µM dNTPs, 5 µl Thermo Scientific Dream Taq Green Buffer, 0.3 µl Dream Taq polymerase (Thermo Fisher Scientific, Dreieich, Germany) and 14.7 µl water. The SSU sequences were obtained in two successive steps. First, the whole SSU was amplified with the general eukaryotic primers, EukA and EukB (Medlin et al. 1988). In the second step, semi-nested re-amplifications were performed by using primers specifically

designed for cercozoans, with the same settings as above and the primer pairs EukA + S963R_Cerco (Fiore-Donno, pers. comm.) targeting the 5' part of the SSU and S616F_Cercomix (Fiore-Donno, pers. comm) + EukB for the 3' part of the gene. One µl of the first PCR product was used as template.

The PCR products were purified by adding 0.15 µl of Exonuclease, 0.9 µl FastAP and 1.95 µl water to 8 µl of the second PCR product. Then heated for 30 min at 37 °C, and subsequently for 20 min at 85 °C.

The Big dye Terminator Cycle sequencing Kit (Thermo Fisher Scientific, Dreieich, Germany) and an ABI PRISM automatic sequencer were used for the sequencing.

Phylogenetic analyses

The partial sequences were manually checked for sequencing errors before they were assembled into one sequence contig. To create a dataset for phylogenetic analyses sequences were blasted (blastn 2.3.0) against the NCBI GenBank database (last date of accession: 14.06.2016). Representative sequences belonging to the Filosa and to the Endomyxa (outgroup) were downloaded and manually aligned in SeaView (V4.5.3, Gouy et al. 2010). 1587 sites were used for the alignment, which were to 45.19 % without polymorphism. The best fitting model GTR+I+G was found by using the program jmodeltest (V.2.1.5, Darriba et al. 2012), testing 88 different models (settings: Substitution schemes 11; add Base frequencies +I+G rate variation nCat=4 resulting in 88 models, ML optimized NNI as base tree). With this model phylogenetic trees were calculated in PhyML 3.1 (Guindon and Gascuel 2003) and MrBayes (settings: mcmc ngen = 10 M, sample freq = 100, print freq = 100, diagn freq = 500; Altekar et al. 2004; Ronquist and Huelsenbeck 2003). The sequences were submitted in the NCBI database under the accesstion numbers: KX580626, KX580624, KX580623, KX580625 and KX580622.

Preparation of the hapantotype

1.5 ml of culture material was mixed 1:4 with Schaudinn's fluid and was heated for 5 minutes at 60°C. A serial dehydration in an ascending series of ethanol solutions with increasing concentration was conducted. Finally, the preserved cells were mixed 1:2 with glycerin, transferred to a glass slide and a cover slide was glued on top.

Acknowledgements

The study was supported by the DFG research group FOR 918. We thank the Culture Collection of Algae at the University of Cologne (CCAC) for their service and the provided algal strains.

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Part 3 - Eukaryvorous protists, their capabilities and dispersal.

Chapter 7: What does it take to eat a fungus? A case study with the eukaryvorous amoeba *Fisculla terrestris*.

What does it take to eat a fungus? A case study with the fungivorous amoeba *Fisculla terrestris*.

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Running title: Fungivory in the Fiscullidae

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Keywords

Soil food web; mycophagy, protist; yeast; chemical sensing; *Fusarium*

Abstract

Protists are commonly suggested to regulate bacteria abundance in soils, whereas fungi are considered to be consumed by arthropods and fungivorous nematodes. Nevertheless, some protist taxa have been found to consume fungi and despise bacteria. Since these protists are difficult to culture little is known about their (a) ecological impact, e.g. grazing selectivity, growth rates and preferred prey organisms and (b) adaptation to fungal food sources, e.g. enzyme production and chemical sensing abilities. We demonstrated that the eukaryvorous amoeba *Fisculla terrestris* senses fungal presence and in fungal presence responds with an increased (5-fold) grazing activity. To test for grazing selectivity, a food choice experiment including three different prey species: 1) a yeast species (*Saccharomyces cerevisiae*), 2) a green alga (*Chlorella vulgaris*) and 3) a diatom (*Navicula* sp.) was conducted. Although *F. terrestris* consumed all offered food sources, the predator selected for the yeast and showed the highest growth rate (about 125 ± 40 times increase of cell density over three days) and shortest generation time (5.9± 1.5 h) when feeding on the fungal food source. In addition, *F. terrestris* produced enzymes specialized to degrade C- and N-sources, i.e. chitinases. We conclude that *F. terrestris* is a eukaryvorous protist with strong preference for fungal cells and should therefore be referred to as fungivorous.

Importance

Fungivorous protists have been researched in very few often very basic studies. Especially in soil ecology, protists are generally referred to as bacterivores. Nevertheless, as we show, protists occupy various ecological niches such as the niche of fungivores. Some protist lineages are specialized and harbour potential for enzyme analyses, experiments on chemical sensing and bio regulators for potentially harmful fungi. To our knowledge this is the first study giving growth curves, generation times, a direct relation of consumed individuals to predator growth and further first evidence for chemical sensing in fungivorous protists. Thus enabling more detailed soil system modelling.

Introduction

Free-living protists are usually considered to feed on bacteria with low impact on fungal abundance (1). Since protists have traditionally been cultivated on bacteria-rich media, those that did not grow under these conditions are still poorly studied and probably many of non bacterivorous protists are not even known to science yet. Therefore, only very few and often old studies about fungivorous protists are available. Those were conducted by taxonomists and most lack ecological data, like their grazing selectivity, the resulting growth rates and the mechanisms by which they locate and digest their fungal prey (2-5).

Fisculla terrestris, a small amoeba that was recently isolated from agricultural soils in Eastern Germany could not be cultivated on bacteria, but under lab conditions has been shown to feed not only on yeasts (e.g. *Cryptococcus laurentii*), spores of plant pathogen fungi (e.g. *Fusarium culmorum*) but also on unicellular algae (6, 7, Dumack, Mausbach, Hegmann and Bonkowski, submitted for publication). According to observations and the assumption that fungal food sources are much more abundant in terrestrial habitats than algae (8, 9) Dumack et al. (6) hypothesised that *F. terrestris* feeds primarily on fungi. Selectivity for fungal nutrition should lead to a series of adaptations, e.g. the ability to (a) sense fungal cells, (b) identify and select for them as a food source and (c) degrade or penetrate chitin since this is the main cell-wall component of fungi.

Chemical sensing in protists has been reported already more than hundred years ago (10). Since then numerous studies identified diverse attractants (e.g. volatile organic compounds (VOCs) and amino acids) as well as chemosensory transduction in protists in a range of distantly related taxa (11-13). These studies, however, exclusively focused on bacterivorous protists. Chitinase-coding genes have been already found in the genome of protists (Amoebozoa: *Acanthamoeba,* Cercozoa: *Plasmodiophora*; 14, 15) and chitinase activity has been confirmed by direct observation of lysed chitin walls or lab experiments for several protist taxa (Amoebozoa: *Hartmannella*, Cercozoa:

Leptophryidae spp.; 5, 16, 17). However, we are not aware of reports on chitinase production in *Fisculla*, its order Tectofilosida or the whole class Thecofilosea.

Accordingly, we performed (a) chemical sensing experiments with a fungal food source, (b) food choice experiments in which we offered one representative of the three dominant possible prey groups, a yeast (*Saccharomyces cerevisiae*), a green alga (*Chlorella vulgaris*) and a diatom (*Navicula sp.*) and (c) enzyme analyses of intact and homogenized *F. terrestris* cells.

Materials & Methods

Preparation of axenic cultures

Cultures of the algae *C. vulgaris* (strain CCAC 3272) and *Navicula* sp. (strain CCAC 1772) were cultivated axenically in Waris-H+Si medium (18) at room temperature, with a light regime of 14 h light to 10 h dark with a light-intensity of about 5 mol photons/m^2/sec. The yeast *S. cerevisiae* was cultivated axenically on potato agar plates after the manufacturer's instructions (*Fluka Analytical*) and incubated for 2-3 days at room temperature. *S. cerevisiae* cells were subsequently transferred to sterile Waris-H+Si medium. With this, a monoxenic (bacteria-free) co-culture of *F. terrestris* (strain CCAP 1943/1) was established by cell sorting as described by Hess & Melkonian (17). *F. terrestris* was also cultivated in Waris-H+Si to control for medium effects.

Set up of the chemical sensing experiment

Erlenmeyer flasks (n=3) were prepared with (a) axenic *S. cerevisiae* in Waris-H and (b) sterile Waris-H as a control. Both treatments were incubated for 5 days at room temperature and then 15 ml of the suspension were subsampled and centrifuged for 1 h by 4,500 U/min (Hettich, Rotina 420R). One ml of axenic culture of *F. terrestris* were transferred into wells of 24-well plates (n=6) and incubated for 2 hours then the base level of activity was determined by counting inactive cells and active grazing cells, giving the activity in percentage. To check a response of *F. terrestris* in activity, out of each Erlenmeyer flask 100 µl of the supernatant was added to the culture, incubated for 30 min and again the grazing activity in percent was calculated.

Set up of the food choice experiment

Cell numbers of each of the three food source cultures (*Saccharomyces cerevisiae*; *Chlorella vulgaris* and *Navicula* sp.) were counted in a Neubauer Counting Chamber (Hecht-Assistant, Sondheim Deutschland) Based on these values, $10*10^4$ algae and yeast cells were pipetted together with 100 individuals of *F. terrestris* into separate wells of a 96-well-plate (n=6). Control treatments contained each organism separately as well as the predator with each prey species individually. The final volume was adjusted to 200 µl Waris-H+Si. Changes in the numbers of cells were monitored by direct counting with a Neubauer chamber once a day over the three days run time of the experiment.

Chitinase assay

A starving culture of *F. terrestris* (4 weeks of culturing, 14 days of starving) was used for the analyses. To control for possible remaining yeast cells, 500 ml of the culture were filtered through a 5 µm pore glass fibre filter. The protist cells were resuspended in Waris-H, examined for possible remaining yeast cells by light microscopy and incubated for additional 4-5 days before analyses. The determination of enzyme kinetics (chitinase) included three treatments, (1) non-treated medium (2) starving, but intact cells of protists and (3) starving and homogenized cells of protists. The homogenized cells were destroyed by pulsed sonication with 50 J s^{-1} for 120 s.

We used 4-methylumbelliferyl N-acetyl-β-D-glucosaminide to determine the enzyme activity of N-acetyl-β-glucosaminidase (chitinase; EC 3.2.1.52) (18). Aliquots (50 µl) of liquid protist culture (3 analytical replicates) were withdrawn and dispensed into 96-well microplates (Brand pureGrade, black). The 4-methylumbelliferyl N-acetyl-β-D-glucosaminide was dissolved in 300 µl dimethyl sulfoxide (DMSO) and then diluted with 80 µl of 0.1 M, pH 6.1 MES to obtain 1 mM of working solution (20, 21). A concentration series of 20, 40, 60, 80, 100, 200, 400 µmol was then prepared and 100 µl of the substrate solution was added to the wells.

Microplates were kept at 21°C, agitated and measured fluorometrically (excitation 360 nm; emission 450 nm) after 1 h, 2 h, and 3 h incubation with an automated fluorometric plate-reader (Wallac 1420, Perkin Elmer, Turku, Finland).

Statistical analyses

Food choice

Changes in protist and prey abundances were analyzed by multivariate analysis of variance (MANOVA) according to Hurlbert (22). The data were tested for sphericity before the MANOVAs were calculated and tested for differences in R (V.3.1.0).

Day 0 was excluded to correct for a possible adaptation phase of the predator, when the generation times of *F. terrestris* were determined. With *S. cerevisiae* as food source, *F. terrestris* reached on the third day food limitation, therefore day 3 was also excluded from the calculation, restricting the values to the exponential growth phase of *F. terrestris*.

Chemical sensing

The data of the chemical sensing experiment were tested for normality (Shapiro Wilk test) and variance homogeneity (Levene's test). Differences between the two treatments and the control were calculated in R with the settings described above since requirements for an ANOVA were given.

Chitinase assay

The substrate-dependent rate of reaction mediated by hydrolytic enzymes, followed Michaelis-Menten kinetics (20, 21, 23). The parameters of the equation were fitted by minimizing the least-square sum using GraphPad Version 6 software (Prism, USA). Parameter optimization was restricted to the applied model equation as indicated by maximum values of statistic criteria (r^2) (19, 20).

Results

Chemical sensing experiment

Starving cultures of *F. terrestris* contained an average of 5.2 $\pm$ 3.5% actively grazing cells (Fig. 1). The activity did not change significantly after incubation in fresh medium (control), but increased about 5-fold, to 26 $\pm$ 9.6%, when supernatant of yeast cells was added ($F_{[2,9]}$=18.38; p<0.001).

