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Morphological and molecular taxonomy of aquatic and terrestrial protists as a prerequisite for studies of evolution, biodiversity, biogeography, bioindication and ecosystem functioning –examples from euglyphid and arcellinid testate amoebae.

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Titre:

“Morphological and molecular taxonomy of aquatic and terrestrial protists as a prerequisite for studies of evolution, biodiversity, biogeography, bioindication and ecosystem functioning –examples from euglyphid and arcellinid testate amoebae”

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Neuchâtel, le 16 décembre 2022

Le Doyen, Prof. R. Bshary

“Absorb what is useful, discard what is useless and add what is specifically your own.”

Bruce Lee, on pursuing a PhD

“Why change? Everyone has his own style. When you have found it, you should stick to it.”

Audrey Hepburn, on her love for taxonomy

“To defy the laws of tradition is a crusade only of the brave”

Primus, on taxonomic revisions

Abstract

Macro-eukaryotes (i.e., organisms that do not need magnification to be seen) appear to being the dominant component of ecosystems, but they are not the only driving forces of ecological processes. Microorganisms are known to play important roles in ecological processes such as elements, and nutrients cycling and are also known to shape macro-eukaryotic communities via parasitism or symbiosis (e.g., mycorrhiza). Now we are starting to assess the diversity and the impact on ecological processes of freshwater and marine micro-organisms but little is known of the soil-dwelling microbes, especially non-fungal and non-plant unicellular microeukaryotes (known as the protists). Maybe because most do not form recognizable colonies like the bacteria and the fungi, soil protists have been overlooked even by microbiologists. Some protists such as the diatoms, radiolarians, foraminiferans and coccolithophores have proved to be potent proxies and are commonly used to evaluate current or past environmental changes, but it concerns only a fraction of the protistan diversity. Furthermore, those are mostly marine organisms. Among the plethora of soil protists and their possible uses, only testate amoebae are regularly used as proxies to monitor the evolution of peatlands. And probably, one of the major reasons that could be invoked is the largely incomplete taxonomy of soil protists. The use of protists as bioindicators implies a sound taxonomic framework. However, the diversity and the taxonomy of these organisms is still far from being understood, as most species have not been described. Protists were mainly characterized with light microscopy, but the absence of characteristic morphological traits - and the fact that the phenotypical plasticity of protists was considered as being extremely high - has led naturalists to underestimate their diversity for a long time. It is only recently with the appearance of staining protocols, electron microscopy and molecular biology that we start to assess the true diversity of these organisms. With these new tools, it appears that many described species were in fact morphospecies complexes including more than one biological species that, sometimes, could be completely unrelated. Among these tools, High Throughput Sequencing (HTS) allows to evaluate more easily the micro-eukaryotic community of an environmental sample, revealing that many clades of protists have not been characterized and are still to be discovered. There is then a need for taxonomists to describe this hidden diversity and to update old descriptions in order to build a sound taxonomy. The aim of this thesis is to cover the several steps required to improve the taxonomy of soil protists, with a focus on testate amoebae, so that they can be used in larger surveys to study their ecology, diversity and evolution.

Keywords : Protists ; Taxonomy ; Testate amoebae

Résumé

Les macro-eucaryotes (les organismes qui peuvent être observes à l'œil nu) semblent être les acteurs dominants des écosystèmes, mais ils ne sont pas les seuls à jouer un rôle dans les processus écologiques. On sait maintenant que les microorganismes jouent aussi un rôle dans divers processus écologiques comme le cycle des nutriments ou la régulation des communautés de macro-eucaryotes via le parasitisme ou la symbiose (par exemple les mycorhizes). La diversité et les fonctions écologiques des micro-organismes d'eau douce ou marins sont de mieux en mieux connues, mais ils existent encore peu d'information sur les microbes du sol, particulièrement les espèces non fongiques et non végétales de micro-eucaryotes (les protistes). Les protistes du sol ont été négligés mêmes par les microbiologistes, possiblement parce qu'ils ne forment pas de structures reconnaissables à l'œil nu comme les champignons ou les bactéries. Certains protistes comme les diatomées, les radiolaires, les foraminifères et les coccolithophores sont couramment utilisés comme bioindicateurs pour évaluer des changements environnementaux passés ou présents, mais cela concerne seulement une fraction de la diversité des protistes. De plus, ce sont principalement des organismes marins. Parmi la pléthore de protistes du sol and de leurs usages potentiels, seulement les amibes à thèques sont utilisées régulièrement comme bioindicateurs pour évaluer l'évolution de tourbières. Et probablement, une des principales raisons est que la taxonomie des protistes du sol est très lacunaire. L'utilisation de protistes comme bioindicateurs implique des connaissances taxonomiques rigoureuses. Cependant, la diversité et la systématique de ces organismes est encore loin d'être connues car la plupart des espèces n'ont pas encore été décrites- Les protistes on principalement été décrits à l'aide de microscopes optiques, mais l'absence de traits morphologiques caractéristiques – et le fait que la plasticité phénotypique des protistes était considérée comme très grande – à mener à grandement sous-estimé leur diversité pendant longtemps. Ce n'est que récemment, avec l'apparition de protocoles de teinture, de la microscopie électronique et de la biologie moléculaire que l'on commence à se rendre compte de la véritable diversité de ces organismes. Avec ces nouveaux outils, il apparait que beaucoup d'espèces décrites sont en fait des complexes incluent plus qu'une véritable espèce qui, parfois, ne sont absolument pas apparentées. Parmi ces outils, le séquençage massif permet d'évaluer plus facilement les communautés de micro-eucaryotes dans un échantillon environnemental et révèle que beaucoup de clades de protistes n'ont pas encore été décrits. Il y a donc besoin de taxonomistes pour décrire cette diversité cachée et mettre à jour les vieilles descriptions afin de construire un cadre taxonomique convenable. Le but de cette thèse est de couvrir les différentes étapes requises pour améliorer la taxonomie des protistes du sol, on se focalisant sur les amibes à thèques, afin qu'ils puissent être utilisés dans des études plus générales pour évaluer leur écologie, diversité et évolution.

Mots-clés: Protistes ; Taxonomie ; Amibes à thèque

Summary

Introduction

1. What are protists

While macro-eukaryotes (i.e., organisms that do not need magnification to be seen) appear to be the dominant component of ecosystems, the vast majority of the eukaryotic diversity (i.e., the organisms with a nucleus by opposition to bacteria and archaea which are lacking membrane-bound organelles) is represented by the so-called protists (Pawlowski et al. 2012): mostly microscopic and unicellular organisms that cannot be classified as animals, plants or fungi (Figure 1) (O'Malley et al. 2013). As they constitute most of the eukaryotic tree of life, they are morphologically and functionally highly diversified and present in virtually every ecosystem (Geisen et al. 2017), which makes them difficult to categorize [Box 1]. They can be sessile or motile, crawling using extensions of their membrane ("the amoebae"), propelled by a single or few flagella ("the flagellates") or rows of hundreds of ciliae ("the ciliates"), they can be naked or protected by shells made of proteins and minerals, their size ranges from a few micrometres up to tens of meters, they can be photosynthetic or predators, symbionts and even parasites. Some well-known protists are the siliceous algae diatoms (Bacilliarophyceae) and Radiolaria, the lab rat *Paramecium* (Ciliophora) and the parasite responsible for the malaria *Plasmodium* (Apicomplexa).

Protists were first categorized into two functionally distinct main groups depending on their mode of nutrition, with phagotrophic protists being referred to as protozoans and phototrophic ones being referred to as algae (Geisen et al. 2018). However, thanks to molecular methods [Box 2], we currently know that these two groups do not correspond to monophyletic clades, and the current model of the eukaryotic tree of life presents phagotrophic and phototrophic species scattered throughout its branches. Currently, the eukaryote tree of life is divided into at least nine major groups of which plants, animals and fungi are only short and isolated branches, the rest of the tree being populated by protists (Burki et al. 2020).

[Box 1] Protists are not well defined

Because protists are either grouped together based on what they are not (i.e., not an animal, plant, or fungus) (O'Malley 2012) or their unicellular nature (Geisen et al. 2017), the boundaries of what can be considered a protist tend to fluctuate. As an example, it can be debated whether brown algae, which are not plants, but which can form structure up to tens of meters should be considered as protists. The same issue can be brought for unicellular fungi like yeasts, or the red and green algae which are part of the Archaeplastida (i.e., the plants *sensu lato*) but are usually not considered as plants, which generally includes only the Embryophyta (i.e., the land plants). Furthermore, green and red algae are mostly studied by phycologists and as such are sometimes considered separately from the other protists. Lastly, some authors have criticized the recognition of non-monophyletic groups because they are not natural and they do not share the same evolutionary history, which might thus slow down the understanding of their evolution (Donoghue and Cantino 1988). Nonetheless grouping these organisms under the term "protists" remains useful, as we need a working concept to discuss the assemblage of these mostly unicellular and highly diverse eukaryotes (Andersen 1998).

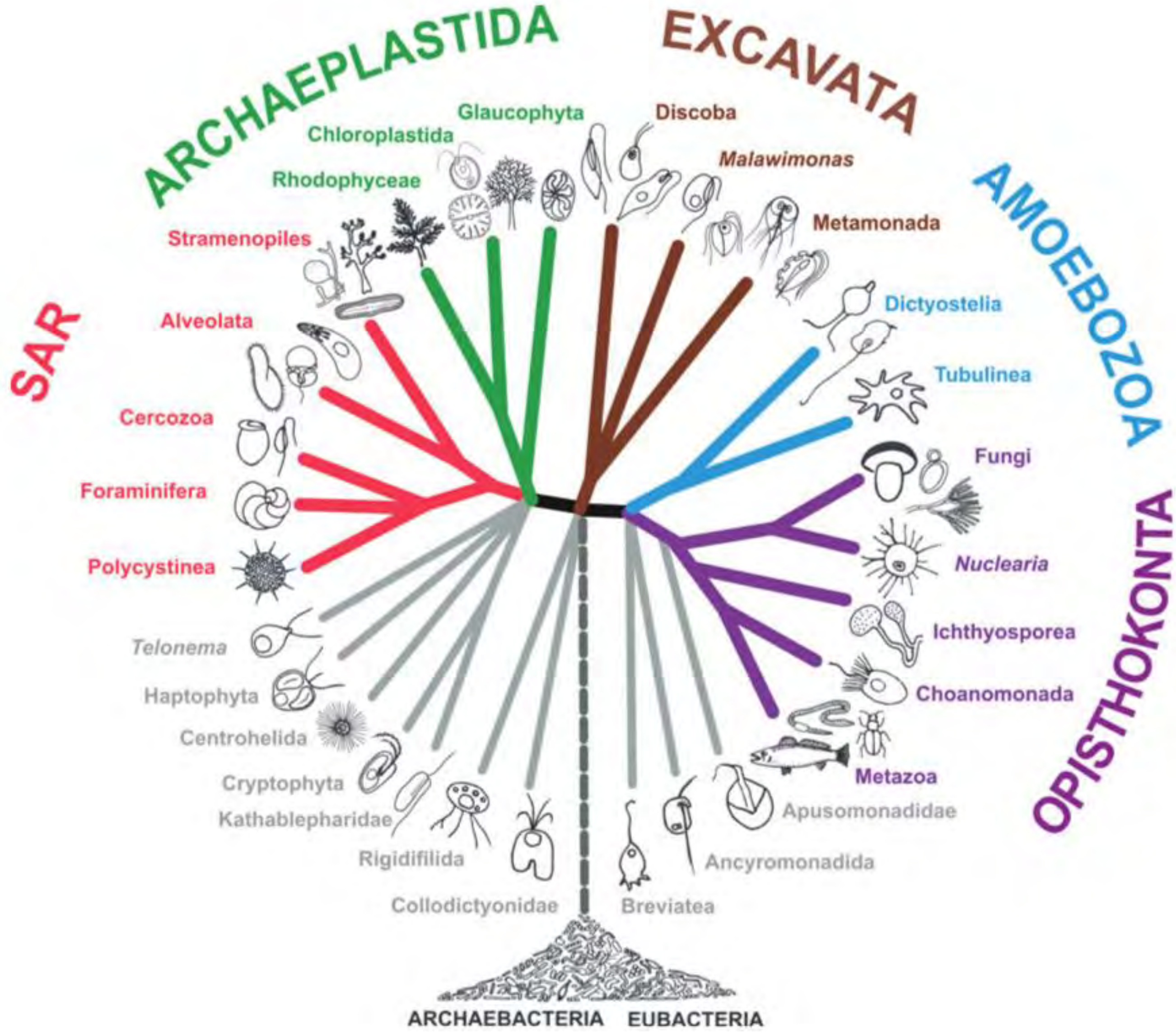

Figure 1: The eukaryotic tree of life. Most branches are populated by protists while plants (in the Chloroplastida), animals (Metazoa) and fungi constitute only isolated and relatively short branches. Adl et al. 2012.

[Box 2] DNA barcoding, phylogenetic reconstruction, and taxonomy

Due to genetic drift, species accumulate random mutations in their genomes leading them to diverge genetically over long periods of time. Thus, by sequencing a genomic fragment that has varied enough between species of a group, it is possible to associate each species with a unique DNA sequence, a “barcode”. This barcode can then be used for species identification by sequencing the same fragment in an unknown specimen and comparing it to a database of previously barcoded species. This method is sometimes considered as more objective than morphology for the identification of species, as living organisms are subject to natural variation and the traits that should be treated as diagnostic can be debated (Lee 2004). Morphological traits that look similar can also be non-homologous and be caused by evolutionary convergences rather than a shared evolutionary history. Furthermore, as each of the nucleotide in a barcode (which usually counts at least hundreds) can be considered as an independent trait, DNA barcoding offer much more traits onto which base identification than morphology (Lee 2004). Because DNA sequencing is increasingly cheaper and easier to use, and to compensate for the shortage of taxonomic expertise, DNA barcoding has also been proposed as the main way to identify species (Hebert et al. 2003). This has however been criticized, as genomes are subject to intraspecific variation too, sequences can be misaligned, the choice of DNA markers can affect the results, and some species that diverged only recently might share the same barcode (Mallet and Willmott 2003, Lee 2004). Nonetheless, DNA barcoding has been proven especially useful for the taxonomy of protists, which often present very few morphological characters and whose most species can hardly be distinguished based on their morphology alone (Pawlowski et al. 2012, Andersen 1998).

While DNA barcodes were originally designed to identify taxa, they are currently the main data used to reconstruct phylogenetic relationships (i.e., molecular phylogenies). Each position in a DNA sequence is a trait that can be used to build phylogenetic reconstruction, and given the usually high number of nucleotides in a single barcode and their discrete possible state that are easy to code (A, T, G, C, or a gap), they have proven to be much more reliable than morphology to infer phylogenetic trees (Lee 2004). Also, since the appearance of Darwinian theories of evolution (Darwin 1859), taxonomists tend to reflect evolutionary relationships by defining taxa that are monophyletic (i.e., a taxon must include all the descendants of a given ancestor). With this approach, which has been referred to as “cladism”, organisms are grouped based on their evolutionary history instead of their traits (Hennig 1950, de Queiroz and Gauthier 1992). As such, phylogenetic reconstructions based on DNA sequences are becoming increasingly important in taxonomic decisions - leading to the revision of many taxa described previously and to some resistance by taxonomists used to the traditional classification (Cronquist 1987). Interestingly with the cladistic approach, phylogenetics and taxonomic classification can be considered as synonym, as both would ultimately group organisms in the same way (Patterson et al. 1993).

2. Ecological roles and diversity of protists

While phototrophic protists (the "algae") did receive some attention, protists were originally omitted from ecological studies (Caron et al. 2009, Wilkinson and Mitchell 2010) which focused more on prokaryotes, fungi or even viruses, and are only little known from the public, and even microbiologists (O'Malley 2012). However, thanks to modern tools that offer a clearer picture of the microbial communities and ecological processes, protists are increasingly shown to have a distinct ecological role to that of other microbes (Caron et al. 2009), and even to be major actors in ecosystems, compensating for their microscopic size with their sheer number (Adl and Gupta 2006, de Vargas et al. 2015) and very short generation time (Payne 2013).

Photoautotrophic protists are key players in carbon fixation and primary production. Marine phytoplankton has been shown to be responsible for carbon fixation as much as terrestrial plants (Falkowski et al. 1998, Field et al. 1998). Even on dry earth, phototrophic protists play an important role in soil formation (Bamforth 2008) and are considered to participate significantly to primary production, especially in ecosystems with little vegetation (Seppey et al. 2017, Geisen et al. 2018). Heterotrophic protists are an indispensable component of the so-called microbial loop in which, by regulating the population of bacteria, they redistribute nutrients which become available to other organisms, making them essential to soil fertility and plant growth (Azam et al. 1983, Bonkowski and Clarholm 2012). Mixotrophic (both heterotrophic and phototrophic) organisms are also important in the carbon cycle and primary production in nutrient poor habitats such as peatlands where they can constitute more than 70% of the microbial biomass (Gomaa et al. 2014, Jassey et al. 2013, Jassey et al. 2015). Besides primary production and nutrients cycling, protists are essential in biogeochemical cycles such as the cycles of silicon and carbon (Jordan and Chamberlain 1997). As an example, in a temperate forest, silicon-metabolizing testate amoebae have been shown to participate to the turnover of silicon as much as the trees themselves (Aoki et al. 2007). Lastly, protists are known to shape the community structures of micro- and macro-organisms via symbiosis (de Vargas et al. 2015) and parasitism (Mahé et al. 2017), some noteworthy examples being the algal symbionts of corals (*Symbiodinium* spp.) or the plant pathogen (*Phytophtora infestans*) that caused the Great Famine in Ireland. However, we are just starting to understand these ecological processes and the underlying mechanisms are, for the most part, a black box (Keeling and del Campo 2017).

Protists are largely under-described compared to macro-organisms (Pawlowski et al. 2012) which renders estimating their diversity a difficult task. The number of described species has been estimated around 21'000 (Mora et al. 2011), but it is understood that this is only a fraction of their true diversity and that most species have not been described (Pawlowski et al. 2012). Retrieved diversity based on environmental sequencing [Box 3] gives numbers of 110'000 operational taxonomy units (OTUs) in oceans' surface alone (de Vargas et al 2015), and soil protists seem to be even more diverse (Singer et al. 2021), however this still pales in comparison with the several millions of species of protists that have been estimated (Orgiazzi et al. 2016).

[Box 3] Metabarcoding

While DNA barcoding is the sequencing of a DNA fragment in a single organism (to associate it with a genetic "barcode"), metabarcoding is the sequencing of a given barcode in (ideally) all organisms of interest in a given samples, usually after a targeted amplification (Figure 2). It is often referred to as "environmental sequencing" as it often aims at capturing the total diversity in an environment (Burki et al. 2021). Metabarcoding has been made possible by new sequencing technologies, often referred to as high-throughput sequencing (HTS) or next generation sequencing, which can yield millions of sequence reads in one run, Illumina being the tool of choice. These methods are however currently limited producing only short reads up to ca. 500 nucleotide long which have good discrimination power but a weak phylogenetic signal. The marker most-often used in environmental sequencing are the V4 and V9 variable regions of 18s rRNA gene, which are in the sweet spot between being conserved enough across all eukaryotes to allow retrieving most of the diversity with generic primers, but variable enough to allow discriminating taxa with a somewhat good taxonomic resolution. The method can also be adapted to target or exclude specific groups (Williams et al. 2018). One of the main advantages of metabarcoding is that it gives a glimpse of the diversity in a sample without requiring any cultivation or direct observation, which are time consuming and yield a much narrower overview of the diversity as most species cannot be cultivated, are elusive or cannot be distinguished morphologically.

However, sequences generated with metabarcoding cannot be directly interpreted in terms of diversity as, due to intraspecific genetic variability and sequencing errors, a species is often represented by more than one amplicon (Caron and Hu 2019). As a result, HTS amplicons must be processed to be clustered into operational taxonomic units (OTUs) or amplicon sequence variants (ASVs). Both OTUs and ASVs are groups of similar, but distinct, amplicons of which a central, often more abundant sequence is chosen as the representative, however the means of grouping differ significantly between OTUs and ASVs. While OTUs are clusters of amplicons grouped according to a certain threshold, such as a percentage similarity match (e.g., VSEARCH by Rognes et al. 2016) or single nucleotide differences (SWARM by Mahé et al 2014), ASVs are clusters of amplicons based on the modelling and correction of sequencing errors (Callahan et al. 2017). It is known that these methods only approximate the true diversity as they can either pool distinct species together into the same OTU/ASV or yield several OTU/ASV for a single species (Forster et al. 2019). Furthermore, several clades are often not recovered in metabarcoding studies because they are too genetically divergent (Williams et al. 2018). However, even given these limitations, metabarcoding still remains the best tool to assess protistan diversity.

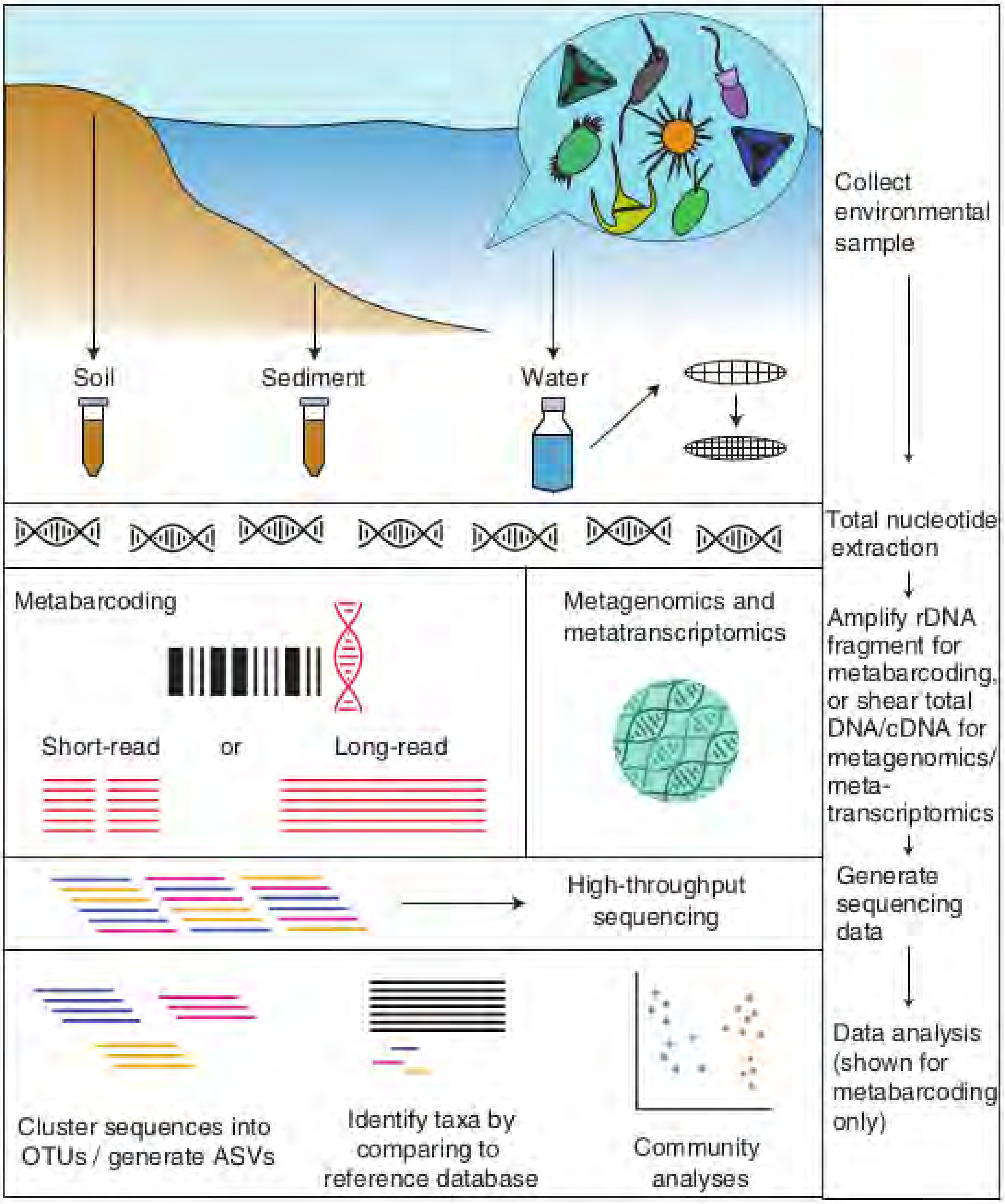

Figure 2: Overview of a classic metabarcoding workflow. Burki et al. 2021

3. Protists as bioindicators

Protists have proved to be potent bioindicators and are commonly used to monitor ecosystems or evaluate past environmental changes over millions of years. Diatoms are commonly used to infer freshwater quality (Lange-Bertalot et al. 2017) and have been proposed to monitor coastal waters as well (Desrosiers et al. 2013); communities of protists, especially ciliates are routinely sampled in wastewater treatment plants to monitor activated sludge (Foissner 2016); marine benthic and planktonic protists such as coccolithophores, radiolarians and foraminiferans are often used as proxies in paleo-climatic studies to infer, among others, past changes in sea surface temperature, oceanic current, primary productivity or atmospheric CO_2 concentration (Abelmann et al. 1999; Garcia et al. 2015; Katsuki et al. 2005; Marino et al. 2014).

However, this concerns only a fraction of the protistan diversity, and protists that are reliably used as proxies are mostly marine or freshwater organisms. Among the plethora of soil protists and their possible uses, only testate amoebae are used somewhat regularly (and not without issues) as proxies to monitor the evolution of peatlands (Amesbury et al. 2016 for an example) and, probably, one of the major reasons that could be invoked for this lack of usage is their deficient taxonomy due to methodological issues related to soil samples.

4. What is taxonomy, its goals, and roles

The term taxonomy is used to designate both the classification system of living organisms and the discipline that establishes this classification. As taxonomy is a fundamental and very broad field in biological science, it is difficult to describe it except in very general terms, but it could be summarized like this: taxonomy aims to define a common language to discuss and understand the diversity of organisms, the patterns of this diversity and its history, by delineating taxa (sets of individuals, e.g., a species) and describing them so as to be able to recognize and communicate about them. As such it is essential for gathering data about any groups of living organisms and accumulate any knowledge in biodiversity science.

While it is indispensable to fundamental biological science, for example to assess the physiology, evolution, ecology, or biogeography of a species, taxonomy also has an important role in more practical fields. With the recognition of the current biodiversity crisis, taxonomy is increasingly recognized as central for conservation. First in a pragmatical way, because it is not possible to protect something that we cannot recognize or understand, and to assess the ecological requirements of a species it is essential to define it correctly (Mace 2004, O'Hara 1994). Then practically because, among others, it allows identifying areas with high level of endemism and in need of protection, it can be used as tool to monitor habitats, and to put in place conservation plans according to the requirements of the ecosystem and its species (Cracraft 2002). Moreover, taxonomic research can have economical and societal benefits by discovering and describing species that, for example, can raise the interest of the public and attract tourism, produce compounds that are of medical or industrial interests, or help developing more resistant and better-yielding crops (Cracraft 2002). An edifying example is the case of Yuan Longping, a Chinese agronomist who, by identifying and collecting unusual hybrids of wild rice in nature, developed new varieties that yielded up to 30% more grains than previous varieties (Normile 1999, Jiming and Longping 2000). This would not have been possible without pre-existing taxonomic knowledge on rice species, as it is what allowed recognizing the opportunity and seizing it when it presented itself.