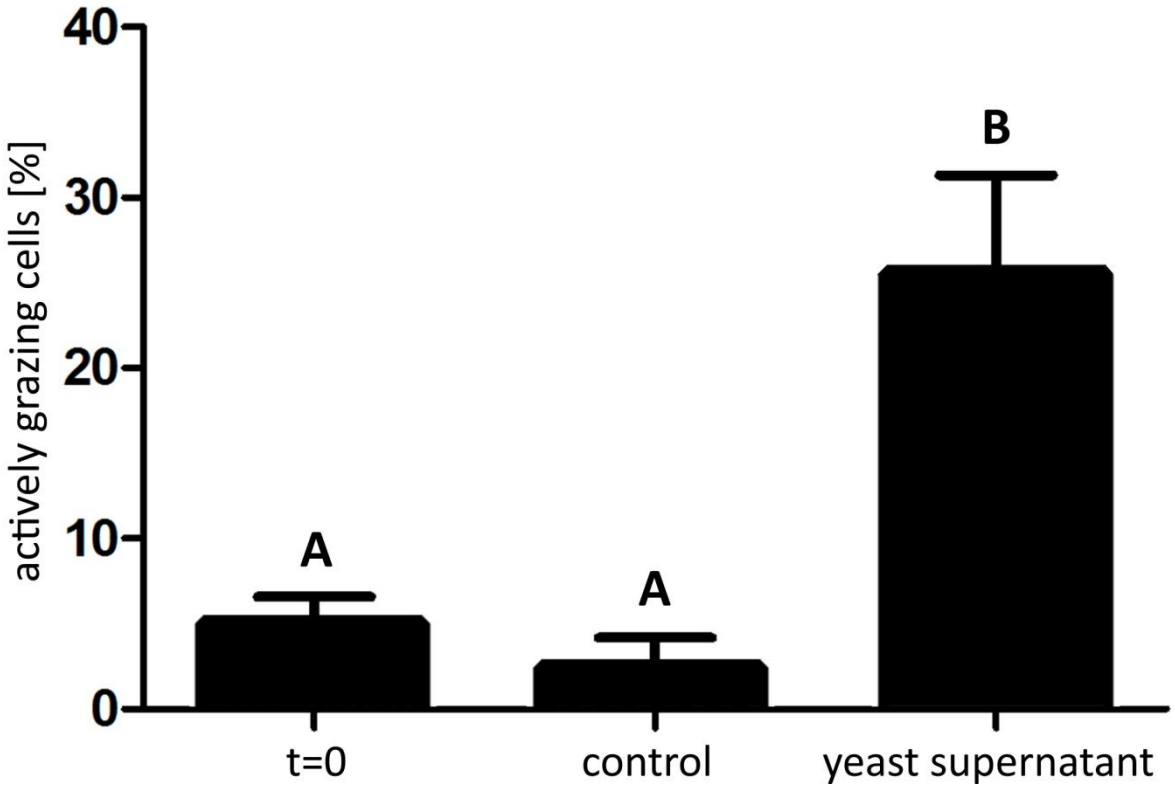

Figure 1: Results of the chemical sensing experiment. Numbers of actively grazing *F. terrestris* cells are shown on the y-axis, the x-axis shows the grazing activity of a starving culture t=0, the activity after addition of culture medium (control) and the activity after addition of culture medium from yeast cells.

Food choice experiments

All three food sources in control treatments showed a stable abundance during the run time of the experiment ($F_{[3,20]}$=1.151; p=0.34; not shown). Only the abundance of *F. terrestris* increased marginally about 2.5-fold to initial density, likely due to nutrient and energy reserve ($F_{[3,20]}$=21.64; p<0.001).

With only one food source offered, *F. terrestris* consumed each of it (*S. cerevisiae*: $F_{[3,20]}$=43.46; p<0.001; *C. vulgaris*: $F_{[3,20]}$=5.58; p<0.01; *Navicula* sp.: $F_{[3,20]}$=4.48; p<0.05; Fig. 2).

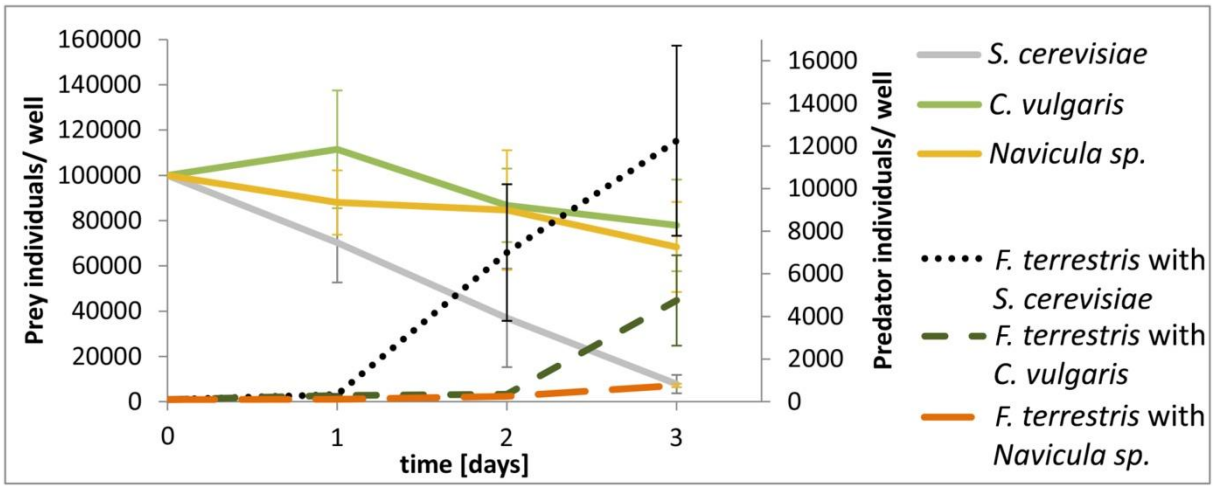

Figure 2: Results of the monoxenic single food-choice experiments; cell abundance of the food sources are shown on the left y axis (grey line: *S. cerevisiae*; green line *C. vulgaris* and yellow line *Navicula* sp.), cell abundance of the predator *F. terrestris* is shown on the right y axis (dotted line: *F. terrestris* + *S. cerevisiae*; short-dashed dark green line *F. terrestris* + *C. vulgaris* and long-dashed orange line *F. terrestris* + *Navicula* sp.).

Over three days the abundance of *S. cerevisiae* declined on average to 7.5± 4% of initial density. The abundance of the algae *C. vulgaris* and *Navicula* sp. declined to 78± 20% and 65± 14% of initial density, respectively. The abundance of *F. terrestris* increased with *S. cerevisiae, C. vulgaris* and *Navicula* sp. as food source about 125 ± 40, 40 ± 20 and 7.5 ± 1 fold, respectively (*F. terrestris* + *S. cerevisiae*: $F_{[3,20]}$=30.41; p<0.001; *F. terrestris* + *C. vulgaris*: $F_{[3,20]}$=25.96; p<0.001; *F. terrestris* + *Navicula* sp.: $F_{[3,20]}$=310.19; p<0.001) compared to initial density.

The mean generation times of *F. terrestris* were about 5.9± 1.5 h, 12.8 ± 3h and 16.9± 1.7 h with *S. cerevisiae, C. vulgaris and Navicula* sp. as food source, respectively (Fig. 2). For each division *F. terrestris* was feeding an average 9.05± 4.7 yeast cells, 5.71± 6.25 green alga cells and 41.65± 36.27 diatom cells, indicating that the average food quality of *S. cerevisiae*, combined with the low handling time lead to the highest growth efficiency.

When *S. cerevisiae* and *C. vulgaris* were offered together (Fig. 3), the abundance of *F. terrestris* grew about 82.5 ± 29 fold in three days. *S. cerevisiae* declined to 6.8± 4% of initial density while *C. vulgaris* stayed constant in density (*S. cerevisiae*: $F_{[3,20]}$=49.28; p<0.001; *C. vulgaris*: $F_{[3,20]}$=1.39; p=0.276).

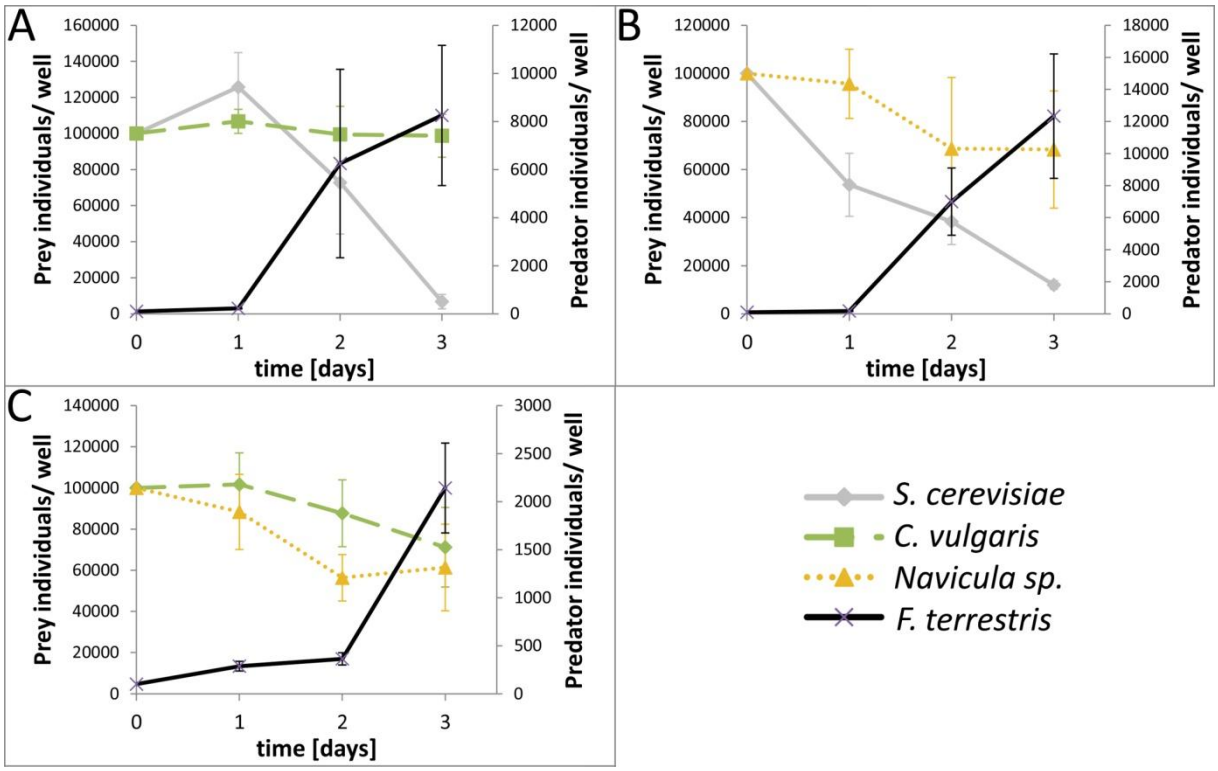

Figure 3: Results of the double food-choice experiments; cell abundance of the food sources are shown on the left y axis (grey line: *S. cerevisiae*; green dashed line *C. vulgaris* and yellow dotted line *Navicula sp.*), cell abundance of the predator *F. terrestris* is shown on the right y axis (black line). (A) *C. vulgaris* + *S. cerevisiae* as food sources, (B) *S. cerevisiae* + *Navicula* sp. as food sources and (C) *C. vulgaris* and *Navicula* sp. as food sources.

When a mixture of *S. cerevisiae* and *Navicula* sp. was offered, both food sources were consumed (*S. cerevisiae*: $F_{[3,20]}$=123.3; p<0.001; *Navicula* sp.: $F_{[3,20]}$=3.85; p<0.05; Fig. 3), and the abundance of *F. terrestris* grew 120 ± 39 fold. When a mixture of *C. vulgaris* and *Navicula* sp. was offered, also both food sources were consumed (*C. vulgaris*: $F_{[3,20]}$=5.47; p<0.01; *Navicula* sp.: $F_{[3,20]}$=11.49; p<0.001; Fig. 3), but the abundance of *F. terrestris* increased up to 20 ± 4.7 fold.

When all three different food sources were offered (Fig. 4), *F. terrestris* fed on all of them (*S. cerevisiae*: $F_{[3,20]}$=102.01; p<0.001; *C. vulgaris*: $F_{[3,20]}$=3.77; p<0.05; *Navicula* sp.: $F_{[3,20]}$=4.4; p<0.05) and the abundance of *F. terrestris* had increased after three days about 133 ± 24 fold.