Unfortunately the taxonomy of protists, especially soil protists, is infamously unstable: how species should be delimited is not clear (Schlegel and Meisterfeld 2003), the vast majority of species are yet to be described (Pawlowski et al. 2012), DNA reference databases are largely incomplete (Keeling and del Campo 2017, Burki et al. 2021), the shortcomings of previous taxonomic efforts (Kosakyan et al. in press) and classification attempts (Adl et al. 2007, Adl et al. 2019) still pollute databases, and their nomenclature is not consistently regulated (Lahr et al. 2012). While it is likely that these issues prevent the usage of more protists in economically relevant processes (see Andersen 1998 for the economic importance of protists), above all these issues prevent protists from being used as reliable scientific tools.

5. A brief history of the taxonomy of protists

The history of protist taxonomy is tightly linked to that of the technology available to microbial naturalists, and major advances in the understanding of microbial diversity often coincided with the appearance of new tools. The majority of protists do not form structures visible to the naked eye, and they had to wait until the 1670's to be observed for the first time by Leeuwenhoek who used his rudimentary microscope to scour water samples (Lane 2015). Their taxonomy originally relied exclusively on the usage of optic microscopy, and protists were classified according to their trophic level (knowable due to the presence or absence of chlorophyll) and their overall morphology (Geisen et al. 2018). Phototrophic species were placed in the algae, whereas the heterotrophic species were placed in the "protozoa" and further classified according to their mode of locomotion into ciliates, flagellates and rhizopods (amoebae). Taxonomic efforts were (and still are) directed towards mostly freshwater and marine species, as soil protists present the additional difficulties that they must be found among the soil particles (Wilkinson et al. 2012), cannot be easily cultivated unlike algae (del Campo et al. 2014) are often very small, inconspicuous, and present fewer morphological characteristics than their aquatic counterparts (see Ernst Haeckel's (1899-1904) drawings of marine protists as an example).

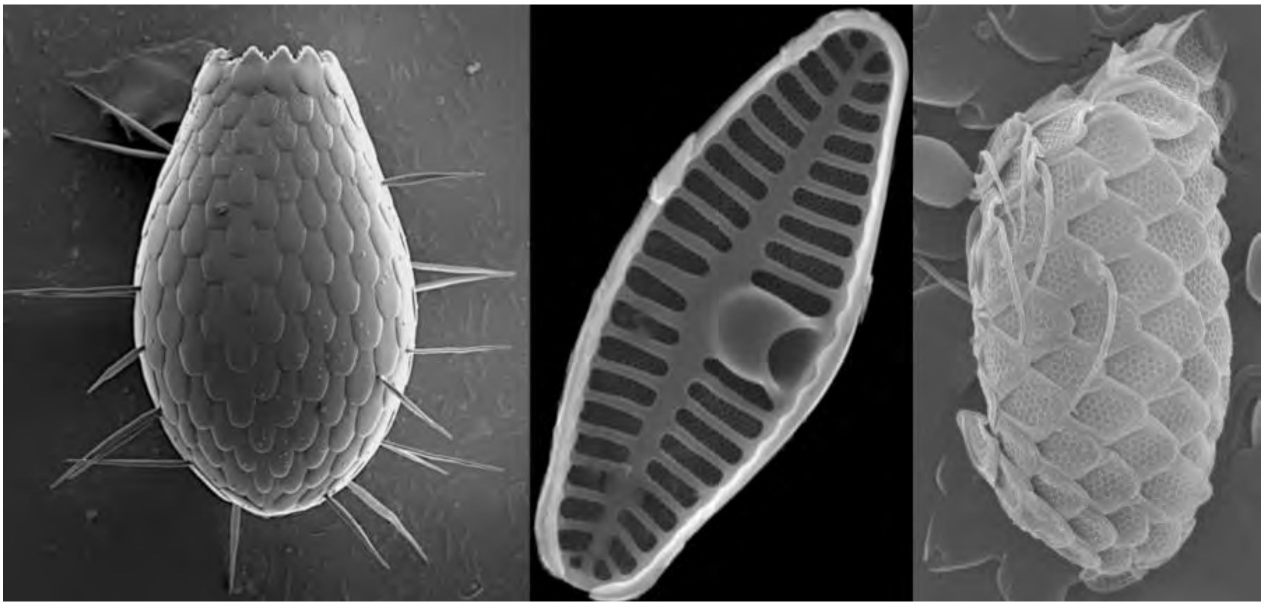

*Figure 3: Scanning electron microscope pictures of a testate amoeba (*Euglypha*), the inside of a diatom (*Planothidium*) and a synurid (*Mallomonas*). Penard.de, diatoms.org, Gusev and Kullkovskly 2020*

The appearance of electron microscopy and staining methods during the 20th century, which allowed the observation of morphological traits that were invisible before, revealed new criteria onto which base taxonomy and allowed distinguishing taxa that were very similar in their overall morphology but differed in minute details. Ciliate taxonomists benefited the most from the development of staining methods, as it reveals the ultrastructure of the ciliary patterns and of the nuclei, both traits being potent taxonomic tools (Abraham et al. 2019, Warren et al. 2017), whereas scanning electron microscopy was especially useful for taxa with hardened shells such as testate amoebae [BOX 4], synurids or diatoms (Figure 3), whose fine morphology has been shown to be useful for species delineation and identification (Coûteaux et al. 1979, Ogden and Hedley 1980, Siver et al. 2015 and Morales et al. 2001).

While the improvement of optic microscopy and the usage of electron microscopy contributed greatly to the taxonomy of protists, it was the appearance of DNA sequencing that revolutionized it (Keeling 2017). DNA barcoding and molecular phylogenies allowed to replace protists in an evolutionary context and revealed, first, that the eukaryotic tree of life is mostly composed of microbial lineages (Burki et al. 2020) and second, that the diversity of macro-organisms to which we are used (plants, animals, and fruiting fungi) are eclipsed by the diversity of the protists (Burki et al. 2021). Additionally, environmental sequencing revealed hyper-diverse clades that were not previously isolated or observed by traditional means like optic microscopy and cultivation (Massana et al. 2014).

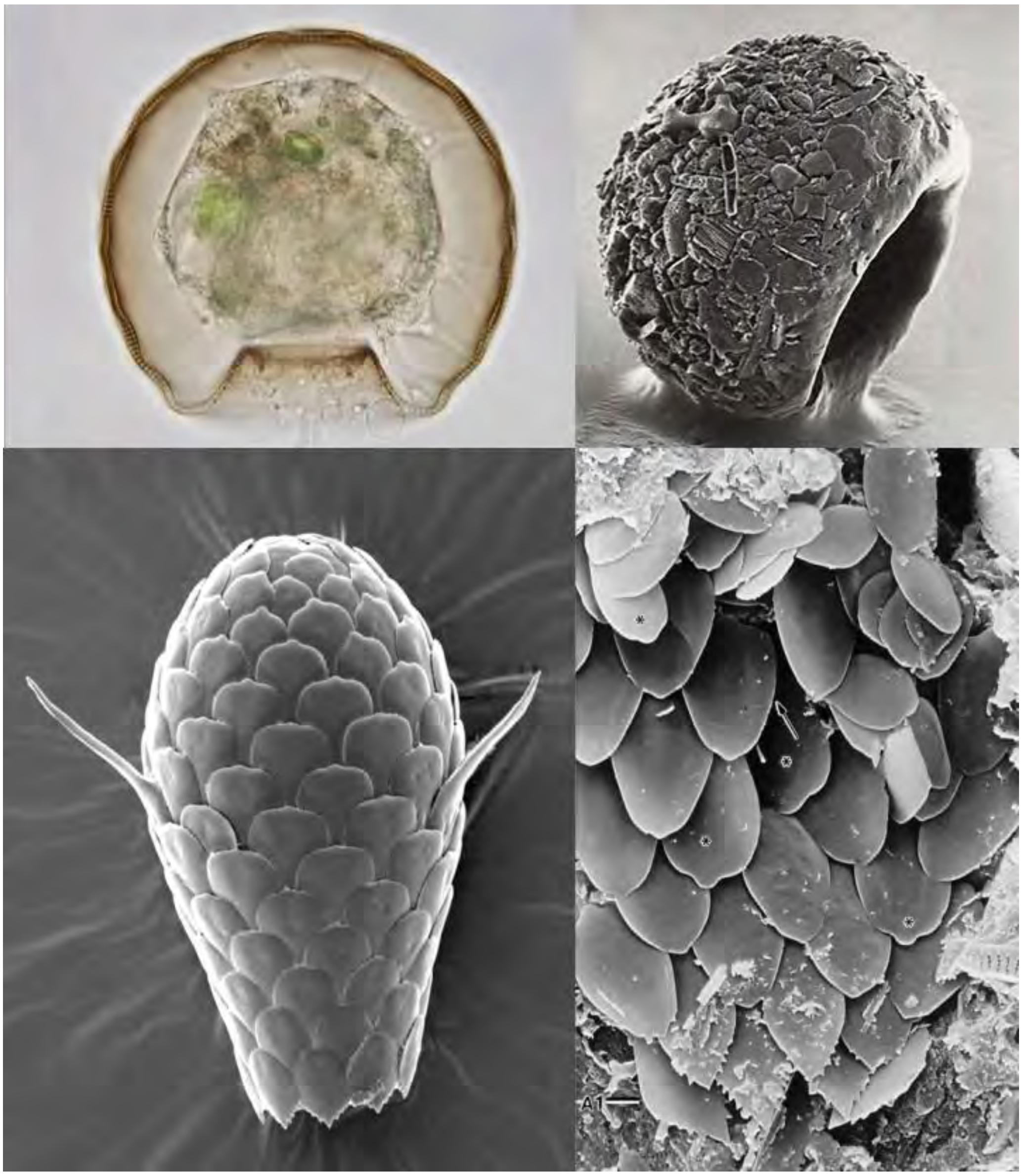

Figure 4: Top left: Arcella mitrata*, the cell inside a proteinic, transparent shell is clearly visible (arcella.nl). Top right:* Cyclopyxis sp. *which builds its shell using soil particles (Ogden and Hedley 1980). Bottom left:* Euglypha sp. *which builds its shell with self-secreted silica scales (Todorov and Bankov 2019). Bottom right: Fossilised scales of euglyphids (Foissner and Schiller 2001).*

[Box 4] Testate amoebae

Testate amoebae (Figure 4) are amoeboid organisms which enclose their single cell in a test (a shell). This test most often has one, sometimes two, pseudostome (i.e., aperture) from which pseudopods (i.e., extensions of the cellular membrane) can protrude for locomotion and feeding. The morphology and dimensions of the test and its aperture are the main taxonomic criteria for identification, and because the test can persist in the environment it can be identified even after the death of the amoeba. The function of the test is not entirely clear, but it is assumed that it provides protection against predators and desiccation. The base material of the test is a proteinaceous layer that can be left bare (as in *Hyalosphenia*), or which can be reinforced with elements secreted by the amoeba (i.e., idiosomes), or extragenous elements gathered from preys or from the environment (i.e., xenosomes). Idiosomes are mostly siliceous or calcareous plates, and xenosomes are mostly diatom frustules or sand grains (Meisterfeld 2002a, Meisterfeld 2002b). Some species can produce tests which harbour both idiosomes and xenosomes (e.g., *Heleopera*).

Because testate amoebae are being grouped together on the base of a very simple morphological trait, the test, they include species which do not share an evolutive history, they are thus polyphyletic and placed in at least three major groups. Most testate amoebae are phylogenetically placed within two orders: the Euglyphida (Rhizaria) and the Arcellinida (Amoebozoa). The Euglyphida are filose (i.e., with thin, needle-like pseudopodes) amoebae of which most species build their test with siliceous idiosomes (Wylezich et al. 2002), whereas the Arcellinida are lobose (i.e., with thick pseudopodes) amoebae (Nikolaev et al. 2005). Every type of shell composition can be found within the Arcellinida, however a given species usually presents only one. The third order (Gomaa et al. 2013), the Amphitremida, harbours filose species characterized by two pseudostomes but are much less frequent. Currently more than 2000 taxa (species and below-species level) have been described, mostly based on morphology alone. However, several of these species have been shown to be complexes of distinct species with sometimes complex distribution patterns (Heger et al. 2013, Singer et al. 2019) or ecology (Singer et al. 2018) and their diversity is likely very much underestimated.

They are present in virtually all ecosystems but are most abundant in moist habitats with a high organic matter content such as humic soil and mosses where they often are the dominant group (Wilkinson and Mitchell 2010, Ogden and Hedley 1980). Despite being highly diversified they form a uniform ecological group as most species are heterotrophic feeding on bacteria or microeukaryotes (mostly fungi and algae), however a non-negligible number of species harbour photosymbionts and are at least partially phototrophic (Wilkinson and Mitchell 2010). Their communities are highly structured by ecological gradients such as soil moisture (Koenig et al. 2017, Harder et al. 2016), pH and nutrient content making them good bioindicators (Meisterfeld 1997, Foissner 1999). Because of their usual abundance in soil and short generation time, they are increasingly being shown to be major actors in ecological processes such as carbon and nitrogen turnover (Schröter et al. 2003) or silica cycling (Aoki et al. 2007).

Due to their ease of observation and manipulation, the testate amoebae are an excellent model group to study the protistan diversity, ecology, and evolution. Most protists are difficult to observe and identify correctly, as they often present very few morphological characters, move quickly (at least under a microscope), need to be stained or to be alive to allow for a correct identification. Conversely, testate amoebae are generally easier to spot, manipulate and identify due to their relatively large test (Wilkinson 2008, Foissner 1999), present morphological traits that are easier to observe (Meisterfeld 2002a), and can be identified even after their death due to their test being preserved in soil, peat, and sediments. Lastly, thanks to their decay-resistant test, testate amoebae are potent proxies for palaeoecological or evolutionary studies. Subfossil tests are often used to infer past ecological conditions in peatlands where peat accumulated for up to thousands of years (Amesbury et al. 2016) or, less frequently, in lacustrine sediments (Ellison and Ogden 1987) while fossils dating up to the Neoproterozoic helped dating events such as marine-freshwater transitions (González-Miguéns et al. 2022) or the appearance of biomineralization (Morais et al. 2017) and eukaryovory (Porter 2016).

6. The issue with past work, as revealed by modern tools

Not only did molecular tools unveil the diversity of protists, but they also revealed the shortcomings of the traditional taxonomic framework and classification. First, it revealed that most major taxonomic groups based on their overall morphology, locomotion and feeding strategy were polyphyletic (the ciliates being a notable exception). This presented a much more complicated view of eukaryotic tree of life, as several clades retrieved by molecular phylogenies were so morphologically and functionally diverse that they were unified by very few shared morphological characteristics and sometimes none at all (Burki et al. 2020).

Additionally, molecular tools revealed that descriptions of morphospecies (species described only based on their morphology) made before the appearance of high-resolution microscopy were often too vague to accurately represent a species and allow a subsequent identification. In many cases, morphospecies were often too genetically diverse to constitute a single species, and DNA sequencing coupled with high resolution microscopy often showed a correlation between genetic differences and minute variations in morphology that were overlooked, suggesting the existence of several species (see Daugberg et al. 1994, Lavau et al. 1997, Bass et al. 2009 and Dumack et al. 2016 as examples). These morphological differences were mostly overlooked because the microscopic size of the protists and some of their diagnostic characteristics were not visible with the technology of the time. However, when morphological variations that could suggest the existence of more than one species were observed, they were often interpreted as plasticity or adaptations to ecological conditions (Chapter 8, revision of the genus *Euglypha*). Furthermore, some morphospecies did not appear as monophyletic at all (Wylezich et al. 2002), and in some extreme cases they were shown to include completely unrelated organisms (Potter et al. 1997, Howe et al. 2009), challenging species delineation based on overall morphology alone [Box 5] (Andersen 1998). This strongly suggested that morphospecies described without the use of modern tools (i.e., DNA sequencing and high-resolution microscopy) should be considered as aggregates of distinct species that can differ significantly in their ecology (Singer et al. 2018), and that a large proportion of the old taxonomic literature is unfortunately unreliable (Adl et al. 2007).

[Box 5] Species concept

How should organisms be grouped and based on what criteria are central questions in taxonomy, and the answers have fluctuated greatly over time. What is a species, the basic unit of taxonomy (Cracraft 2002) and evolution (Mayr 1996), can even be considered as one of the main issues of biology as a whole given the importance of correctly defining a species (de Queiroz 2005; Wheeler and Meier 2000). As a result, several species concepts (i.e., criteria on which to delineate species) have been proposed depending on current tools and knowledge, the more prevalent ones being summarized here.

At the beginning of the modern taxonomic system erected by Linnaeus, the morphological species concept prevailed, and taxa were defined based on shared characteristics, mostly morphological, and organisms were grouped based on their similarity according to these characteristics (Cronquist 1987). Mayr later proposed the biological species concept (though it was anticipated before him by several authors), in which two species can be considered as distinct when they are reproductively isolated (Mayr 1942, 1996). This concept recognizes the existence of “cryptic” or “sibling species”, species that are distinct as they do not interbreed, but are morphologically identical. Due to the existence of many hybrids, Mayr later revised his original definition, considering then that a complete reproductive isolation is not necessary for two species to be distinct as long as they limit gene flows enough to avoid a complete fusion (Mayr 1970). A phylogenetic concept in which species are the smallest monophyletic clusters of similar individuals (Cracraft 1983), and a genetic species concept in which species manifest as clusters of DNA sequences (Hebert et al. 2003a-b) were also proposed. Those have been criticized because they are too vague with unclear delimitation and can lead to mere populations being considered as species (O’Hara 1994). An ecological species concept which aims to delimit species based on their ecological range has also been proposed (Van Valen, 1976). Mayr however argues that, whichever of these species concepts is used to delineate species, they are based on evidences that suggest distinct gene pools and thus reproductive isolation, otherwise these species would not have accumulated differences (morphological, ecological, or genetic) that would have allowed delineation, and as such they are only particular cases of the biological species concept (Mayr 1996). He nonetheless acknowledged that it cannot be directly applied to asexual organisms as each individual should be regarded as a distinct species, but argues that asexual organisms belong to the same gene pool can be considered together as a species.

However, while it was previously considered that the vast majority of protists reproduced asexually, meaning that the biological species concept could not be applied (Schlegel and Meisterfeld 2003), it has recently been argued that protists should be considered as sexual until proven otherwise, as most seem to have a meiotic machinery (Lahr et al. 2011, Hofstatter et al. 2018, Hofstatter et al. 2019). The function of these meiotic genes is however debated and they might be unrelated to sex (Dunthorn et al. 2017).

7. Taxonomy of soil protists is still lagging behind

Compared to aquatic protists, or even other soil microbes such as prokaryotes or fungi, the taxonomy of soil protists is still lagging, and the reasons why are plentiful. The taxonomic literature of many groups is old and scattered (Foissner 2006), many morphospecies have not been re-evaluated using modern tools despite their inadequacy being generally accepted (Adl et al. 2007) and invalid taxon names still pollute databases (Boscaro et al. 2014). The nomenclatural [BOX 6] mess has not been addressed either, and synonyms (different names applied to the same species) and names encompassing distinct (and often only vaguely similar) species are frequent. Furthermore, most of the identification literature has been developed in Europe and North America while other regions are comparatively underexplored, but it is most often this literature that is used for identification worldwide leading to a very biased view of the protistan diversity outside of the Northern Western world (Foissner 2006).

This has several practical consequences. First, as species identification often relies on the subjective interpretation of a mostly old and scattered literature, misidentifications are frequent, which can strongly skew ecological or biogeographical studies (Payne et al. 2010, Mitchell and Meisterfeld 2005, Mitchell et al. 2014), and observations of a species without proofs are unreliable (Foissner 2006). Additionally, as this old literature does often not allow discriminating closely related species, this has led to strongly underestimating the protistan diversity and to a biased view of their ecology and biogeography, such as the presumed absence of biogeographical patterns (see Finlay and Esteban 1998, Finlay and Clarke 1999, Finlay et al. 1999).

Also, because most species of protists remain to be characterized using molecular tools and the coverage is very uneven between taxonomic groups, it is difficult to make sense of metabarcoding data which is the current tool of choice to study protist communities (Keeling and del Campo 2017). This is especially true for studies focused on soil protists where most environmental sequences match only loosely with databases (Mahé et al. 2017), rendering unreliable their taxonomic assignation and the interpretation of the diversity and functions of the organisms behind the DNA sequences (Geisen and Bonkowski 2018).

Finally, the issues with the old taxonomic literature render taxonomic revisions excruciating, as a significant amount of time has to be dedicated to compiling all past descriptions, and as such hinders any tentative to improve the situation (Godfray

[Box 6] Nomenclature

As taxonomy depends on the judgement of researchers, it is subjective. However, nomenclature, i.e., what name should be attached to a taxon, obeys strict and objective rules (ICZN 1999). Together those rules are often referred to as the code of nomenclature or just “the code”, whose goal is to preserve the stability of the naming of taxa and avoid any confusion by ensuring the uniqueness of the scientific name of any taxon. Most protists are treated by the International Code of Zoological Nomenclature (the “zoological code”) which is regulated by the International Commission on Zoological Nomenclature (both are often referred to as ICZN), however some photosynthetic protists such as green algae and diatoms are treated by the International Code of Nomenclature for algae, fungi, and plants (the “botanical code”). As those two codes are independent, it must be noted that the same name can be used twice if the two taxa in question are not treated by the same code. While the code includes some provisions on the naming of new species, most of it is dedicated to ensuring the continuity when a taxon is revised and its scientific name changes (e.g., when a species is moved into another genus) or when it is described twice (Mallet and Willmott 2003). It is important to understand that taxonomy and nomenclature are two distinct matters, and that nomenclature only regulates the technicalities of the naming process without interfering with the classification work itself. Furthermore, the code is not inflexible, and exceptions can be made when the application of its rules would invalidate a name that prevails in usage but is technically invalid. During this thesis each taxonomic action were done accordingly to the International Code of Zoological Nomenclature.

2002). This is something that is not unique to protistology, but it is very likely more prominent in this field. It is thus essential to build a sound taxonomic framework in order to properly delineate species, or at least ecologically and evolutionary consistent units that can be identified reliably, so as to allow the development of protists as a research tool.

8. The goals of this thesis

The aim of this thesis is to cover the several steps required to build a sound taxonomic framework so as to be able to use soil protists reliably in ecology, biodiversity or evolution studies, with a focus on testate amoebae. As such, I aimed during my PhD, first, to improve the taxonomy of testate amoebae using molecular phylogeny and high-resolution microscopy. An important part is the revision of the past taxonomic work and the reassessment of its validity. Then I explored how these results and techniques could be used in larger studies to assess the diversity and evolutionary patterns of protists. My thesis chapters and its appendices can be separated into three major themes:

1) Re-characterization of known taxa using modern tools and revision of their taxonomic history and nomenclature (Chapters 1, 2, 3, 8; Appendix A)

2) Description of new taxa (Chapters 4, 5, 8; Appendices B, F)

3) Use taxonomic by-products to assess the ecology, diversity and evolution patterns of soil protists (Chapters 9, 10; Appendices B, C, D, E, G, H)

Summary of chapters

1. En garde! Redefinition of *Nebela militaris* (Arcellinida, Hyalospheniidae) and erection of *Alabasta* gen. nov. - published

The position of *Nebela militaris* within the genus *Nebela* was well accepted despite its elongated test and curved pseudostome that is not present in other species of this genus. Here based on a phylogenetic reconstruction on the mtCOI gene we show that it is genetically distinct from other species of the genus *Nebela* and cannot be placed in any other genera of Hyalospheniidae. We thus erected the genus *Alabasta* to accommodate this species.

Designed and supervised the laboratory and optic microscopy work, made the phylogenetic reconstruction, wrote the taxonomic revision, made the figures, co-wrote the manuscript with Quentin Blandenier.

2. Case 3782–*Nebela militaris* Penard, 1890 (Arcellinida, Hyalospheniidae): proposed conservation of the specific name by giving it precedence over *Nebela bursella* Taranek, 1881 - published

During the revision of the taxonomy of *Alabasta (Nebela) militaris*, we discovered that it was first described under the name *Nebela bursella*. According to the principle of priority of the ICZN the name *Nebela bursella* should be considered as the valid name for the species even if *Nebela militaris* was the only name in usage. Here we provide a complete review of the taxonomic history of this species and ask the ICZN to use its plenary power to conserve the well-known name *Nebela militaris* over *Nebela bursella*.

Did the taxonomic revision, wrote the manuscript.

3. Superficially described and ignored for 92 years, rediscovered and emended: *Apodera angatakere* (Amoebozoa: Arcellinida: Hyalospheniformes) is a new flagship testate amoeba taxon from Aotearoa (New Zealand) - published

Based on high resolution microscopy and DNA barcoding we redescribed *Nebela pernadi* (Brehm, 1928), a conspicuous species of testate amoebae from Aotearoa (New Zealand) that was poorly described under an invalid name and overlooked for almost a century. We renamed it *Apodera angatakere* with the help of Māori Language Commission and discuss its potential as flagship species for island biodiversity conservation.

Participated in the sampling, designed and participated to the laboratory work, did the morphometric analyses, did some figures, co-wrote the manuscript with Edward Mitchell.

4. Description of *Phaeobola aeris* gen. nov., sp. nov (Rhizaria, Cercozoa, Euglyphida) Sheds Light on Euglyphida's Dark Matter (second author) - published

Here we describe *Phaeobola aeris*, a species of euglyphid testate amoebae that lack silica scales. As it is inconspicuous and might be easily overlooked by protistologists, and because of its diverging 18S rRNA sequence that might escape metabarcoding studies, we further discuss its belonging to a potential "dark matter" of testate amoebae, an unseen but diverse group that could make up for the majority of euglyphids.

Did the phylogenetic reconstruction, made the phylogenetic tree figure, co-wrote the manuscript with Kenneth Dumack and Enrique Lara.

5. *Hyalosphenia papilio paynei subsp. nov. - a highly consipicuous, likely recently evolved and localized Sphagnum peatland testate amoeba*

In this study we describe *Hyalosphenia papilio* subsp. *paynei*, an unusually broad form of *H. papilio* known only from two peatlands, one in Wales and one Ireland, where the regular *H. papilio* is absent. We furthermore explore the factors behind its unusual morphology, its current distribution and the rarity of the regular *H. papilio* in the peatland where it was found.

Designed and supervised the laboratory and optic microscopy work, made the phylogenetic reconstruction, did the taxonomic act, wrote the manuscript.

6. *Certesella larai* (Amoebozoa: Arcellinida: Hyalospheniformes) a new soil testate amoeba species from the Dominican Republic and Chile challenges the definition of genera *Certesella* and *Porosia* (second author) - published

Here we describe *Certesella larai*. Most specimens of this new species lack the characteristic internal teeth that distinguish the genus *Certesella* from *Porosia* and thus questions the validity of the distinction between the two genera. Moreover, as *Certesella* has never been found North of the Cancer tropic while *Porosia* is found worldwide, we call for phylogeny based taxonomical work to be directed towards these genera, as a phylogenetic reconstruction could shed light on both their relation and their distribution patterns.

Participated to the morphometry analyses, did the taxonomic assignation, made some figures, co-wrote the manuscript with Edward Mitchell.

7. Comment on “Amoebae Assemble Synthetic Spherical Particles to Form Reproducible Constructs” - published

This is a comment on a publication that misidentified its model organism, which happened to be a testate amoeba. As the taxonomy of testate amoebae is fast evolving and misidentification is frequent, this comment was published with the goal to avoid identification errors from spreading in the scientific literature.

Wrote the manuscript.

8. Put away during the 70s’, the disco ball is back. A taxonomic revision of the genus *Euglypha*.

Based on the morphological and molecular characterization of 41 strains and wild populations in addition to an extensive review of the taxonomic literature, we propose a complete revision of the testate amoeba genus *Euglypha*.