After three days over 90% of the yeast cells were consumed with only 9 ± 4% *S. cerevisiae* cells remaining, whereas the abundance of the algae *C. vulgaris* and *Navicula* sp. slightly decreased to 90 ± 9% and 69 ± 20% of initial cell density, respectively.

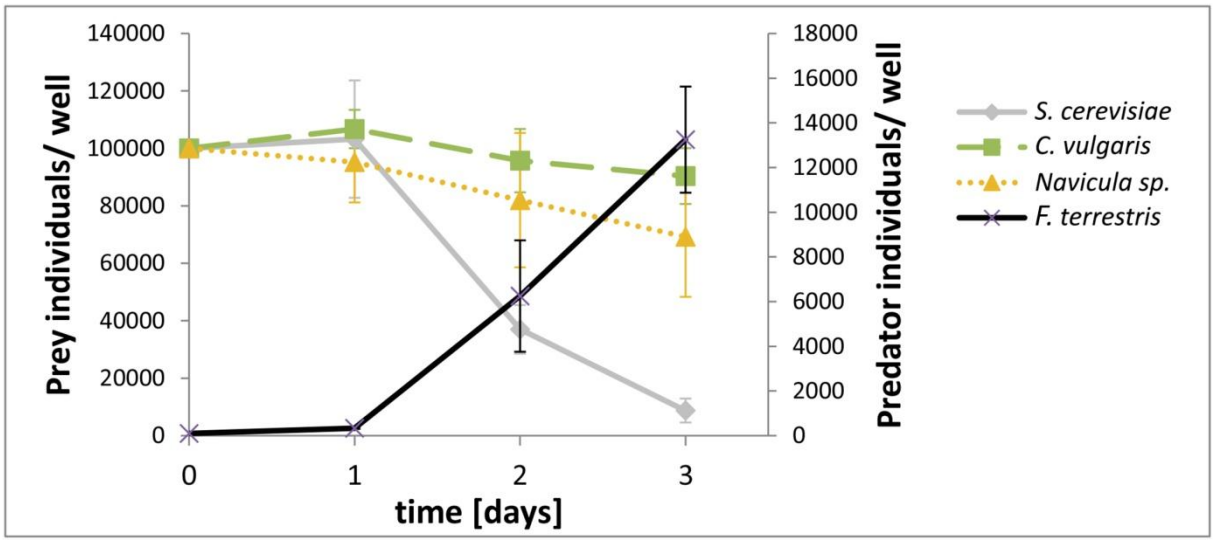

Figure 4: Results of the triple food-choice experiment; cell abundance of the food sources are shown on the left y axis (grey line: *S. cerevisiae*; green dashed line *C. vulgaris* and yellow dotted line *Navicula sp.*), cell abundance of the predator *F. terrestris* is shown on the right y axis (black line).

Chitinase analyses

The potential enzyme activity (V_{max}) increased by 32% in homogenised samples ($F_{[1,32]}$=15.95; p<0.001; Fig. 5), indicating the production of intra-cellular chitinases. Furthermore, the K_m was 40% higher when cells were destroyed by sonication compared to intact cells, reflecting lower enzyme affinity to the substrate (Fig. 6). The control medium did not show chitinase activity (not shown).

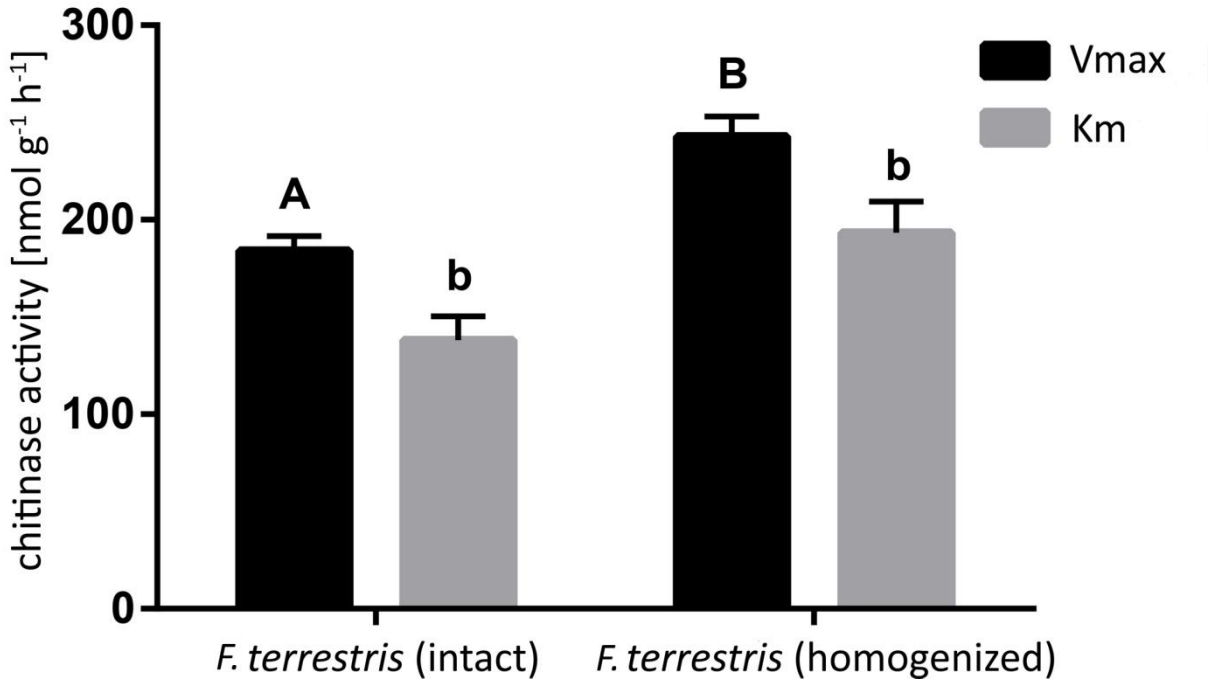

Figure 5: Results of the chitinase activity measurements, Enzyme kinetic properties: Vmax (black) and Km (grey) are given for intact *F. terrestris* cells (left) and homogenized ones (right), the difference in Vmax is considered as intracellular chitinase.

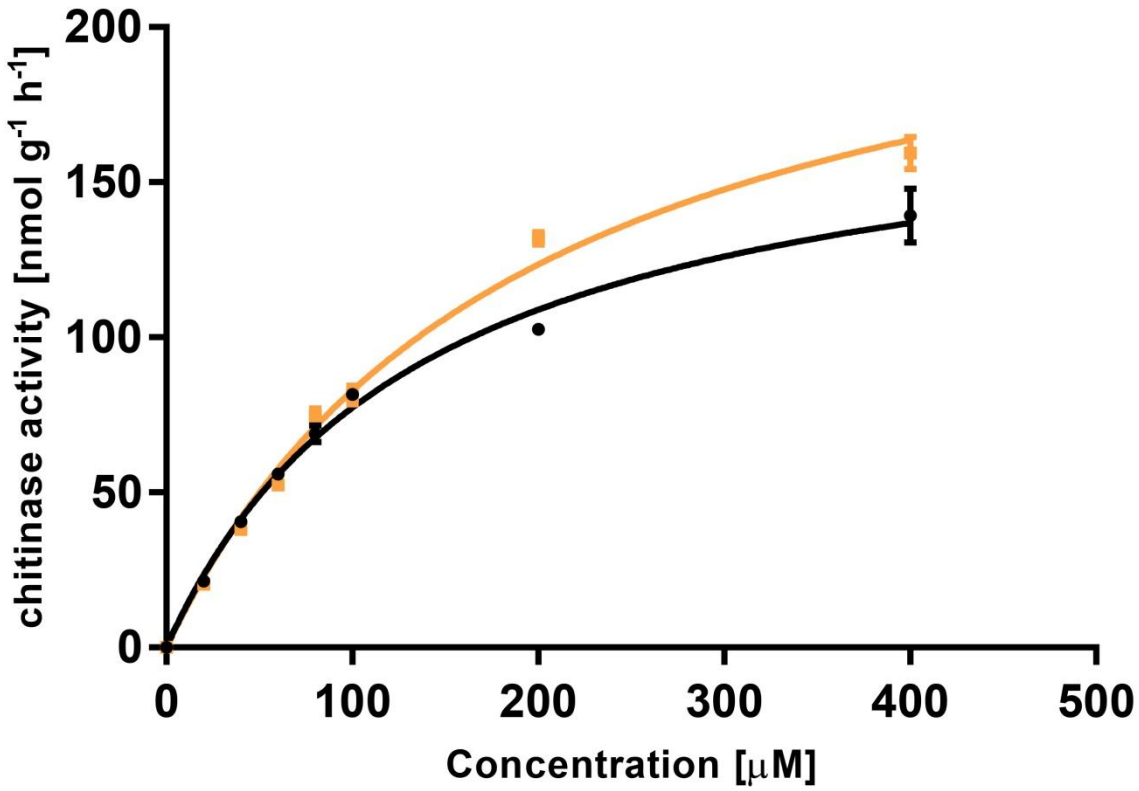

Figure 6: Chitinase activity in dependence of substrate concentration. The orange line shows the homogenized protists, the black line intact *F. terrestris* cells.

Discussion

Food choice

F. terrestris preferred yeast cells although being able to grow on all offered eukaryotes. This corroborates our hypothesis that *F. terrestris* as a soil dwelling organism should prefer abundant terrestrial food sources such as fungal cells.

Despite similar cell abundance, size and motility of all offered prey organisms, the growth rates of *F. terrestris* in monoxenic cultures differed drastically. The yeast *S. cerevisiae* caused the highest reproduction of *F. terrestris* compared to both offered algae, indicating that *F. terrestris* is well adapted to digest fungal cells.

Algae are known as the main food source of aquatic *Fisculla* isolates (6, 7). Both algae, *C. vulgaris* and *Navicula* sp. were consumed under monoxenic conditions which implies that this terrestrial *Fisculla* species has not entirely lost the ability to feed on algae. We consider *F. terrestris* as an omnivorous eukaryote predator with strong preference for fungal cells. Therefore *F. terrestris* should be referred to as fungivorous. Experiments with the terrestrial members of the Leptophryidae have shown that these fungivorous protists can also consume algae under experimental conditions (2, 5).

Fungivory in F. *terrestris*

How does *F. terrestris* select for food cells?

Based on microscopical observation, we suggest that there are at least two steps in prey recognition of *F. terrestris* (see Figure 7):

Starving *F. terrestris* cells form immotile resting stages, similar to cysts but without an additional cyst wall. After addition of every tested fungal material (including spores of *Fusarium* spp. and *Rhizopus* sp. or yeast like *Cryptococcus* spp. and *Saccharomyces cerevisiae*, for more details see 6) *F. terrestris* started to show active grazing behaviour within minutes. Also some algae triggered this behaviour (e.g. *Chlorella* spp. and *Stichococcus bacillaris*) others (e.g. *Kirchneriella* sp. and *Chromulina nebulosa*) did not. We consider this as the first step to recognize suitable prey. No direct contact with the prey is needed to cause the induction of activity; we suggest a sensing of water soluble signals by the predator as the chemical sensing experiment has shown.

F. terrestris, after catching potential prey organisms with its filopodia, ‘inspects’ them by contact of several seconds to minutes before either ingestion or a release of the potential prey organism takes

place (6). This second step in the process of prey recognition might include a direct contact or short range sensing of cell wall compounds.

Moreover, the results exhibited a trade-off between the consumption of optimal and sub-optimal food sources. Additional, Supplementary Videos 3+4 (6) showed that less suitable food sources will be ingested if too many unsuccessful scanning events have taken place. This explains the small proportion of less suitable food sources (e.g. *C. vulgaris and Navicula* sp.), which is grazed together with high amounts of the preferred food sources (e.g. *S. cerevisiae* and *Cryptococcus laurentii*).