Characterized 12 strains morphologically and molecularly, performed the review of the literature, made the phylogenetic analyses, made the figures, wrote the manuscript.

9. High-throughput sequencing of litter and moss eDNA reveals a positive correlation between the diversity of Apicomplexa and their invertebrate hosts across alpine habitats (co-first author) - published

The Apicomplexa are a group of parasitic protists whose diversity is thought to reflect the diversity of their invertebrate hosts in the Swiss Alps. Here we compare the genetic diversity of Apicomplexa and Metazoa in litter and moss samples from the Furka Pass to discuss the use of metabarcoding as a tool to explore host-parasite interactions.

Processed the metabarcoding data from the raw sequences up to the statistical analyses (not included, this step was carried out by David Singer), did the phylogenetic reconstruction, did the phylogenetic placement, made the figures, co-wrote the manuscript with David Singer.

10. Generalized diversification of euglyphids during the Paleocene and Eocene

Here we analysed the magnitude and timing of the diversification rate of self-silicifying amoebae (Euglyphida: Rhizaria), a group of mostly terrestrial micro-organisms. A time calibrated phylogeny revealed a significant increase in euglyphid diversification rate between 60 and 50 million years ago during a period when global temperature peaked and decreased several times.

Did the reference phylogenetic tree and alignment, the phylogenetic reconstruction, the molecular clock analysis, the placement of short reads on the phylogenetic tree, the diversification analyses, the figures, wrote the manuscript.

Summary of appendices

Peer-reviewed publications

A. A taxonomic monograph of hyalospheniid testate amoebae (Amoebozoa: Arcellinida: Hyalospheniformes)

This is a taxonomic revision of the infra-order Hyalospheniformes.

Made sure the taxonomic acts were done in accordance with the ICZN, actively participated in the writing.

B. Dispersal limitations and historical factors determine the biogeography of specialized terrestrial protists

It is usually considered that distribution patterns of micro-eukaryotes are not shaped by dispersal limitation and thus fundamentally different from that of macro-organisms. Based on both pre-existing and new data we studied the phylogeographical patterns in *Hyalosphenia papilio*, a testate amoeba restricted to Holarctic peatlands. Here we show that its current distribution pattern can be explained by dispersal limitation and the contraction and expansion of its habitat, adding new evidence that the distribution patterns of micro and macro-eukaryotes are, at least partially, shaped by the same processes.

Did some phylogenetic analyses, actively participated in the writing.

C. The testate amoebae of New Zealand: A checklist, identification key and assessment of biogeographic patterns

The diversity of Aotearoa (New Zealand) micro-eukaryotes is only poorly documented compared to that of macro-organisms, despite knowledge of the existence of endemic microbial species. This study includes a checklist of the species of testate amoebae of New Zealand based on all written records of testate amoebae since the early 20th century, and a visual identification key.

Actively participated in the elaboration of the online identification key, reviewed the manuscript.

D. Phylogenetic divergence within the Arcellinida (Amoebozoa) is congruent with test size and metabolism type

This study combined phylogenetics and morphometrics to assess the phylogenetic signal of morphological traits within species of the genus *Difflugia*.

Supervised the molecular laboratory work with Quentin Blandenier, the phylogenetic reconstruction and reviewed the manuscript.

E. Assessing the ecological value of small testate amoebae (< 45 µm) in New Zealand peatlands

Small testate amoebae are often not taken into account in ecological surveys, and when they do, they are usually lumped into broad categories reducing considerably their potential as bioindicators. Here we studied the ecological value of these species by comparing their signal in analyses where they were either lumped into categories or considered separately. We showed that small species of testate amoebae add ecological information that are not conveyed by bigger species, and that lumping them into categories decreases the quality of the signal.

Participated in the writing, reviewed the manuscript (mostly the taxonomy).

F. *Mycamoeba gemmipara* nov. gen., nov. sp., the First Cultured Member of the Environmental Dermamoebidae Clade LKM74 and its Unusual Life Cycle

Here we describe *Mycamoeba gemmipara*, the first species of the environmental Dermamoebidae (Amoebozoa) clade LKM74 to be cultured and morphologically characterized. We also present its unusual life cycle which include yeast-like buds and ramifications, which had previously not been observed in other species of Amoebozoa.

Took the pictures of the trophozoite stage of *Mycamoeba gemmipara*, reviewed the manuscript.

G. Protist taxonomic and functional diversity in soil, freshwater and marine ecosystems

This is a comparative study of the protist diversity in soil, freshwater and marine habitats.

Participated in the drafting of the manuscript in the original stages of the writing, reviewed the manuscript.

H. Deconstructing *Difflugia*: the tangled evolution of lobose testate amoebae shells (Amoebozoa: Arcellinida) illustrates the importance of convergent evolution in protist phylogeny

This study challenges the presumed morphological stasis in testate amoebae and shows an important variability between species of family Difflugiidae

Contributed DNA sequences, reviewed the manuscript.

Non peer-reviewed publications

- **State of knowledge of soil biodiversity–Status, challenges and potentialities. Summary for policy makers. GSBI, S. (2020).**

As a contributing author. Contributed pictures of testate amoebae and text box 2.3.5 | Testate amoebae.

Chapter 1 - En garde! Redefinition of *Nebela militaris* (Arcellinida, Hyalospheniidae) and erection of *Alabasta* gen. nov. (published)

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Keywords

Amoebozoa; Biometry; DNA barcoding; Molecular phylogeny; Protist; Taxonomic revision

Abstract

Molecular data have considerably contributed to building the taxonomy of protists. Recently, the systematics of Hyalospheniidae (Amoebozoa; Tubulinea; Arcellinida) has been widely revised, with implications extending to ecological, biogeographical and evolutionary investigations. Certain taxa, however, still have an uncertain phylogenetic position, including the common and conspicuous species *Nebela militaris*. A phylogenetic reconstruction of the Hyalospheniidae using partial sequences of the mitochondrial Cytochrome Oxidase Subunit 1 (COI) gene shows that *N. militaris* does not belong to genus *Nebela*, but should be placed in its own genus. The morphological singularities (strongly curved pseudostome and a marked notch in lateral view) and phylogenetic placement of our isolates motivated the creation of a new genus: *Alabasta* gen. nov. Based on their morphology, we include in this genus *Nebela kivuense* and *Nebela longicollis*. We discuss the position of genus *Alabasta* within Hyalospheniidae, and the species that could integrate this new genus based on their morphological characteristics.

Introduction

Testate amoebae are ubiquitous microorganisms in many terrestrial and freshwater to brackish aquatic environments (Meisterfeld 2002; Tsyganov et al. 2016). Their ecological sensitivity, key functional role in microbial food-webs (Jassey et al. 2012), and the good preservation of their test in sediments and peat designates them as excellent bioindicators for present and past environmental conditions (Amesbury et al. 2016; Mitchell et al. 2008), ecotoxicology (Amacker et al. 2018), and even forensic science (Seppey et al.

2016). Clear taxonomy is a prerequisite for sound ecological work. However, most original descriptions of testate amoeba species were based only on morphological characters (e.g., test composition, test and pseudostome dimensions) and the validity and phylogenetic position of many taxa are currently unclear. On one hand, several studies have revealed cases of cryptic or pseudo-cryptic diversity and proved that morphological differences of the shell indeed corresponded to distinct species (Dumack et al. 2016; Heger et al. 2011; Kosakyan et al. 2013; Singer et al. 2015). On the other hand, phenotypic plasticity of the test has also been demonstrated experimentally (Mulot et al. 2017; Wanner 1999; Wanner and Meisterfeld 1994).

Hyalospheniidae (Amoebozoa, Arcellinida) are a widespread family of testate amoebae. They build their test from a proteinaceous matrix (*Hyalosphenia*) (Meisterfeld 2002), which can be reinforced by self-secreted siliceous plates (*Quadrulella*) or by small silica elements taken from preys (e.g., *Nebela*, *Padaungiella*, etc.), a process referred to as kleptosquamy (Lahr et al. 2015). They are mostly found in forest litter (Krashevska et al. 2018), fens and *Sphagnum* dominated peatlands (Singer et al. 2018), but also in more extreme environments like cryptogamic crusts in arid environments (Pérez-Juárez et al. 2017). They have been a key element in the debate over protist cosmopolitanism, showing several examples of biogeographic “flagship species”, i.e., organisms with a conspicuous morphology only found in a limited part of the world (Heger et al. 2011; Smith et al. 2008; Smith and Wilkinson 2007). Molecular approaches on Hyalospheniidae opened new horizons in microbial biogeography; indeed, cryptic species showed non-overlapping geographic distribution areas (Heger et al. 2013), disproving the cosmopolitan hypothesis, for testate amoebae at least. Cryptic species also showed diverging ecological preferences for different micro-niches in Sphagnum peatlands (Singer et al. 2018), thus further demonstrating the need for improved taxonomy. Consequently, there is currently an active effort to do a systematic revision of testate amoeba taxonomy using combined molecular and morphological approaches (Lahr et al. 2017).

The first molecular surveys of arcellinid testate amoebae were focused on the Small Subunit Ribosomal RNA (18S rRNA) gene and permitted to place the Hyalospheniidae among the Arcellinida (Nikolaev et al. 2005). As the 18S rRNA gene is very conservative (Pawlowski et al. 2012), it is only of limited use for species delineation in testate amoebae. Other markers based on mitochondrial genes (Blandenier et al. 2017; Hebert et al. 2003) were developed to overcome this limitation. The mitochondrial Cytochrome Oxidase Subunit 1 (COI) gene was shown to be useful to explore the cryptic diversity of hyalospheniid species and to resolve phylogenetic relationships within the family (Kosakyan et al. 2013). This resulted in splitting genus *Nebela*, the most species-rich genus of the family, into genera *Padaungiella* (Kosakyan et al. 2012), *Gibbocarina*, *Planocarina*, *Cornutheca*, *Longinebela* and *Nebela* (sensu stricto) (Kosakyan et al. 2016).

However, the taxonomic validity and phylogenetic position of many hyalospheniids, including several common taxa, remain unclear. Our focus here is on *Nebela militaris*, an emblematic species in peatlands, where it typically occurs in relatively dry microhabitats (Sphagnum hummocks) with generally low pH (Diaconu et al. 2017; Väliranta et al. 2012). Its characteristic morphology and good preservation in peat designates it as a valuable bioindicator in ecological and palaeoecological studies of peatlands (Amesbury et al. 2016; Mitchell et al. 2008). Despite its unmistakable morphology, several morphologically similar taxa have been described, but as these descriptions are lacunar (e.g., poor biometry or illustrations, lack of differential diagnosis) their validity is unclear. Although *N. militaris* clearly belongs to the Hyalospheniidae, its morphological characters do not allow a clear assignation to one of the genera as defined by Kosakyan et al. (2016). Its phylogenetic position among the Hyalospheniidae thus remains mysterious. Here, we characterized isolates of *Nebela militaris* from a Swiss peat bog based on detailed morphological observation and a single cell barcoding approach and we clarify its phylogenetic position and propose the new genus *Alabasta*.

Material and Methods

Single cell isolation

Cells corresponding to the original description of "*Nebela militaris*" (Penard 1890) were isolated from *Sphagnum* mosses in a wooded peatland (*Pinus mugo uncinata*) in the Swiss Jura Mountains (Le Cachot bog, 47°00′15.23′′N, 6°39′52.83′′ E). Testate amoeba cells were extracted from ca. 30 g of fresh *Sphagnum* after filtration through a 200 μm mesh filter. Cells were isolated individually under an inverted microscope (Olympus IX81) with a narrow pipette. Light microscopy pictures were taken at 600× magnification (Fig. 1) and morphometric characteristics (length, breadth and width of the aperture) were measured on 20 isolated cells (Fig. 2).

DNA extraction

We used a specific procedure developed to extract DNA from a single cell of testate amoeba adapted after Chomczynski and Sacchi (1987). We prepared a thiocyanate guanidinium (TG) solution with the following protocol: We first dissolved 60 g of TG in 20 ml of EDTA (0.5 M, pH 8) and 20 ml of H2O under agitation at 65 °C then when the solution reached room temperature, we added 5 ml of Sarkosyl 10% (Na N-lauroylsarcisinate). Finally, the solution was topped up to 100 ml with H_2O and filtered at 0.2 μm before being stored in the dark.

Each single cell was rinsed several times with distilled water to eliminate contaminants and then transferred into individual PCR tubes containing 50 μl of TG solution. The tubes were heated at 65 °C during 30 min, then 50l of isopropanol was added and the tubes then left at −20 °C during 12 h. The cleaning step of the DNA consisted of a first centrifugation at 15,000 rpm during 20 min. The supernatant was removed before two additional washing steps with 180 μl of ethanol (70% and 99% respectively, 15,000 rpm during 5 min). The residual ethanol is evaporated during 2 h under a fume hood.

PCR amplification and DNA sequencing

The PCR were processed by adding the components and reagents directly into the PCR tubes used for the extractions. Partial sequences of the mitochondrial COI gene were obtained using a nested PCR protocol. The first PCR was conducted using the Arcellinida-specific forward primer ArcelCox (CAA AAT CAT AAA GAT ATT GGD AC) (Kosakyan et al. 2012) and the eukaryote-general reverse primer HCO (TAA ACT TCA GGG TGA CCA AAA AAT CA) (Folmer et al. 1994). The PCR conditions were: Denaturation step at 95 ◦C for 5 min, then 45 cycles with a denaturation step at 95 ◦C for 15 s, an hybridization step at 43 ◦C for 15 s, an elongation step at 72 ◦C for 1 min, and a final elongation at 72 ◦C for 10 min. The second PCR was done using hyalospheniid-specific primers HPCOIF (GTT ATT GTT ACT GCT CAT GCC) and HPCOIR (ATA CAA AAT AGG ATC ACC TCC ACC) (Gomaa et al. 2014) with the following conditions: Denaturation step at 95 ◦C for 5 min, then 40 cycles with a denaturation step at 95 ◦C for 15 s, an annealing step at 55 ◦C for 15 s, an elongation step at 72 ◦C for 1 min, and a final elongation at 72 ◦C for 10 min. PCR products were purified using a Millipore kit and sequenced with an ABI3730XL DNA sequencer (Applied Biosystems) at Macrogen, Amsterdam NL. Sequences were deposited in GenBank with the following accession numbers: MH616621–MH616624. Light microscopy pictures of the DNA barcoded cells are shown in Fig. 1.