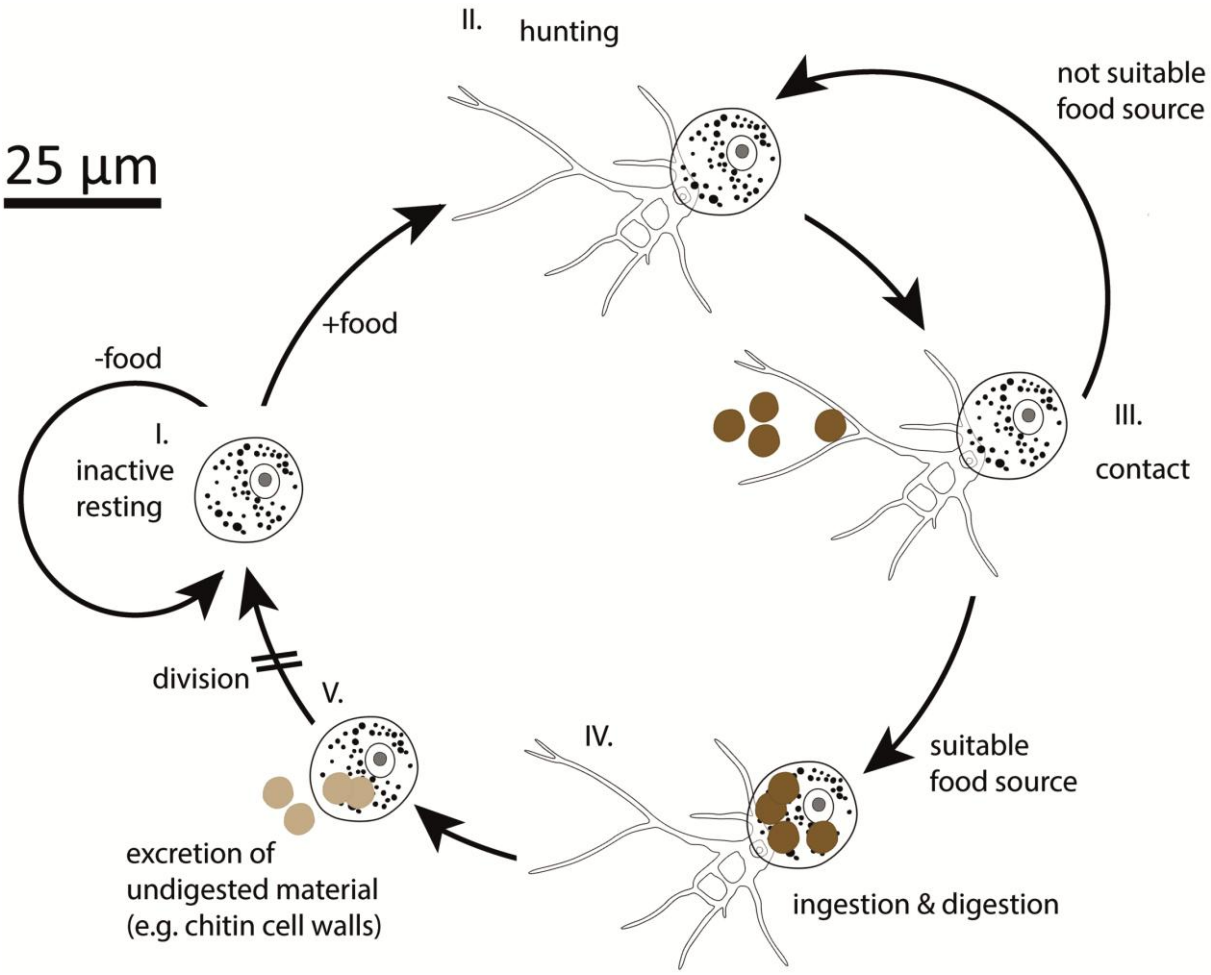

Figure 7: A simplified, hypothesised hunting cycle of *F. terrestris*. Inactive resting stages start grazing when suitable food is added. The chemical signals serve as long-distance cues that induce this behaviour.

How does *F. terrestris* digest its food?

Fungivorous protists like *F. terrestris* have to be able to mechanically penetrate (like the Grossglockneriidae, 3, 4) or enzymatically degrade (like the Vampyrellida, 24) the protective fungal cell wall (1). Since no hints for a mechanical penetration of fungal cells were observed, an enzymatic degradation of the fungal cell wall was likely. To our knowledge, this is the first report of catalytic properties of chitinases in Filosea. It is likely that many more Cercozoa are able to produce chitinases, for example omnivorous cercomonads that occasionally may feed on yeast cells (1).

Conclusion

Our results confirmed that *F. terrestris* is a highly selective grazer and selects for fungal cells. *F. terrestris* produced enzymes, such as chitinases, which are able to performed the degrade for instance fungal cell walls to aquire C- and N- sources.

F. terrestris although being able to multiply to a much lower extend on algae and, therefore must be considered as an omnivorous predator of eukaryotic cells, with strong preference for fungi, despite other described close relatives are known to feed mainly on algae.

Therefore, *F. terrestris* as one of very few known fungivorous soil protists might be an ideal organism for chemosensing as well as chemotaxis experiments in fungivorous soil protists.

With its easily maintained bacteria-free culture, the short generation time (~ 8 h) and the trait to stay inactive until food is sensed it provides a convenient opportunity for bioassay experiments that might simply be performed by addition of solved compounds in medium, a subsequent filming of the culture and counting the percentage of activated individuals per area.

Acknowledgements

We thank the Culture Collection of Algae at the University of Cologne (CCAC) for providing the *Chlorella vulgaris* strain CCAC 3272 and *Navicula sp.* strain CCAC 1772 and for their very professional support.

Funding information

This study was supported by the German Research Foundation (DFG) within the Research Unit “Carbon Flow in Belowground Food Webs assessed by Isotope Tracers”.

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Chapter 8: The soil food web revisited: Diverse and widespread mycophagous soil protists.

Soil Biology & Biochemistry 94 (2016) 10–18

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

The soil food web revisited: Diverse and widespread mycophagous soil protists

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ARTICLE INFO

Article history:
Received 3 October 2015
Received in revised form
9 November 2015
Accepted 11 November 2015
Available online 27 November 2015

Keywords:
Soil protists
Soil food web
Trophic interactions
Fungi
Soil metatranscriptomes
Microcosm studies

ABSTRACT

Soil protists are commonly suggested being solely bacterivorous, serving together with bacterivorous nematodes as the main controllers of the bacterial energy channel in soil food webs. In contrast, the fungal energy channel is assumed to be controlled by arthropods and mycophagous nematodes. This perspective accepted by most soil biologists is, however, challenged by functional studies conducted by taxonomists that revealed a range of mycophagous protists. In order to increase the knowledge on the functional importance of mycophagous protists we isolated and initiated cultures of protist taxa and tested eight for facultative feeding on diverse fungi in microcosm experiments. Two different flagellate species of the genus *Cercomonas*, the testate amoeba *Cryptodifflugia operculata* and four genera of naked amoebae (*Acanthamoeba* sp., *Leptomyxa* sp., two *Mayorella* spp. and *Thecamoeba* spp.) fed and grew on yeasts with four taxa (*Cercomonas* sp., *Leptomyxa* sp., *Mayorella* sp., and *Thecamoeba* sp.) also thriving on spores of the plant pathogenic hyphal-forming fungus *Fusarium culmorum*.

To identify the potential importance of mycophagous protists in the environment we applied a data-mining approach targeting small subunit (SSU) rRNA data obtained in metatranscriptomes of five fundamentally different terrestrial samples. We focused our analyses on the distribution and relative abundances of two well-studied mycophagous protist groups, vampyrellid amoebae and grossglockneriid ciliates. Both groups were detected in all of the highly contrasting terrestrial samples, comprising up to 3% of all protist SSU rRNA transcripts. SSU transcripts of these two groups, in contrast to all remaining protist SSU transcripts, showed strong correlations with the relative abundance of fungal sequences indicating close direct trophic interactions.

Taken together, this study provides evidence that mycophagy among soil protists is common and might be of substantial but hitherto overlooked ecological importance in terrestrial ecosystems. Future studies should aim at evaluating taxon-specific (facultative) mycophagy, decipher changes caused in the fungal community and quantitatively evaluate the functional importance of this trophic position in soil ecosystems.

1. Introduction

Soil biologists generally discriminate the nutrient flows in soil food webs into a bacterial and a fungal-based energy channel (e.g. Moore and Hunt, 1988; Holtkamp et al., 2011). Heterotrophic protists are considered as major consumers of bacterial biomass supplementing higher trophic levels with nutrients bound in these microbes; in contrast, protists are supposed to be of marginal importance in the fungal energy channel where microarthropods and mycophagous nematodes are suggested to be the predominant consumers (Hunt et al., 1987; de Ruiter et al., 1995; Bonkowski, 2004).

A major reason why soil biologists largely treat protists as bacterivores derived from traditional extraction and cultivation methods that select for bacterivorous protists (Page, 1988; Berthold and Palzenberger, 1995; Ekelund, 1998). Protist taxonomists have, however, long realized that diverse facultative and obligate mycophagous protist taxa are common in soils (Old and Darbyshire, 1978; Petz et al., 1985; Ekelund, 1998). For example, all described

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http://dx.doi.org/10.1016/j.soilbio.2015.11.010
0038-0717/

ciliates of the family Grossglockneriidae are obligatory mycophagous (Foissner and Didier, 1983; Petz et al., 1985, 1986). Facultative mycophagous protists (omnivores) that feed on a range of soil eukaryotes are vampyrellid amoebae (Old and Darbyshire, 1978; Hess et al., 2012), *Thecamoeba* spp. (Bamforth, 2004), few testate amoebae (Coûteaux and Darbyshire, 1998; Mitchell et al., 2008; Wilkinson, 2008; Wilkinson and Mitchell, 2010) and eumycetozoans (Stephenson and Feest, 2012). All of the aforementioned taxa are usually large (often >100 μm), but mycophagy has even been reported in small flagellates (Hekman et al., 1992; Flavin et al., 2000). Still, detailed knowledge on diversity and functional importance of mycophagous protists in terrestrial ecosystems remains limited, despite the fact that mycophagous protists can reach biomasses similar to those of bacterivorous protists (Ekelund, 1998).

Recent methodological advances using molecular techniques provided means of investigating the largely unknown diversity of soil protists. These sequence based studies have revealed that cultivable protists only represent a small fraction of the total protist community in soils (Foissner, 1999b; Bates et al., 2013; Kamono et al., 2013; Geisen et al., 2015b). The ecological function of uncultivated protists remains, however, largely unknown, as it is usually impossible to reliably link molecular phylogenetic information with realized ecological functioning. Molecular sequence-information needs to be supplemented with functional information on ecological traits of the respective taxon, which relies mainly on *in-vitro* studies on cultivated protists. Experiments on cultivated species revealed not only a variety of mycophagous protists, but also that even closely related protists differentially feed on bacterial prey (e.g. Böhme et al., 2009; Glücksman et al., 2010; Saleem et al., 2012). Mycophagous protists might also feed differentially on fungi, which could lead to shifts in fungal communities in soils. Some evidence for this hypothesis came from former studies showing that yeasts are a preferred food source for protists, while hyphae forming fungi were a less suitable prey (Heal, 1963; Bunting et al., 1979; Allen and Dawidowicz, 1990).

In order to increase the knowledge on the diversity and importance of mycophagy among soil protists we (1) cultivated soil protists and tested if facultative mycophagy is common for "bacterivorous" protists. Further (2) we used a data mining approach on SSU rRNA sequences obtained in metatranscriptomes of five highly diverse soils (Geisen et al., 2015b) to investigate the presence and relative abundance of mycophagous protists.

2. Materials and methods

2.1. Isolation of protists from soil and microscopic observation on facultative mycophagy

Soil samples were taken in Pulheim Stommeln (Germany; 51°01′N, 6°45′E); Müncheberg (Germany; 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 three locations (upper 2 cm in Pulheim Stommeln, 10 cm in Müncheberg and 10 cm in Cologne). Additionally, soil from earthworm burrows (2 mm around burrows) was sampled at Les Verrines (Table 1).

Enrichment cultures were established to isolate (facultative) mycophagous protists. From each soil sample, 1 g dry weight of soil was suspended in 250 ml Neff's Modified Amoeba Salina (NMAS) according to Page (1988). After shaking on an orbital shaker (Köttermann, Uetze, Germany) at 100 rpm for 10 min and fourfold dilution with NMAS, 20 μl of the suspension was added to wells of a 24 multiwell-plate (Sarstedt, Nümbrecht, Germany). A mixed fungal inoculum of 80 μl of a 0.4 g l^{-1} NMAS solution of dried *Saccharomyces cerevisiae* (Ruf, Quakenbrück, Germany) and 160 μl of a *Fusarium culmorum* spore solution with a concentration of four spores * μl^{-1} were added to each well. 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 TS-100, Japan) at 100× and 400× 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 0.5% non-nutrient agar followed by autoclaving (122 °C, 20 min). MEA plates were inoculated with 100 μl suspension of *F. culmorum* spores and hyphae in H_2O_{dest} to establish active fungal cultures. Cultures of the yeast *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).

Amoebae and amoeboflagellates that showed indications of facultative mycophagy (Table 1) were cultivated monoxenically on bacteria for subsequent microcosm experiments. For that, individual protists were transferred from enrichment cultures to 60 mm Petri dishes filled with NMAS using a tapered glass pipette under an inverted phase-contrast microscope (Nikon TS-100, Japan). 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× magnification.

Protist cultures obtained were tested for their feeding preferences on three fungal taxa, i.e. two yeasts *C. laurentii* and *S. cerevisiae* and spores of the hyphal-forming fungus *F. culmorum*. 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 grown on accompanying bacteria in a 60 mm petridish for one week. The cultures were microscopically investigated for uptake of fungal material 2 and 24 h after inoculation, and microphotographs of protists ingesting fungal material were recorded.

2.2. Facultative mycophagy of the bacterivorous protist *Acanthamoeba castellanii*

Further microcosm tests were conducted using the model protist *Acanthamoeba castellanii* Neff strain to test potential for facultative mycophagy on four different fungi. Two strains of the single celled yeasts *S. cerevisiae* and two filamentous fungi, *Neurospora crassa* and *Coprinus cinerea* were presented as potential prey for *A. castellanii* grown axenically (proteose peptone–yeast extract–glucose medium, 4:2:1 mixture, respectively) (Rosenberg et al., 2009). 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). 100 spores of all four fungi were inoculated either alone or together with *A. castellanii*. Control treatments contained only NB-NMAS or *A. castellanii* in NB-NMAS. All treatments were replicated eightfold. Before use, *A. castellanii* cultures were washed three times with sterile NMAS after centrifugation at 800 rpm for 3 min and 100 amoebae were added to each well of the *A. castellanii* treatments. Control plates received equivalent amounts of NMAS. Plates were sealed with Parafilm and directly placed in an automated microplate reader (Varioscan, Thermo Scientific, Waltham, USA) at room temperature. Optical density (OD) as an estimate of changes in fungal density/growth was measured every hour for a total of four days. Plates were additionally examined microscopically for amoebae growth and potential contamination every second day.

Table 1
Overview of potential facultative mycophagous amoebae and amoeboflagellates investigated in this study. Taxonomic affinities according to Smirnov et al. (2011) and Adl et al. (2012); l = large taxon; s = small species.

Protist taxon	Order	Class	Supergroup	Morphotype	Length [μm]	Sampling site	Coordinates of sample site
Acanthamoeba sp.	Centramoebida	Discosea	Amoebozoa	Naked amoeba	~40	Müncheberg	52°30′N; 14°07′E
Acanthamoeba castellanii	Centramoebida	Discosea	Amoebozoa	Naked amoeba	~30	Pacific Grove	36°60′N; 121°93′W
Cercomonas sp. (s)	Cercomonadida	Sarcomonadea	SAR	Amoeboflagellate	~12	Müncheberg	52°30′N; 14°07′E
Cercomonas sp. (l)	Cercomonadida	Sarcomonadea	SAR	Amoeboflagellate	~30	Müncheberg	52°30′N; 14°07′E
Cryptodifflugia operculata	Arcellinida	Tubulinea	SAR	Testate amoeba	~18	Pulheim Stommeln	51°01′N; 6°45′E
Leptomyxa sp.	Leptomyxida	Tubulinea	Amoebozoa	Naked amoeba	>100	Cologne	50°55′N; 6°55′E
Mayorella sp. (s)	Dermamoebida	Discosea	Amoebozoa	Naked amoeba	~40	Les Verrines	46°25′N; 0°7′E
Mayorella sp. (l)	Dermamoebida	Discosea	Amoebozoa	Naked amoeba	~100	Cologne	50°55′N; 6°55′E
Thecamoeba sp.	Thecamoebida	Discosea	Amoebozoa	Naked amoeba	~50	Cologne	50°55′N; 6°55′E

Table 2
Feeding experiment of isolated mycophagous protists on the three different fungi (yeast species *C. laurentii* and *S. cerevisiae* and the hyphae forming *F. culmorum*). X = ingestion of fungal material, protist growth; – = no ingestion, no protist growth.

Protist genus	Supergroup; class; order	*C. laurentii*	*S. cerevisiae*	*F. culmorum*
Acanthamoeba	Amoebozoa; Discosea; Centramoebida	X	X	–
Cercomonas (s)	SAR; Sarcomonadea; Cercomonadida	X	X	–
Cercomonas (l)	SAR; Sarcomonadea; Cercomonadida	X	X	X
Cryptodifflugia	Amoebozoa; Tubulinea; Arcellinida	X	X	X
Leptomyxa	Amoebozoa; Tubulinea; Leptomyxida	X	X	X
Mayorella (s)	Amoebozoa; Discosea; Dermamoebida	X	X	–
Mayorella (l)	Amoebozoa; Discosea; Dermamoebida	X	X	X

2.3. Screening of soil metatranscriptomes

Protist communities in diverse, replicated soil and litter samples were investigated using a metatranscriptomic approach (Geisen et al., 2015b) and mined for vampyrellid and grossglockneriid sequences.

Details about sampling, extraction of nucleic acids and 454 pyrosequencing are described in Geisen et al. (2015b) and references therein. Processing of raw reads is also described in Geisen et al. (2015b) and references therein. In short, sequences were filtered using LUCY (Chou and Holmes, 2001) to remove 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 (Lanzén 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 of all vampyrellid and grossglockneriid sequences was verified by manual BLASTn searches against the NCBI GenBank nt database.

2.4. Statistical analyses

Data obtained in the experiment testing facultative mycophagy of *A. castellanii*, evaluating density changes for *N. crassa*, *C. cinerea* and strains of *S. cerevisiae*, were analysed daily by analyses of variance. SAS 8.0 (Statistical Analysis System, SAS Institute Inc., Cary, USA) software package was used for statistical analyses.

3. Results

3.1. Isolation of protists from soil and microscopic observation on facultative mycophagy

Eight protist taxa of different morphology and taxonomy were examined microscopically to evaluate feeding and growth on fungi. All thrived on the yeast taxa *S. cerevisiae* and *C. laurentii*, while *Cryptodifflugia operculata*, *Cercomonas* sp. (l), *Leptomyxa* sp. and *Mayorella* sp. (l) also fed on spores of *F. culmorum* (Table 2, Fig. 1). Along with ingestion of fungi, all protists reproduced in presence of the respective fungal prey.

3.2. Facultative mycophagy of the bacterivorous protist *Acanthamoeba castellanii*

A. castellanii inhibited growth of *C. cinerea* and both *S. cerevisiae* strains resulting in significantly reduced density as a proxy for biomass gain of those fungi compared to controls without amoebae (Fig. 2; Table 3). 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 could not be conducted as cells were masked by fungal material. In contrast, neither trophozoits nor cysts of *A. castellanii* were found in presence of *N. crassa*. In line, biomass of *N. crassa* was unaffected by amoebae (Fig. 2; Table 3).

The observed effects were always highest when fungal growth started and fungi gradually compensated for feeding losses. Due to differential growth of fungi, this differed between species; *A. castellanii* reduced the density of the fast growing yeast *S. cerevisiae* most strongly (by 30%) at day 1, which was reduced to 15% at day 4 (Fig. 2; Table 3). In contrast, *A. castellanii* most strongly impacted *C. cinerea* only after day 1, in line with strongest density increases of this fungus, resulting in a density reduction of 90% at day 2 and 82% at day 4 (Fig. 2, Table 3).

3.3. Presence and relative abundance of metabolically active mycophagous protists in soils using metatranscriptomics

Vampyrellid and grossglockneriid SSU rRNA transcripts were found in all investigated samples, representing 0.1–3.0% of all protist 18S rRNA transcripts. While Vampyrellida and Grossglockneriidae contributed little to the diversity of protists in artic peatlands (<0.4% of all protists), higher fractions were found in

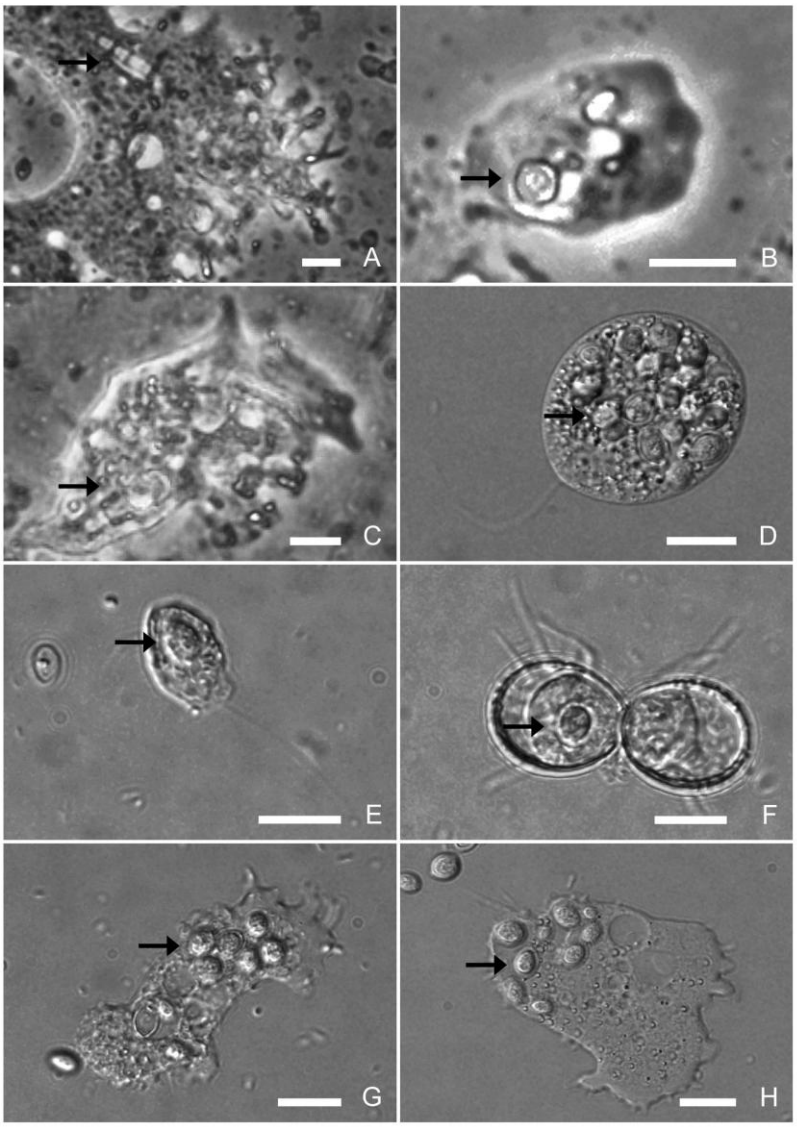

Fig. 1. Isolated soil protists with ingested fungi; (a) *Leptomyxa* sp. with ingested spores of *F. culmorum*; (b) *Thecamoeba* sp. with ingested *S. cerevisiae*; (c) *Mayorella* sp. feeding on spores of *F. culmorum*; (d) *Cercomonas* sp. (l) with ingested *S. cerevisiae*; (e) *Cercomonas* sp. (s) with ingested *C. laurentii*; (f) *Cryptodifflugia operculata* feeding on *S. cerevisiae*; (g) *Mayorella* sp. (s) with engulfed *S. cerevisiae*; (h) *Acanthamoeba* sp. with ingested *S. cerevisiae*; arrows indicate ingested fungal material; scale bar = 10 μm.

forest soil and litter (1.5% of all protists) with highest relative abundance in grassland (3.0%; Fig. 3).

Most grossglockneriid sequences most closely resembled *P. nana* and *M. terricola*, with especially short sequences showing identical similarities to both species. Some transcripts more closely resembled uncultivated grossglockneriids rather than the identified taxa *P. nana* and *M. terricola*.

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

The metatranscriptomic data further revealed a strong positive correlation between the relative abundances of SSU rRNA of mycophagous protists and the relative abundances of fungi (as fraction of all eukaryotic SSU rRNA transcripts), while the entity of protists showed the opposite trend (Fig. 4).