Phylogenetic analysis

The sequences obtained were 482 nucleotides long and were aligned using BioEdit (Hall 1999) with an exhaustive reference database composed of COI sequences belonging to the Hyalospheniidae family in the

GenBank database (Heger et al. 2013; Kosakyan et al. 2012, 2013, 2016; Pérez-Juárez et al. 2017; Singer et al. 2015; Qin et al. 2016). Phylogenetic reconstruction was conducted using the CIPRES Portal (Miller et al. 2010). A maximum likelihood phylogenetic tree was built using the RAxML v.8.2.10 algorithm (Stamatakis, 2014) with the GTR + GAMMA + INVARIANT model and 1000 bootstraps. A Bayesian reconstruction was built with MrBayes (Ronquist and Huelsenbeck 2003) using the GTR + GAMMA + INVARIANT model and ran on two independent chains for 200,000 generations sampled every 100 generations resulting in 4,000 trees, of which 25% were discarded as the burn-in. The trees were rooted using genera *Alocodera* and *Padaungiella* as outgroup based on previous Hyalospheniidae phylogenies (Kosakyan et al. 2016).

Results and Discussion

The morphology of the studied cells is congruent with the original description of "*Nebela militaris*"

The cells described in this study correspond well to the original description of *Nebela militaris* (Penard 1890). They have the same morphology (hyaline, yellowish or colourless and rigid test) and the shape of an ancient cannon (hence the species name given by Penard). The test is proteinaceous and can incorporate silica scales taken from prey. Lateral pores are frequently observed in the first anterior third of the test. The pseudostome is fan-shaped i.e., strongly curved in broad view, with a notch in narrow view, and shows a flare just before the pseudostome (Fig. 1A–D). We recorded the following measurements: Length: 61–77.5 µm (mean 67.9 µm), width: 31.5–53 µm (mean 37.7 µm), aperture width (pseudostome long axis): 15.5–20.5 µm (mean 18.1 µm) (Fig. 2, Table 1). However, our isolates differ slightly from the original description because of their wider test (Fig. 2, Table 1). Also, Penard (1890) originally described the pores on each side of the pseudostome to be infrequent but later stated that they are present in most cases (Penard 1902) which is confirmed by our observations. It is possible that the pores were not visible with the microscope Penard used for the original description as they can be difficult to observe. Nevertheless, a thorough comparison between the original permanent slides made by Penard (pictures available in Wikimedia Commons: https://commons.wikimedia.org/wiki/Category: *Nebela militaris*) and the investigated cells confirm that those isolates are the same morphospecies. Moreover, the original types were isolated from the same environment, i.e., Sphagnum dominated peatland of the Swiss Jura Mountains (Penard 1890). We thus consider our isolates to belong to the same taxon as "*Nebela militaris*" described by Penard.

Clarification of the taxonomic status of *Nebela militaris*

The taxonomic history behind *Nebela militaris* is rather complicated as *N. militaris* is morphologically similar to two other taxa: *Nebela bursella* and *N. americana* var. *bryophila*. It is thus unclear whether *N. militaris*, *N. bursella* and *N. americana* var. *bryophila* represent distinct taxa and the confusion encompassing those names comforts the idea that they are merely the same morphospecies.

Nebela bursella was originally described by Taranek (1881) as *Nebela bursella* Vejdovsky, described again by Vejdovský (1882) and was later reported in Taranek's monograph (1882). In this monograph, the illustrations that Taranek includes in the description of *N. bursella* are confusing, since some of them clearly correspond to the two first descriptions of *N. bursella* made by himself and Vejdovsky while other depicted specimens are ambiguous.

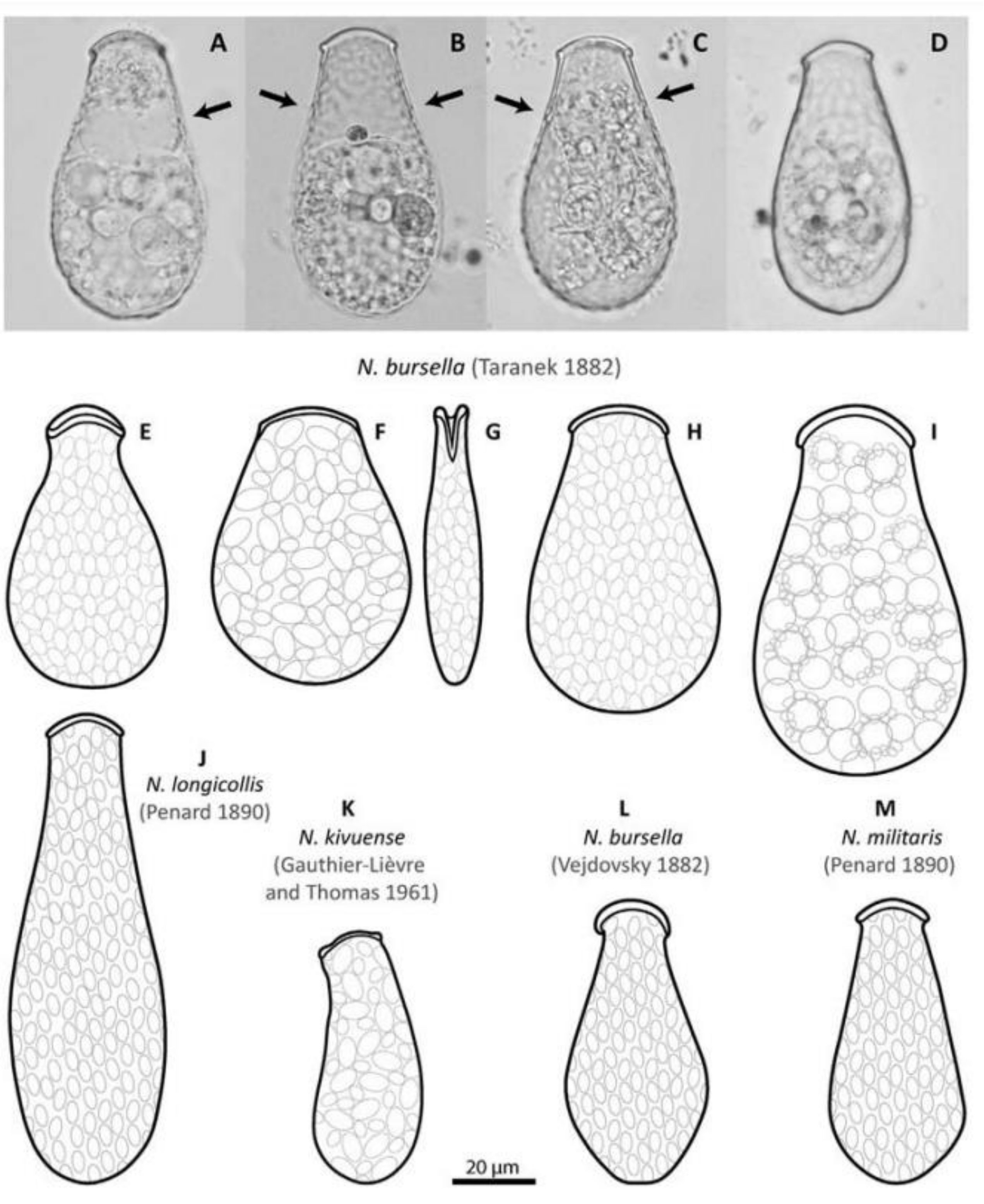

Fig. 1. A–D Light microscopy pictures of the barcoded specimens of Alabasta militaris *(GenBank accession number A: MH616621; B: MH616622; C: MH616623; D: MH616624). The arrows point to the position of the lateral pores. E-I Reproduction of* Nebela bursella *sensu Taranek as it appears in Plate III Fig. 8, 9, 10, 7 and Plate IV Fig. 16 (Supplementary material), respectively, in Taranek (1882). J-M Reproduction of* N. longicollis*,* N. kivuense*,* N. bursella*, and* N. militaris*, respectively, as they appear in their original description. The size of E-I and L was approximated as no formal scale was given in the original drawing, but the proportions of E-I were kept. Scale bar = 20 µm*

Indeed, while two illustrations (Plate III Fig. 8 and 12 by Taranek (1882) (Supplementary material 1), Fig. 8 here reproduced as Fig. 1E) resemble Vejdovsky′′ s and Taranek's original drawings of *N. bursella* with its characteristic curved pseudostome, the other illustrations correspond to specimens that are clearly broader. Based on modern knowledge of Hyalospheniidae systematics, these illustrated specimens should rather be assigned to another species within genus *Nebela* (Plate III Fig. 7 and Plate IV Fig. 16 by Taranek (1882) (Supplementary material 1), reproduced here as Fig. 1H, I respectively) or, possibly even, given the slit-like aperture, to genus *Heleopera* (Plate III Fig. 9–11 by Taranek (1882), reproduced here as Fig. 1F,G), resulting in one description possibly corresponding to three species. This situation led to the misinterpretation that *N. bursella* corresponded to the broader morphotype rather than to the cells illustrated by Vejdovsky. This error was not corrected and *N. bursella* was ultimately considered as a synonym to *Nebela tincta* (Awerintzev 1906). In his description of *N. militaris* Penard (1890) states that his new species could be considered by some as mere aberrant specimens of *N. bursella*, thus suggesting that he too was misled. However, unfortunately Penard did not explain what features could be considered as aberrant. He actually based his concept of *N. bursella* on Taranek's erroneous lumping and thus confused it with *Nebela tincta* (see Penard 1902). Penard then described *N. militaris*, being certain that it was not only an aberrantly slender form of *N. tincta*, resulting in the redescription of *N. bursella* as *N. militaris*.

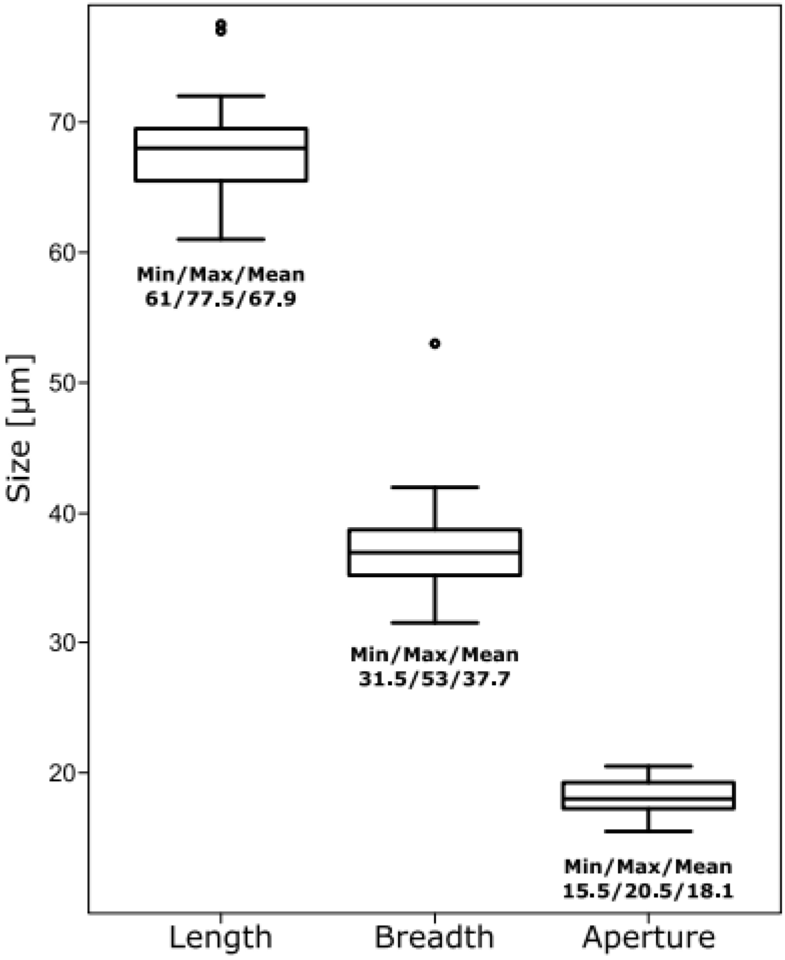

Fig. 2. Length, width and aperture of Alabasta militaris *(n = 20) taken from the same population as the four barcoded specimens (Le Cachot peatland, Swiss Jura Mountains).*

Nebela americana var. *bryophila* Van Oye (1933) was described as a smaller morphospecies of *Nebela americana* (Taranek 1882). The dimensions and shape of the test are comparable to *N. militaris* but Van Oye (1933) noted the absence of lateral pores on his isolates. As the pores remain difficult to observe, we consider that the original description of *Nebela americana* var. *bryophila* is too lacunar to allow distinguishing it from *N. militaris* and we consider them as synonyms.

For these reasons, we propose to synonymize *N. militaris*, *N. bursella* and *N. americana* var. *bryophila*. Although we are aware that the name *Nebela bursella* precedes the others and thus should prevail, *N. militaris* has been consistently used since Penard's description and is well known to the scientific community working on testate amoebae. Moreover, we performed an extensive literature search and found no report for *N. bursella*, after 1964 (Sudzuki 1964). For these practical reasons, we will invoke article 23.9.3 of the International Code of Zoological Nomenclature to keep *N. militaris* as the valid name even though it is technically a junior synonym.