4. Discussion

In this work we highlight the hitherto neglected role of soil protists as mycophages. The functional group of mycophages among protists is almost exclusively recognized by taxonomists, who characterized highly specialized fungal feeders (e.g. Grossglockneriidae (Foissner, 1980; Petz et al., 1985; Foissner, 1998, 1999a)). We found that diverse soil protist taxa, previously

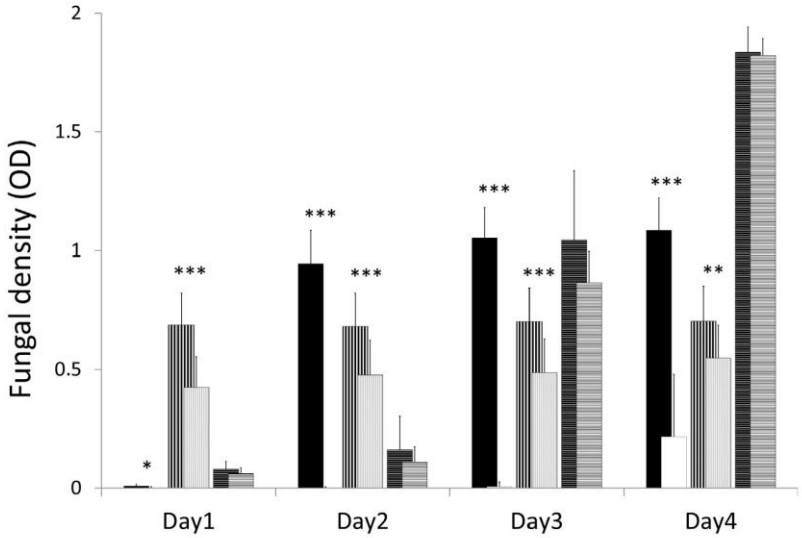

Fig. 2. Fungal density (OD_{450}) of fungi 1, 2, 3, 4 days after starting the experiment. *Neurospora crassa* (filled bars), *Coprinus cinerea* (vertical lines), and *Saccharomyces cerevisiae* (horizontal lines) are shown for each day; background darker bars represent non-*A. castellanii* treatments, foreground brighter bars depict fungal cultures with *A. castellanii*; differences between fungal only and fungi with *A. castellanii* are indicated ($^* = p < 0.05$, $^{**} = p < 0.01$; $^{***} = p < 0.001$); error bars represent the standard derivative (SD).

Table 3
ANOVA table of *F*- and *p*-values on the effect of *A. castellanii* on the fungal density (OD_{450}) of three different fungi 1, 2, 3, 4 days after starting the experiment (yeast species *S. cerevisiae* and the hyphae forming *C. cinerea* and *N. crassa*).

	Day 1		Day 2		Day 3		Day 4	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
C. cinerea	7.01	<0.05	376	<0.001	520.65	<0.0001	68.5	<0.0001
S. cerevisae	31.65	<0.0001	16.39	<0.001	18.56	<0.001	9.34	<0.01
N. crassa	1.57	0.23	0.88	0.363	2.46	0.14	0.09	0.769

classified as bacterivores, selectively fed on yeasts and on spores of soil fungi. Therefore, facultative mycophagy seems to be a widespread evolutionary feeding characteristic among soil protists. Unselective phagotrophy, characterized by random uptake of suitable sized objects, might partially explain this phenomenon. Engulfed potential prey items are then subject to extreme conditions in food vacuoles such as low pH (Laybourn-Parry, 1984) and a battery of enzymes (Bowers and Korn, 1973; Laybourn-Parry, 1984; Khan, 2009). Among the latter can be chitinases, which are encoded in the genome and can be expressed by some protist taxa (Tracey, 1955; Anderson et al., 2005; Fouque et al., 2012). Therefore, it is not unlikely that many protists can access fungal-bound nutrients from engulfed prey.

In line with size-dependent uptake of potential prey, our study revealed that yeasts were commonly taken up by morphologically and taxonomically diverse protist taxa. Transport of yeasts inside amoebae without damage of the cells has been reported (Heal, 1963; Chakraborty and Old, 1982); here, however, we observed active digestion of fungal material inside food vacuoles of protists resulting in an inhibition of fungal growth and increases in protist growth rates. All eight protists tested in this study consumed yeast cells. *C. operculata* and *Cercomonas* sp. (s) ingested single yeast cells, whereas the other, larger protists ingested several cells simultaneously. Fungal hyphae were never observed inside protists. This is in line with former studies that reported protist uptake of yeasts, while hyphae were not taken up (Heal, 1963; Bunting et al., 1979; Allen and Dawidowicz, 1990). This suggests that these fungal structures are mechanically protected against grazing of facultative mycophagous protists, that do not have specifically adapted mechanisms to perforate hyphae such as shown for vampyrellid amoebae (Old and Darbyshire, 1978; Old and Oros, 1980; Chakraborty and Old, 1982) or specialist feeding structures reported in grossglockneriid ciliates (Petz et al., 1985; Foissner, 1998, 1999a). Increased or decreased cell size and changes in cell shapes, such as through the production of filaments or colony formation, have been shown to result in mechanical protection adopted by certain bacteria to avoid protist grazing (Matz and Kjelleberg, 2005; Jousset, 2012).

Size of potential prey fungi does, however, not ultimately determine whether protists can feed on fungi; fungal spores and yeasts are often of similar size, but we found that *A. castellanii* almost entirely prevented growth of the filament-forming *C. cinerea*, strongly reduced yeast biomass, while the filament-forming *N. crassa* was unaffected. Secondary metabolites against protist grazing might protect *N. crassa*, as genes encoding unknown, putative secondary metabolites have been found in the genome of *N. crassa* (Galagan et al., 2003). Generally, toxin production adds grazing resistance to both fungi (Stotefeld et al., 2012) and bacteria (Matz and Kjelleberg, 2005; Jousset et al., 2006, 2009). Nevertheless, four out of eight protist species studied here, the amoeboflagellate *Cercomonas* sp. and the amoebae *C. operculata*, *Leptomyxa* sp. and *Mayorella* sp. ingested and grew on spores of the plant pathogenic, toxin-producing *F. culmorum* (Scherm et al., 2013). This rather applied aspect has been investigated before

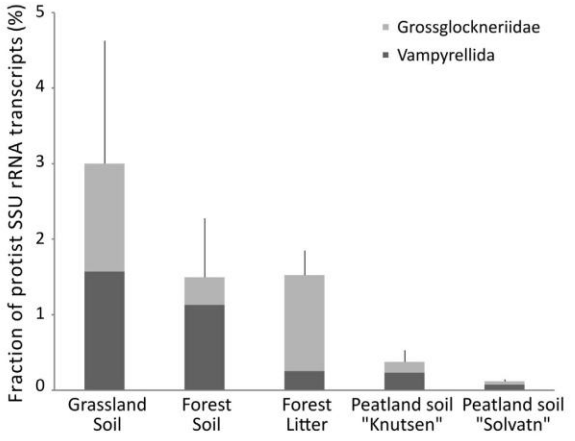

Fig. 3. SSU rRNA sequences assigned as grossglockneriids and vampyrellids shown as relative abundance of all protist SSU rRNA sequences obtained from metatranscriptomes of different soils; error bars represent standard derivative of both mycophagous groups.

showing that different protists consume other plant pathogenic fungi (Tapilskaja, 1967; Old and Oros, 1980; Chakraborty and Old, 1982; Chakraborty et al., 1983), suggesting that toxins protect fungi from grazing in a species specific manner. Taken together, no universal protection mechanism to mycophagy can be assumed and future studies need to include a wider range of fungi and mycophagous protists to investigate species-specific interactions. Nevertheless, it seems that mycophagy is highly widespread among soil protists and the differences in feeding can promote changes in fungal communities.

That size is not an ultimate characteristic that determines nutrient accessibility is also nicely illustrated by the widespread amoeba *C. operculata*, which has recently been shown to prey on several-fold larger nematodes (Geisen et al., 2015a). The fact that *C. operculata* also feeds on yeasts and can even live solely on bacteria as a nutrient source makes this amoeba particularly interesting, as it represents a true omnivore with a wide food range; furthermore, it complicates classical food web theory as it not only feeds on the source of both the bacterial and fungal energy channel, but also on higher trophic levels. Further studies are needed that study this or similar cases that represent exceptions to existing theories of the food web and if these should be included in an updated soil food web.

Interestingly, we found that the model protist *A. castellanii* Neff strain took up and reduced biomasses of *S. cerevisiae* and *C. cinerea*. Amoebae of this ubiquitous and highly abundant genus *Acanthamoeba* (Page, 1988; Rodríguez-Zaragoza, 1994; Geisen et al., 2014), especially those species identified as *A. castellanii* are commonly treated as obligatory bacterivores (Chakraborty et al., 1983; Bamforth, 1988). *A. castellanii* has been used as a model protist to study bacterivory in a number of experiments (Weekers et al., 1993; Bonkowski and Brandt, 2002; Rønn et al., 2002; Neidig et al., 2010; Koller et al., 2013b), and was consistently found to alter bacterial community composition in the plant rhizosphere (Kreuzer et al., 2006; Herdler et al., 2008; Rosenberg et al., 2009; Koller et al., 2013a). Our results show that *A. castellanii* also exhibits selective feeding preference for fungal species, even suppressing the growth of the hyphae forming fungi *C. cinerea*. Unlike larger mycophagous amoeba such as vampyrellids that perforate hyphae (Old and Darbyshire, 1978; Old and Oros, 1980; Chakraborty and Old, 1982), *Acanthamoeba* spp. rely on phagocytosis, which excludes long hyphae from the food spectrum. Engulfed potential prey is subjected to an immense potential enzymatic repertoire such as chitinases detected in the genome of *A. castellanii* Neff strain (Anderson et al., 2005; Clarke et al., 2013), suggesting that *A. castellanii* has the intrinsic capacity to thrive on fungi. The potential for facultative mycophagy is also present in other *Acanthamoeba* spp. as chitinases were found in *A. culbertsoni* (Krishna Murti and Shukla, 1984), *A. polyphaga* was reported to grow on *Cryptococcus neoformans* (Bunting et al., 1979) and chitinolytic activity was described in *A. glebae* (Tracey, 1955). The enormous species-diversity of *Acanthamoba* spp. (Gast et al., 1996; Stothard et al., 1998; Gast, 2001; Corsaro and Venditti, 2010; Qvarnstrom et al., 2013; Geisen et al., 2014) their wide enzymatic repertoire and obvious omnivory of some acanthamoebae might explain their ubiquity and high abundance in basically all environments (Sawyer and Griffin, 1975; Page, 1988; Rodríguez-Zaragoza, 1994). This highlights the need to carefully study ecological functions not only of one or few model taxa, but investigating ecological roles of a higher diversity of taxa.

4.1. Ubiquitous presence in significant relative abundances of metabolically active mycophagous protists in soils

Our environmental metatranscriptomics approach focussing on SSU rRNA transcripts revealed that the known mycophagous vampyrellids and grossglockneriids (Old and Darbyshire, 1978; Foissner, 1980; Petz et al., 1986; Hess et al., 2012) were present in all soils investigated. Grossglockneriidae have been described in 1980 with the family now containing nine species in six genera, all being obligate mycophages (Foissner, 1980; Petz et al., 1985, 1986; Foissner, 1999a). Cultivation based studies indicated their (sporadic) presence in a wide range of soils (Foissner, 1998, 1999a), but still little is known on the distribution and ecological importance of these ciliates. Our molecular data showing the presence of grossglockneriid sequences in all investigated samples support and advance the findings of Foissner (1998, 1999a). Despite an enormous diversity of other soil protists, grossglockneriids represented up to 2.5% of all protist sequences, indicating not only substantial abundances but also their potential functional importance in soil systems. Several sequences showed highest similarity to undescribed grossglockneriid species and the molecular diversity of grossglockneriids in samples generally appeared high. This indicates that grossglockneriids are undersampled and the true diversity remains unknown. The ancestral evolutionary origin of grossglockneriids around 280 million years ago, even predating those of the better studied dominant soil ciliates, i.e. colpodids (Foissner, 1999a), strongly reinforces their likely importance and potential diversity in soils. Currently only *Mykophagophrys terricola* and *Pseudoplatyophrya nana* have been sequenced (Lynn et al., 1999; Dunthorn et al., 2008) urging for cultivation and sequencing efforts to supplement the remaining described (and potentially undescribed) grossglockneriid species to investigate the full extent of diversity in these mycophagous ciliates.

Vampyrellid amoebae have been the focus of a recent detailed investigation including data-mining of HTS datasets showing that vampyrellids are highly diverse both in sediments and soils (Berney et al., 2013). Our HTAS and metatranscriptomic data clearly support the findings of Berney et al. (2013), as vampyrellid sequences were ubiquitously retrieved, seemed diverse, and abundant (up to 1.8% of all protist sequences) in all analysed samples. Sequences perfectly matching described species, such as *Theratromyxa weberi* and *Platyreta germanica* were detected, but sequences also often most closely resembled undescribed and uncultivated species in Vampyrellida. While only cultivation based efforts followed by targeted

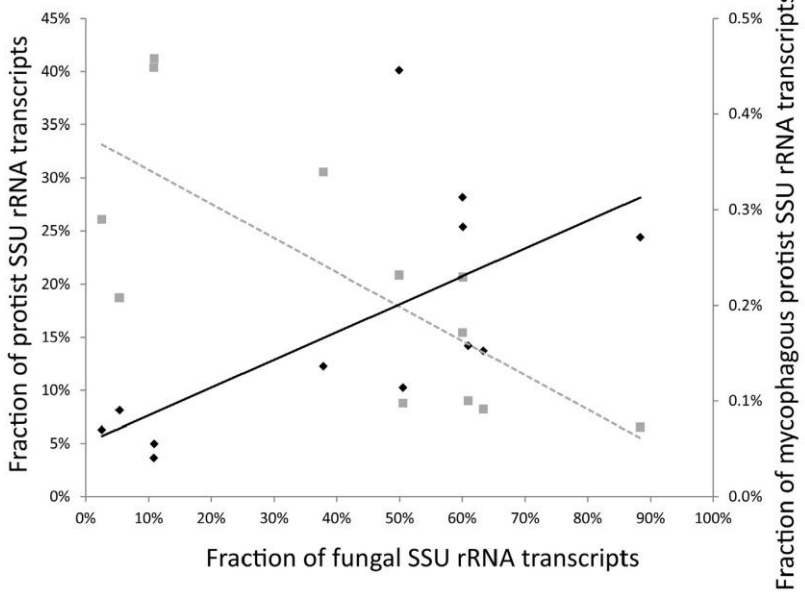

Fig. 4. Relative SSU rRNA abundance of all protists (grey squares, first Y-axis) and mycophagous protists (black diamonds, second Y-axis) to relative abundances of fungi (X-axis) among all eukaryote sequences. Note: positive correlation ($r^2 = 0.43$) between mycophagous protists and fungi (black filled regression line) while all protists and fungi showed a negative correlation ($r^2 = 0.56$; grey dotted line).

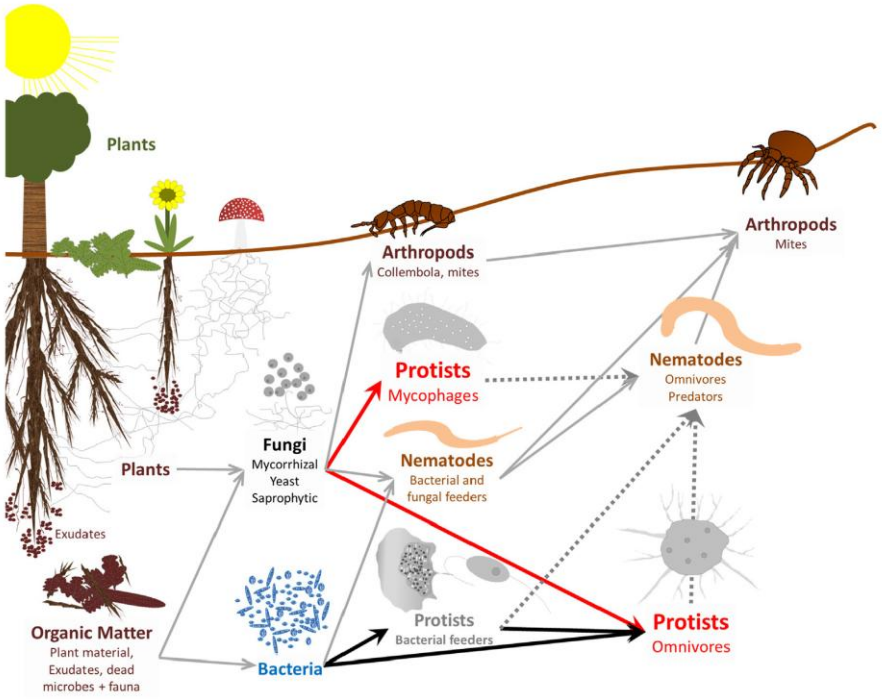

Fig. 5. The soil food web revisited showing that free-living heterotrophic protists are not only bacterivores, but also include mycophages and omnivores; bold black arrows: Widely assumed nutrient flows to protists; dotted arrows: suggested nutrient flow to higher trophic levels; red: formerly largely neglected trophic positions of protists. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiments can reliably unravel the true ecological importance of vampyrellids as mycophages, our data indicate that this functional role could be of importance in the soil food web.

The strong correlation of the two mycophagous protist groups with fungi supports their direct connectedness, likely through a direct trophic interaction as shown before. Further we show that

the entity of protists showed the opposite pattern, indicating that the most abundant protists do not depend on fungi as a prey and competitive interactions between both groups rather than trophic interactions prevail. Further functional studies are, however needed to prove this hypothesis. As abundant soil inhabitants (Berney and Pawlowski, 2006) fungi and protists inevitably co-occur in every soil environment. Therefore, a variety of interactions between these two groups must have evolved. It is not unlikely that these interactions have contributed to the evolution of both groups and increasing pathogenicity in fungal taxa to resist protist predators as suggested for the "arms race" between bacteria and protists (Brüssow, 2007).

5. Conclusions

Detailed knowledge on key drivers of carbon and nutrient fluxes in terrestrial ecosystems is a prerequisite for understanding ecosystem functioning. Here, undoubtedly, protists play a key role due to their importance as bacterivores in the soil food web (Moore and Hunt, 1988; de Ruiter et al., 1995; Crotty et al., 2012) and strongly affect C allocation and nutrient cycling in the plant–soil interface (Trap et al., 2015; Koller et al., 2013b). Results of this study suggest that a significant part of "bacterivorous" protists are actually facultative mycophagous and that known mycophagous protists are common and abundant members of soil communities. This obviously broad diversity of mycophagous protists is likely to harbour promising biocontrol agents protecting plants against pathogenic fungi, which should be included in these kinds of studies. Given their diversity and ubiquity, mycophagous protists could also play an unappreciated role in nutrient and C cycling with major implications for soil food web models, supplementing nematodes and microarthropods as major consumers of fungi (Fig. 5). Furthermore, it seems that the classical separation of the bacterial and fungal energy channel seems artificial, especially when protists are treated as a single trophic node in these schemes.

Acknowledgements

This research was largely funded by the European Commission through the EcoFINDERS project (FP7-264465), with support by the DFG-Research Unit FOR 918 (Carbon flow in belowground food-webs assessed by isotope tracers), subproject BO 1907/3-1.

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Discussion

Unknown Diversity in Cercozoa

Molecular studies hint a large uncharted diversity that needs to be characterized by morphological means (Bass and Cavalier-Smith 2004). We were able to find several of such unknown lineages of Cercozoa. The vast majority of Cercozoa of which morphology and genetical data are available are bacterivores, most predominantly of the Sarcomonadea, in particular the glissomonads and cercomonads (Bass et al. 2009b; Brabender et al. 2012; Cavalier-Smith and Karpov 2011).

The previously undescribed scaled amoebae with a huge network of filopodia turned out to be an independent lineage with cercozoan affiliation, *Kraken carinae*. They group (although with only moderate support) within the class Sarcomonadea, CAVALIER-SMITH, 1993. If *Kraken* turns out to be a true sarcomonadean, the discovery of a scale-bearing flagella-lacking amoeboid with sarcomonadean affiliation contradicts the image of the Sarcomonadea and might be a stepstone for a revision of cercozoan evolution. Moreover, the discovery of *Kraken* underlines that morphologically, phylogenetically and ecologically distinct lineages remain to be discovered even within groups such as Cercozoa and sarcomonads in particular that have been relatively well studied by environmental sequencing and culture-based surveys (Bass et al. 2009b; Brabender et al. 2012; Cavalier-Smith and Karpov 2011; Harder et al. 2016; Howe et al. 2009).

We further were able to contribute to the known diversity of Cercozoa by the characterization of many lineages feeding on eukaryotes. Such protists were common and by far not an exception in Cercozoa. In probably 50% of the examined samples we found unexplored diversity of Cercozoa feeding on eukaryotes. Thus it seems likely that there are still many cercozoan lineages (bacterivorous and eukaryvorous) to discover, thereby confirming our first hypothesis.

During the studies presented in this thesis it turned out that most of the isolated eukaryvorous thecate amoeba genera (*Lecythium*, *Rhizaspis*, ...) obtained from freshwater and terrestrial systems branch in the Thecofilosea, Cercozoa. These findings correspond with previously published phylogenies including morphologically similar testate amoeba genera,

e.g. *Pseudodifflugia*, *Rhogostoma* and *Capsellina*, all of which bear a theca, some with accumulated exosomes (foreign materials, like sand grains or diatom frustules agglutinated or attached to the theca), which all branch in the class Thecofilosea in the phylum Cercozoa although with polyphyletic origin (Howe et al. 2011, Wylezich et al. 2002).

We therefore can confirm our second hypothesis and moreover, those eukaryvorous strains of thecate amoebae now provide the opportunity to improve our understanding of thecate amoeba diversity, test evolution, eukaryvorous feeding behaviour and the resulting ecological impact.

Moreover, a stable taxonomic framework was obtained by combining intensive literature research with morphological and molecular data of such poorly-known lineages of cercozoan diversity. This enables future species descriptions and an easy identification of species by others, e.g. ecologists. The connection of molecular data with morphological and autecological data is still of fundamental necessity for high throughput sequencing studies that can only then be interpreted on a professional basis. Giving information on the genetic margins of morphospecies (and vice versa: morphological margins of operational taxonomic units (OTUs)) is needed to draw conclusions about the investigated diversity, dispersal and ecological function of protists.

Culturing of 'unculturable' protists

Petz et al. (1985) were able to culture an 'unculturable' lineage of fungivores with nutrient agar that was inoculated with varying fungi and partially submerged with culture medium and the desired organism (*Grossglockneria acuta*). This demonstrated with effort and the development of novel techniques even 'unculturable' protists with unusual feeding habits like fungivores are culturable.

Recently more and more 'unculturable' eukaryvorous protists have been cultured as researchers invested more and more effort into protist surveys and the establishment of cultures (Bass et al. 2009; Berney et al. 2013; Dirren et al. 2014; Hess et al. 2012; Hess & Melkonian 2013). This includes a direct enrichment of freshwater samples with putative food organisms (Dirren et al. 2014; Hess et al. 2012; Hess and Melkonian 2013) or the insertion of "traps" with "bait" into soils (Pakzad and Schlösser 1998).

We were able to culture numerous strains of several cercozoan lineages that previously have been (or might have been) declared as unculturable, resulting in the discovery of novel lineages and the molecular characterization of already described species, confirming our third hypothesis.

(1) We could show that there is a novel lineage of protists that is extremely fragile and slow growing: Weak disturbance (i.e. movement of the water body in the Petri dish) leads to damage of the filopodia often followed by cell death. Since compared weak mechanical disturbance of the *Kraken* is most often fatal, the establishment of a single clonal *Kraken* culture was seldom achieved by only few attempts, but most often had to be tried up to 20 times. Furthermore, to colonize the surface of a Petri dish an actively growing culture required several months under our culture conditions. Thus, the establishment of a culture of organisms that easily die off and grow only very slowly needed a year of continuous effort and a tremendous amount of time that not every researcher has, or is willing to invest. Thus, the key to a long-time maintenance of culturing the *Kraken* is therefore immense patience. Moreover, those characteristics hampered not only the process of culturing itself but also culture based work, i.e. morphological analyses.

(2) The cultured Thecofilosea showed different characteristics that required a different set of equipment and expertise. As they are eukaryvorous, they have to be continuously co-cultured (or fed) with a suitable food source. The required culture conditions could not be reliably predicted either by their phylogenetic position, the type of habitat of which the sample originated from or by thorough investigation of the amoebae in natural samples. Although many typical eukaryvorous protists, e.g. the vast majority of Vampyrellida species show highly specialized monophagous feeding preferences that are also reflected in their phylogeny (Hess et al. 2012, Hess pers. comm.), we were not able to find such patterns in the Thecofilosea. For instance, *Fisculla margaritae* and *Fisculla* cf. *margaritae* did not feed on fungi whereas the closely related (and also limnic) *Fisculla asini* and *Fisculla siemensmai* could not be cultured without. Moreover, some strains grew steadily over multiple years of culturing on a single food source, others ceased to grow after a couple days or even months unless being cultured on a variety of (up to four) food organisms.

Moreover, we were able to establish the first known (and probably the first ever established) bacteria-free culture of a thecofilosean amoeba.

Fisculla terrestris cultures contaminated with bacteria were very easy to handle, but bacteria-free cultures of *Fisculla terrestris* were maintainable but unstable. Bacteria, although not consumed, can play an important role in species coexistence in cultures. Potential roles of bacteria in cultures are the degradation and/ or metabolisation of secondary metabolites produced by eukaryotes, that may either just be waste products or even be specifically produced repellents or toxins against eukaryotic predators; or (b) the degradation of extracellular enzymes of fungi that might cause cell death when enriched to high concentration (e.g. chitinases, own observations, unpublished).

What is the ecological impact and dispersal of eukaryvorous protists?

Bacterivory in protists has been extensively studied in terms of nutrient flows and trophic interactions, eukaryvores have received much less attention. It has been shown that eukaryvory plays a dominant role in freshwater systems in terms of carbon flux from primary producers to higher trophic levels (Sherr and Sherr 1994; Sherr and Sherr 2002), but little is known from eukaryvory in terrestrial systems.