Erection of the genus *Alabasta* and its phylogenetic position within the Hyalospheniidae

We obtained four identical partial COI sequences of 482 nucleotides from four different cells. The phylogenetic reconstruction does not place our sequences within the genus *Nebela*, but rather as a sister clade of the genus *Planocarina* (Fig. 3), this position being weakly supported (bs 48, pp 0.97). Moreover, the genetic distance between our sequences and the genetically closest species in genus *Nebela* (i.e., *N. babellulum*, 18% of dissimilarity) is well above the barcoding gaps calculated for Hyalospheniidae and Amoebozoa in general (Kosakyan et al. 2012; Nassonova et al. 2010; Singer et al. 2018). Our isolates cannot be ascribed to *Planocarina* either due (1) to the genetic distance (ca. 20% of dissimilarity with *P. marginata*), and (2) by the fact that genus *Planocarina* is characterized by the presence of a keel on the posterior part of the test (Kosakyan et al. 2016), whereas it is absent in *N. militaris*. This justifies the creation of a novel genus for *N. militaris* here-after named *Alabasta* that incorporates species with an elongated test and strongly curved pseudostome with a flare and a marked notch in narrow view.

Species to include in the genus *Alabasta* gen. nov.

Two species fit the morphological description of genus *Alabasta*: *Nebela kivuense* (Gauthier-Lièvre and Thomas 1961) and *N. longicollis* (Penard 1890). We thus formally move those species to genus *Alabasta*.

Alabasta (*Nebela*) *kivuense* (Fig. 1K) was described by Gauthier-Lièvre and Thomas (1961) from an area near Lake Edward in the Democratic Republic of Congo and was recently observed in two wetland-coniferous forest ecosystems in southern Ontario, Canada (Nicholls 2015). *A. kivuense* may potentially be mistaken for *Alabasta* (*Nebela*) *militaris* as both species having overlapping length (Table 1), similar narrow piriform tests and deeply curved pseudostome, however the pseudostome of *A. kivuense* is less curved than *Alabasta militaris* (Fig. 1).

Alabasta (*Nebela*) *longicollis* (Fig. 1V) has been reported only on a few occasions, albeit from several geographical regions worldwide. *Alabasta longicollis* is larger and more elongated than *A. militaris* (almost twice as long and slightly wider, Table 1).

As the original descriptions present clear morphological distinctions between *A. militaris*, *A. kivuense* and *A. longicollis* we keep them as distinct species. However further investigations using molecular data and precise environmental descriptions are needed to investigate the relationships between these three morphologically similar species and clarify the true diversity within this genus.

Table 1. Measurements and shape of the different species of Alabasta *based on the literature*

Species	General shape of the test	Length (L) (μm)	Breadth (B) (μm)	L/B ratio	Pseudostome shape and width (μm)
Alabasta militaris	compressed, narrow piriform	50–82	25–46	1.8–2	deeply curved, 15-22
Alabasta kivuense	compressed, narrow piriform	49–64	18–26	2.4–2.7	very curved, 12-16
Alabasta longicollis	compressed, elongated piriform	120–140	40–45	3–3.1	deeply curved, n.a.

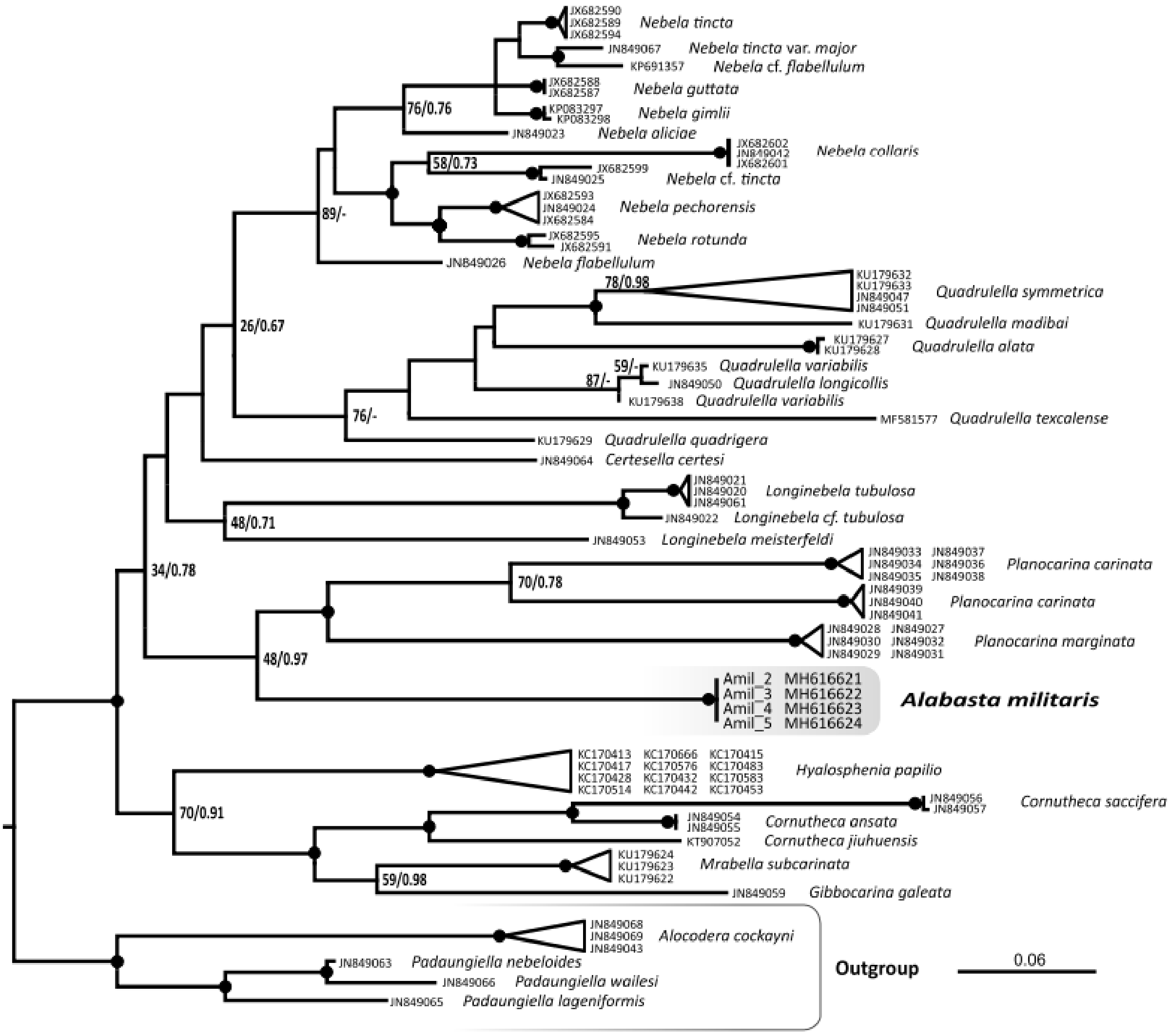

Fig. 3. Maximum likelihood phylogenetic tree of all COI gene sequences available on GenBank with the sequences of four single cells of Alabasta militaris. *Numbers between branches correspond respectively to bootstrap values (bs) and posterior probabilities (p.p.) as calculated with Bayesian inference. The bs and p.p. values are indicated only for supported nodes (bs > 50 and p.p. > 70). Well supported nodes (bs > 80 and p.p. > 90) are marked with a dot.*

Species morphologically related to the genus *Alabasta* gen. nov.

Besides *Alabasta* (*Nebela*) *longicollis* and *A.* (*Nebela*) *kivuense* there are several species that could be considered as morphologically related to *A. militaris*.

Hyalosphenia elegans (Leidy 1879) and *H. insecta* (Harnisch 1938) also share several common morphological characteristics with *Alabasta*. Furthermore, based on the 18S rRNA gene Lara et al. (2008) demonstrated that *H. elegans* does not branch with *H. papilio* as it is placed as a sister clade to genus *Nebela*. Thus, *H. elegans* and *H. insecta* could potentially also be included in genus *Alabasta*. However, two major differences between these species are 1) the inability of *H. elegans* and *H. insecta* to perform kleptosquamy as commonly observed in *Alabasta militaris*, *A. longicollis* and *A. kivuense* and 2) the presence of circular to oval depressions at the surface of the shell of *H. elegans* and *H. insecta* that are not observed in *Alabasta* species. Thus, we believe that *H. elegans* and *H. insecta* likely represent yet another clade (likely a new genus), but further molecular data are required to determine this.

Conclusion

Alabasta militaris is a cosmopolitan species that has been reported in the Northern and Southern Hemispheres and in high to low latitudes (e.g., Fernández et al. 2015; Golemansky 1962; Krashevska et al. 2017, 2018; Van Oye 1956). However, these observations are based solely on rough morphology-based observation, and a study combining morphometry and single cell barcoding may well reveal an unsuspected diversity of cryptic species (Dumack et al. 2016; Kosakyan et al. 2012, 2013; Lara et al. 2011; Singer et al. 2015). In parallel with findings in other Hyalospheniidae, these closely-related species may have different ecological optima (Singer et al. 2018) and may also have somewhat contrasted functional roles in microbial food webs (Geisen et al. 2018). As *Alabasta militaris* in its present definition has a narrow ecological tolerance, i.e., dry microhabitats in *Sphagnum* (Mazei and Bubnova 2007), the distribution of its lineagesis arguably less likely to be structured by geographic distance and barriers to dispersal than larger species such as *Hyalosphenia papilio* (Heger et al. 2013). Finally, single cell transcriptomic studies of amoebae (Kang et al. 2017) may also provide new insight to the evolution of closely related species. The present study of *Alabasta* will pave the way to further studies in this iconic species of protist which may prove a useful model for future studies aiming to understand the general rules that shape ecological, biogeographical and evolutionary process in terrestrial protists.

Taxonomic actions

Description of new genus: *Alabasta* gen. nov. Duckert, Blandenier, Kosakyan and Singer

Taxonomic summary:

Arcellinida Kent 1880.

Hyalospheniidae (Schultze 1877) Kosakyan et Lara 2012.

Alabasta gen. nov. Duckert, Blandenier, Kosakyan and Singer.

Description: Test rigid, colourless or yellowish, elongated in broad view with a maximal width at about two thirds from the aperture and sides then tapering towards the aperture. Test proteinaceous often with incorporated silica scales taken from euglyphid preys. Pseudostome strongly convex with a flare (i.e., fan shaped) in broad view and a deep notch in profile. Lateral pores usually present at about one third of the distance from the pseudostome to the fundus.

Differential diagnosis: *Alabasta* can be distinguished from similar genera such as *Nebela* and *Longinebela* by its fanshaped pseudostome (i.e., strongly curved with a flare) in broad view and a deep notch in profile. Type species: *Alabasta militaris* comb. nov. (Penard 1890) Duckert, Blandenier, Kosakyan and Singer. Included taxa: *Nebela militaris* Penard 1890; *N. kivuense* Gauthier-Lièvre and Thomas 1961; *N. longicollis* Penard 1890.

Etymology: The name of the genus is derived from the Greek word "ἀλάβαστος" (alabaster), in connection with the diaphanous and yellowish aspect of the test of this genus. Also, it appears that *Alabasta* is the name of a fictitious city established in the middle of a desert in the manga One Piece (Oda 1999), reminiscent of the ecological preferences of *Alabasta militaris* which is used as a dry indicator in peatlands monitoring. LSID numbers for the nomenclatural act urn:lsid:zoobank.org:act: 7408C1E9-2DD6-4098- 97DA-3CF743954B5F and for this publication urn:lsid:zoobank.org:pub:72E66220-2093-4765-8D79- 413E9B52071D.

Key to the species

1. Smaller species <100 µm, test narrow piriform in broad view **2**

1*. Larger species: L = 120–140 µm, test elongated piriform in broad view, with an elongated neck. ***A. longicollis***

2. Wider species in broad view (L/B = 1.8–2), pseudostome wide (15–22) µm ***A. militaris***

2*. Slender species, often laterally curved, in broad view (L/B = 2.4–2.7) pseudostome narrow (12–16 µm). ***A. kivuense***

Redefinition of *Nebela militaris* Penard 1890

Alabasta militaris comb. nov. Duckert, Blandenier, Kosakyan and Singer

1879 ***Nebela collaris*** (pars) in Leidy, Freshw. Rhiz. N. America, p. 147, pl. 22 Fig. 11, 12, 16 (misidentiſcation).

1881 ***Nebela bursella*** Vejdovsky in Taranek (1881) Sitzber. böhm. Ges. Wiss. p. 230, Fig. 2

1882 ***Nebela bursella*** Vejdovsky in Thierische Organismen der Brunnenwässer von Prag

1882 ***Nebela bursella*** Vejdovsky (pars) in Taranek Abh. böhm. Ges. Wiss. (6) XI (1882), 8, p. 36, pl. III, Figs. 8 and 12.

1890 ***Nebela militaris*** Penard, Mem. Soc. Phys. Hist. Nat. Genève 31: 164 (creation of *Alabasta* gen. nov. and transfer to the new genus)

1933 ***Nebela americana*** var. ***bryophila*** Van Oye, Arch. Naturgestch. Abt. B., N.F. 2 (overlapping morphology).

Updated description: Test rigid, colourless or yellowish, compressed in broad view, narrow piriform, reminding the shape of an ancient cannon (hence the species name) with a maximal width at about the first third from the posterior part and the sides of the test tapering towards the aperture. Test proteinaceous often incorporating silica scales taken from euglyphid preys. Lateral pores frequently observed at ca. one third of the distance from the pseudostome to the fundus. Pseudostome fan-shaped (i.e., strongly curved) in broad view, with a notch in profile, and a thick organic lip (Fig. 1.A–D). We recorded the following measurements (Fig. 2): Length: 61–77.5 m (mean 67.9m), width: 31.5–53m (mean 37.7 m), pseudostome (aperture) long axis: 15.5–20.5m (mean 18.1m). Dimensions based on previous observations: L = 50–82m, B = 25–46m. Pseudostome 15–22m wide.

Differential diagnosis: *A. militaris* may be confused with *A. longicollis* and *A. kivuense*, from which it differs respectively by a shorter test or by a wider test and pseudostome (see Table 1). Also *A. kivuense* is often laterally curved.