Hess & Melkonian (2013) demonstrated with time-lapse photography what severe effects protist grazing can have on algal cultures. Within few hours to days whole prey populations (green algae of the Zygnematales) are eradicated. Our feeding experiments with the fungivorous *Fisculla terrestris* showed similar patterns in fungi, where a starting population of 100 predator individuals eradicated prey populations of 100.000 yeast cells in less than three days, indicating a importance of fungivores on fungi populations and possibly a top-down control of those.

It is important to note, that such a simplified two-species-system misses predators of higher trophic levels. Protists are usually preyed on by other protists (often ciliates or large omnivorous amoebae), or metazoa (e.g. cladocerans, rotifers, copepods, nematodes; see Geisen 2016, Sherr and Sherr 2002). Accordingly, such experiments show what eukaryvores

are capable of but lack insight in the real ecological impact of eukaryvores. Molecular methods like co-occurence surveys might give insight into inter-specific interactions.

Direct observations of our freshwater samples and the thecofiloseans (and in particular the freshwater *Fisculla*) within, revealed the freshwater inhabiting *Fisculla* to feed on algae, although some fed in laboratory conditions also on fungi, they were not culturable without sufficient algal concentration. Since direct observation of soil protists is not possible, a laboratory approach was used to determine feeding preferences of the terrestrial *Fisculla*. By laboratory experiments we were able to show that the terrestrial relatives of the genus *Fisculla* feed on every of the presented fungal cells, are able to sense fungal presence, produce chitinases and select for fungi, whereas we with diverse experiments could show little (or no) algae ingestion and with different approaches we were not able to detect any bacteria ingestion.

We therefore consider the terrestrial *Fisculla* as fungivorous, giving example of another fungivorous lineage closely related to algivores, which supports our fourth and fifth hypotheses. With sampling and a culture based approach, we could show the fungivorous *Fisculla* in two distinct terrestrial habitats. We further showed that very common amoebae or amoeboflagellates, like *Cercomonas* and *Acanthamoeba,* fed on fungal cells. Moreover a metatranscriptome study focussing on fungivorous soil inhabiting protists contribute to our understanding of less prominent but probably widespread trophic interactions between micropredators and eukaryotic prey like algae and fungi, giving further support to our fourth and fifth hypotheses.

Remaining questions and perspective for future investigations

Although the *Kraken* was only very recently described, it is already topic of ongoing molecular studies (Sapp pers. comm. unpublished), emphasizing that the characterization of novel lineages enables new ways to interpret ecological data, which may lead to a more comprehensive understanding of nature.

Culture-based and molecular studies of the Tectofilosida show their diversity and functional role of eukaryvores. Such feeding behaviour makes them a valuable target in food web studies involving investigations of carbon and nutrient flow studies. It is therefore a

necessity to uncover the large uncharted diversity of the Thecofilosea, especially the Tectofilosida. Moreover, there are still old descriptions of species, like *Lecythium mutabilis, Lecythium spinosum, Lecythium curvus* and more, of which molecular data is missing. All but one of these species descriptions have been made by observing freshwater samples. Molecular analyses already revealed a series of OTUs with Tectofilosida affiliation in terrestrial and marine habitats (Bass and Cavalier-Smith 2004), clearly showing that there is still much to be discovered when further targeting these habitats. These steps will be essential to our understanding of the Tectofilosida in ecological context: (a) an increased sampling of tectofilosidan amoebae of freshwater, marine and terrestrial habitats; (b) the collection of morphological as well as autecological data; (c) the phylogenetic characterization, predominantly targeting the SSU-coding gene and; (d) when those steps have been conducted to a high extend, molecular studies can be performed to get insight into ecological data on a broad scale, including diversity and dispersal surveys and detailed food web (co-occurrence) investigations.

Moreover the Tectofilosida represent a valuable target to study a variety of physiological adaptations of eukaryvorous protists: (a) The genus *Fisculla* comprises eukaryvores feeding on algae and fungi, we therefore assume that *Fisculla* is able to produce a cocktail of enzymes needed to digest β-polysaccharides, like in chitin, cellulose and other biopolymers. The screening for novel enzymes often basically focuses on macroscopic organisms or bacteria (Ferrer et al. 2009; Lee et al. 2010). Distant lineages of protists, like the Tectofilosida in the Cercozoa may harbour enzymes highly valuable for applied purposes. A first insight might be given by harvesting large amounts of bacteria-free *Fisculla terrestris* cells and performing simple protein extractions; (b) such protein extractions might also give insight into testate amoeba evolution as it is still unknown how *Fisculla* (and other thecofiloseans) builds its test. (c) *Fisculla terrestris* showed to be further highly sensitive for chemical signals released by its prey (kairomones). *F. terrestris* responds visibly in behaviour to those signals and can be cultured in bacteria-free conditions, making it a valuable organism for the establishment of a bioassay. Bioassays are necessary in chemical ecology to see the reactivity of extracted and fractioned chemical compounds. A continuous filming of such a culture is very easy and short-timed manageable.

Nevertheless there are still plenty other cercozoan lineages or morphotypes of which barely something is known. Just to name three very promising lineages of which a doctorial thesis could lead to very interesting results: Terrestrial Viridiraptoridae are still not studied, since the freshwater Viridiraptoridae feed on algae, their terrestrial relatives could also show fungivorous behaviour. If so, further evidence for the fourth of our hypotheses could be given. The Clade Y, closely related to the Viridiraptoridae, is still without any morphological data. Since the Viridiraptoridae showed surprising unique morphology and ecology, it is easy imaginable that the closely related Clade Y shows also parasitoid behaviour of unknown extend. All environmental sequences of Clade Y have been obtained from soils, it is therefore not unlikely that the organisms of Clade Y parasitize soil inhabiting organisms. Also agglutinating testate amoebae of the Cercozoa are a yet uncovered field of research. Although there are species known, these morphological descriptions show that *Pseudodifflugia, Diaphoropodon, Capsellina* and more taxa are highly diverse. There are up to now only one (maybe two, see Wylezich et al. 2002 and Howe et al. 2011) sequences assigned to such genera. It is likely, that these genera of agglutinating testate amoebae will be shown to be closely related to the thecate testate amoebae in the Cercozoa, therefore probably also showing eukaryvorous or omnivorous feeding behaviour and interesting ecology.

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Acknowledgements

First and foremost I would like to express my gratitude to my advisor Prof. Dr. **Michael Bonkowski** for giving me the opportunity to do my thesis in his lab. Although funding was scarce Michael always found a way to support me financially. Moreover, in my opinion most importantly, he guided my thesis in a way that I appreciated very much. Instead of giving orders, he showed opportunities, gave opinions and discussed those with me. The very precise Dr. **Anna Maria Fiore-Donno** introduced me into bioinformatic analyses and enabled me therefore to continue my way in protistology with very modern and timely methods. I thank **Thi Anh Duong Nguyen** and **Fionn Clissmann** for the very nice atmosphere in our office and in general I thank the whole terrestrial ecology workgroup as I had very pleasant time with my colleagues.

Additionally, I would like to thank Prof. Dr. **Hartmut Arndt** for being the second assessor of this work. I am aware that his time is precious, thus reading and grading such a thesis is a tremendous amount of work.

During the course of my thesis I contacted several external experts of which many became coauthors of my publications. Foremost, I would like to thank **Ferry Siemensma**, this enthusiastic hobby microscopist was especially valuable in the beginning of my thesis as he enjoyed to discuss my questions. I would like to thank **Sebastian Loeppmann**, as he enabled me to carry out the enzyme analyses of *F. terrestris*, he showed me the whole procedure and taught to such an extent that I am now able to perform them for myself. I also thank Dr. **Alexander Mynikov,** Dr. **Frank Nitsche** and **Eckhard Völcker** for helping with published and yet unpublished electron microscopy.

I enjoyed the work with several bachelor students of which I had the pleasure to work with.

And last of all, many persons that are not named here but were nevertheless important contributors.

Subpublications in Three Parts and Record of Achievement

The Publications correspond to the chapters.

Part 1 - Characterization of the unusual bacterivorous amoeba *Kraken carinae*.

Chapter 1[1, 4]: **Dumack K**, Schuster J, Bass D, Bonkowski M (2016) A novel lineage of 'naked filose amoebae'; *Kraken carinae* gen. nov. sp. nov. (Cercozoa) with a remarkable locomotion by disassembly of its cell body. Protist 167:268-278

Chapter 2[1, 5]: **Dumack K**, Mylnikov AP, Bonkowski M (in review) Cercomonad or archaic Imbricatea? On the hunt for the true taxonomy of the scale-bearing *Kraken* (*incertae sedis*, Cercozoa, Rhizaria): Combining ultrastructure data and a two-gene (SSU + LSU) phylogeny. Protist

Part 2 - Eukaryvorous amoebae of the Thecofilosea, Cercozoa.

Chapter 3[1]: **Dumack K**, Müller ME, Bonkowski M (2016) Description of *Lecythium terrestris* sp. nov. (Chlamydophryidae, Cercozoa), a soil dwelling protist feeding on fungi and algae. Protist 167:93-105

Chapter 4[1, 2]: **Dumack K**, Baumann C, Bonkowski M (2016) Revision of the thecate amoeba genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa, Rhizaria) including a description of four new species and an identification key. Protist 167:440-459

Chapter 5[1, 2]: **Dumack K**, Öztoprak H, Rüger L, Bonkowski M (in review) Shedding light on the polyphyletic thecate amoeba genus *Plagiophrys*: Transition of some of its species to *Rhizaspis* (Tectofilosida, Cercozoa) and the establishment of *Sacciforma* gen. nov. and Rhogostomidae fam. nov. (Cryomonadida, Cercozoa). Protist

Chapter 6[1, 2]: **Dumack K**, Mausbach P, Hegmann M, Bonkowski M (in review) Polyphyly in the thecate amoeba genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa), redescription of its type species *L. hyalinum*, description of *L. jennyae* sp. nov. and the establishment of *Fisculla* gen. nov. and Fiscullidae fam. nov. Protist

Part 3 - Eukaryvorous protists, their capabilities and dispersal.

Chapter 7[1, 6]: **Dumack K**, Pundt J, Loeppmann S, Bonkowski M (in review) What does it take to eat a fungus? A case study with the eukaryvorous amoeba *Fisculla terrestris*. Applied and Environmental Microbiology

Chapter 8[3]: Geisen S, Koller R, Hünninghaus M, **Dumack K**, Urich T, Bonkowski M (2016) The soil food web revisited: Diverse and widespread mycophagous soil protists. Soil Biol Biochem 94:10–18

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

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

[3] The author was involved in the writing of the article and in performing the laboratory experiments.

[4] Phylogenetic analyses were performed with help of the coauthor Prof. Dr. David Bass.

[5] Electron microscopy was performed by the coauthor Dr. Alexander P. Mylnikov.

[6] The enzymatic analyses were performed by the author and the coauthor Sebastian Loeppmann.

Erklärung (gemäß § 4 Abs 1 Punkt 9 der Prüfungsordnung)

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

_____

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Seit 2012	SHK, WHF und WHK Tätigkeit bei Prof. Dr. Michael Bonkowski, Universität zu Köln

Schulische Ausbildung

2006 – 2009	Besuch des Geschwister Scholl Gymnasiums Pulheim
2000 – 2006	Besuch der Marion Dönhoff Realschule, Pulheim

Tagungen und Beiträge

Moscow Forum «PROTIST–2016»
Mündlicher Vortrag "The Chlamydophryidae revisited with special focus on the genus *Lecythium*"
Poster Präsentation "The base of cercomonad radiation is still in for a surprise, *Kraken* gen. nov." **Empfänger des Holz-Conner Preises.**
Unter den ehrenhaften Nennungen für die besten Poster und besten Vorträge.

35th annual meeting of the German Society for Protozoology
Mündlicher Vortrag "A bowl with marbles; the fungal and algal-eating amoeba genus *Lecythium* (Chlamydophryidae, Tectofilosida, Cercozoa, Rhizaria) revisited; phylogeny and the description of four new species"
Poster Präsentation "The base of cercomonad radiation is still in for a surprise, *Kraken* gen. nov."
Empfänger der zweiten Preise für das beste Poster und den besten Vortrag.

Gastvortrag an der Universität Göttingen, eingeladen von Valentyna Krashevska
Titel: "*Lecythium*, the neglected testate amoebae. Insight into phylogeny, morphology and ecology with focus on *L. terrestris* sp. nov."

34th annual meeting of the German Society for Protozoology
Mündlicher Vortrag "*Lecythium terrestris*, its morphology, phylogeny and autecology."
Empfänger des Preises für den besten Vortrag.

33rd annual meeting of the German Society for Protozoology