Neotype: Due to the absence of a type, we declare the Specimen 516-2 isolated by Penard and mounted on a permanent slide, now deposited at the Natural History Museum of Geneva, Switzerland as the name-bearing type (https://commons.wikimedia.org/wiki/Category:Nebela militaris#/media/File:Collection Penard MHNG Specimen 516-2-1 Nebela militaris.tif).

Type locality: Jura Mountains, Switzerland

Etymology: The name refers to its general shape which reminds of an ancient military cannon.

Habitat: Sphagnum mosses (relatively dry microhabitats such as hummocks), brown mosses, litter, sediments (rare).

Geographical distribution: Apparently cosmopolitan41

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejop.2018.08.005.

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Chapter 2 - Case 3782 – *Nebela militaris* Penard, 1890 (Arcellinida, Hyalospheniidae): proposed conservation of the specific name by giving it precedence over *Nebela bursella* Taranek, 1881 (published)

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Abstract

The purpose of this application, under Article 23.9.3 of the Code, is to conserve the specific name *Nebela militaris* Penard, 1890, a junior subjective synonym of *Nebela bursella* Taranek, 1881 – referred to as *Nebela bursella* Vejdovský in the literature. Due to the absence of any type or reference specimen and due to the confusing original description, doubts about the taxonomic status of *N. bursella* persist. A review of the literature revealed that the names *N. militaris* and *N. bursella* originally referred to the same species, with the name *N. bursella* later being applied erroneously to another species. According to the Principle of Priority, *N. bursella* is the valid name of the species generally known as *N. militaris*, but there has been no mention of the former taxon since 1964 and its name is unknown to most active testate amoeba researchers. To avoid confusion, we propose to conserve the widely used species name *Nebela militaris* Penard, 1890 by granting it conditional precedence over *Nebela bursella* Taranek, 1881, and to designate a neotype.

1. In 1880, František Vejdovský found specimens of an unknown species of testate amoeba, which he later described as *Nebela bursella* in Vejdovský (1882: 32, pl. II, fig. 2a-c). Before publishing, he mentioned his finding to a fellow naturalist, Karl J. Taranek, who reported this species in 1881 as *Nebela bursella* Vejdovský (Taranek, 1881: 230-231, fig. 2). In this publication, the description and illustration of *Nebela bursella* are congruent with those of Vejdovský (1882). Neither author designated a type specimen or provided any permanent reference slides, and the original material is considered to be lost (Duckert et al. 2018). Because Taranek (1881) attributed the name *Nebela bursella* to Vejdovský, various authors have subsequently referred to it as *N. bursella* Vejdovský, 1881 or as *N. bursella* Vejdovský, 1882. Despite this, Taranek did not explicitly attribute the text of his description to Vejdovský, and the specific name must be attributed solely to Taranek (1881). In consequence, Vejdovský's specific name is simultaneously a junior homonym and junior subjective synonym of Taranek's name.

2. Taranek (1882, pp. 36-38) reported on *Nebela bursella* again, and his illustrations clearly show that he included in this taxon specimens that differed markedly in morphology. While some of the illustrations (Taranek (1882): pl III, fig. 8, 12) are congruent with those of Taranek (1881) and Vejdovský (1882), others clearly do not match the original description. They depict larger and broader specimens which, based on the current taxonomy of the Arcellinida, should rather be assigned to another species of the genus *Nebela* (Taranek, 1882: pl. III, fig. 7; pl. IV, fig. 16), or potentially even to the genus *Heleopera* (Taranek, 1882: pl. III, fig. 9–11) (Duckert et al. 2018, see also Tsyganov et al. 2016 for a comparison between *Nebela* and *Heleopera*). This error was not corrected and led to the misinterpretation that *Nebela bursella* corresponded to the broader morphotypes, which ultimately resulted in *Nebela bursella* being synonymized with *Nebela tincta* (Leidy, 1879) (see Awerintzev, 1906) – now *Nebela tincta sensu* Kosakyan & Lara (2013).

3. Penard (1890, p.164, pl. VII, fig. 16-22) described *Nebela militaris* Penard, 1890 but he neither designated a type nor mentioned any isolated specimens in his description. However, he did mention in his description the location (Tröllhattan, Rosersberg, Marstrand in Sweden and the Vallée de Joux in Switzerland) of the specimens on which he established his species and several permanent slides that he made, of which the exact year of creation is not known but thought to postdate 1900, are still preserved in two distinct collections in the Natural History Museum of London and the Natural History Museum of Geneva. We found five slides containing specimens of *Nebela militaris* after inspection of Penard's permanent slides in both museums, but it is apparent that none of those specimens can be considered as type material, as the registers of the collections show that none have been isolated in the regions mentioned by Penard in his description of *Nebela militaris*. The registers of Penard's permanent slide collections in both the Natural History Museum of London and the Natural History Museum of Geneva have been transmitted to the Commission Secretariat. Given that Penard did not designate any name-bearing type, that no specimen on which he could have based his description was found in his collections of permanent slides and that the absence of type was (and unfortunately still is) more the rule than the exception in the field of taxonomy of testate amoebae, we are certain that there is not any type material for *Nebela militaris*. The specimens that Penard isolated as well as his original description and illustrations of *Nebela militaris* (Penard, 1890) are very similar to the first description of *Nebela bursella* by Taranek (1881). Nonetheless, Penard stated that his was truly a distinct species while acknowledging that some may consider it to be merely an aberrant form of *Nebela bursella*. He did not explain what features of *N. militaris* could be considered as abnormal in comparison to *N. bursella*, but in a subsequent major monograph Penard (1902) considered *N. bursella* a synonym of *N. tincta*, cited by him as *Hyalosphenia tincta* Leidy, 1879. This allows us to infer that Penard (1890) based his concept of *N. bursella* on Taranek's (1882) misidentified specimens and thought that his new species *N. militaris* might be regarded as an abnormally slender form of *Nebela tincta*.

4. The name *Nebela militaris* was rapidly accepted by the scientific community. A list of 26 works by 53 authors using this name as valid for the taxonomic species in question is held by the Commission Secretariat and available upon request. Among these works, we may mention Amesbury et al. (2016), Payne et al. (2011), Mitchell et al. (2008) and Charman et al. (2007). In contrast, the name *Nebela bursella*, having long been considered a junior synonym of *Nebela tincta* (Leidy, 1879) (Awerintzev, 1906) fell into disuse, being used as a valid name for the last time by Sudzuki (1964). It is no longer found in its original meaning in major compendia such as Grospietsch (1958) and Tsyganov et al. (2016), and to revive it now as the valid senior subjective synonym of *N. militaris* would lead to confusion.

5. Due to the absence of any type and the lacunar state of the taxonomy of testate amoebae, it is also required that a neotype be designated for *Nebela militaris* Penard, 1890. It has been shown that species of testate amoeba can hide complexes of closely related species that differ only by slight variations in their morphology, even in the case of well-known species (see Kosakyan et al., 2013 as an example). As *Nebela militaris* has been recorded worldwide from a variety of different habitats it is likely that it corresponds to such a species complex, with each species potentially having a restricted distribution and ecology. However, the illustrations and the descriptions originally made by Penard are not representative of the morphotype found in Europe, and establishing a species on specimens found in such a large area (Switzerland and Sweden) increases the chance of lumping distinct species

6. To avoid future confusion over the identity of *Nebela militaris* and any potential closely related species yet to be described, we propose to designate a neotype representative of the specimens found in the Swiss Jura mountains and propose this region as the type locality, as it is from this region that the specimens studied by Duckert et al. (2018) and the specimens isolated by Penard originate. Accordingly, we selected the specimen 516-2 from the Penard Collection now deposited at the Natural History Museum of Geneva in Switzerland (https://commons.wikimedia.org/wiki/Category:Nebela_militaris#/media/File:Collection_Penard_MHNG_S peci men_516-2-1_Nebela_militaris.tif) to be set as the neotype.

7. This application is submitted to coincide with the publication of Duckert et al. (2018), who reappraised the taxonomic status of *Nebela militaris* and *Nebela bursella* and thereby became aware of this nomenclatural problem.

8. The International Commission on Zoological Nomenclature is accordingly asked:
   (1) to use its plenary power to give the specific name *militaris* Penard, 1890, as published in the binomen *Nebela militaris*, precedence over the specific name *bursella* Taranek, 1881, as published in the binomen *Nebela bursella*, whenever the two are considered synonyms; and
   (2) to place on the Official List of Specific Names in Zoology the following names:

(a) *militaris* Penard, 1890, as published in the binomen *Nebela militaris* and as defined by the neotype (specimen 516-2 from the Penard collection in the MHNG) designated in para. 7, with the endorsement that it is to be given precedence over the name *bursella* Taranek, 1881, as published in the binomen Nebela bursella, whenever the two are considered synonyms; and

(b) *bursella* Taranek, 1881, as published in the binomen *Nebela bursella*, with the endorsement that it is not to be given precedence over the name *militaris* Penard, 1890, as published in the binomen *Nebela militaris*, whenever the two are considered synonyms.

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Chapter 3 - Superficially described and ignored for 92 years, rediscovered and emended: *Apodera angatakere* (Amoebozoa: Arcellinida: Hyalospheniformes) is a new flagship testate amoeba taxon from Aotearoa (New Zealand) (published)

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Apodera vas; *Gibbocarina*; *Sphagnum*; Gondwana; Hyalospheniidae; Māori language and culture; biodiversity conservation; biogeography; microbial diversity; peatlands; taxonomy.

Abstract

Eukaryotic microbial diversity is known to be extensive but remains largely undescribed and uncharted. While much of this unknown diversity is composed of inconspicuous flagellates and parasites, larger and morphologically distinct protists are regularly discovered, most notably from poorly studied regions. Here we report a new flagship species of hyalospheniid (Amoebozoa; Arcellinida; Hyalospheniformes) testate amoeba from New Zealand and an unusual story of overlooked description under a preoccupied name and subsequent oversight for nearly one century. Through a process involving The Māori Language Commission, we named the species *Apodera angatakere*, meaning "a shell with a keel." This species resembles *Apodera* vas but differs by the presence of a distinctive hollow keel. Cytochrome Oxidase Subunit 1 (COI) sequence data show that this species forms a distinct clade nested within genus *Apodera*. This conspicuous species is so far known only from New Zealand and is restricted to peatlands. It is one of the few examples of endemic microorganisms from this biodiversity hotspot and biogeographer's paradise. As over 90% of New Zealand's peatlands have been lost since European colonization and much of the remaining surfaces are threatened,

Apodera angatakere could be a flagship species not only for microbial biogeography but also for island biodiversity conservation.

ANYONE with a sense for adventure may dream about the age of discovery (15th–17th century period), during which time the New World was (re)discovered and many new inventions made that changed our perception of the world. Traveling back in time to experience such thrill first hand is, unfortunately, not possible. However, when we consider the lack of knowledge of the microbial world, every sample can be an unexplored island for the curious protistologist with an inclination for natural history.

The invention of the microscope in the late 16th century paved a new era of observation and discovery by making the microbial world accessible to scientific researchers. With subsequent improvements in optics, along with the exploration of various habitats and regions of the World a broad range of microbes was gradually described, gaining momentum in the 19th and early 20th century by pioneers such as Christian Gottfried Ehrenberg, Ernst Haeckel, Josef Leidy and Eugène Penard (Ehrenberg, 1838; Haeckel, 1899–1904; Leidy, 1879; Penard, 1902). More recent developments such as high throughput sequencing of environmental DNA (eDNA) are allowing large amounts of genetic data to be generated revealing a huge, mostly unknown, diversity of microorganisms including bacteria (Delgado-Baquerizo et al., 2018), fungi (Nilsson et al., 2019), protists (Mahé et al., 2017) and micro-Metazoa (van den Hoogen et al., 2019). The magnitude of protist diversity is now believed to exceed that of bacteria (Delgado-Baquerizo et al., 2018) and fungi (Tedersoo et al., 2014), at least in the ocean (de Vargas et al., 2015) but recent work also suggests that this may be the case for terrestrial soil (Geisen et al., 2018). This diversity is confirmed by numerous examples of cryptic diversity reported in classical DNA barcoding studies (Fontaneto et al., 2008; Kosakyan et al., 2012; Kumar & Foissner, 2016; Mann, 2010; Pawlowski et al., 2008), as was first documented for insects (Hebert et al., 2004).

These newly available molecular identification tools have stimulated another golden age of protist discovery, comparable to that triggered by the invention of the microscope (Geisen et al., 2015; Heger et al., 2014; Mitchell, 2015). Novel protist diversity can now be found virtually anywhere. DNA barcoding studies are regularly revealing the existence of numerous species within known groups, questioning long-held ideas about their geographical distribution or ecology that were formerly based on only morphological similarities (Singer et al., 2015; de Vargas et al., 1999). Taxa known from ancient morphological descriptions are now being redescribed and placed in the tree of life using the full range of molecular and detailed microscopy techniques (Burki et al., 2019; Dumack et al., 2016, 2018; Lax et al., 2018).

Finding novel protist diversity can be expected when exploring under-studied regions (Yubuki et al., 2009) or poorly studied groups (Dumack et al., 2018; Siemensma et al., 2020). Although there is a strong sampling bias in favor of Europe and North America where most protistologists work, detailed studies of other regions frequently report many new species, as illustrated in South America, Australia, Africa, and Antarctica (Fernández et al., 2015; Foissner, 1997). Twenty-four years ago, Foissner estimated that at least 70% of the world soil ciliates remained to be described (Foissner, 1997), which is still an underestimation of their true diversity as revealed by eDNA sequencing (Boscaro et al., 2018).

New Zealand is a biogeographer's paradise but has barely been explored for soil protists and especially testate amoebae. Eugène Penard reported 45 testate amoeba species, including three new taxa, *Nebela certesi*, *Hyalosphenia cockayni*, and *Nebela griseola*, from samples collected during the 1907–09 British Antarctic Expedition (Penard, 1911). All three species have since been transferred to new genera, *Certesella certesi*, *Alocodera cockayni*, and *Physochila griseola*. Since then, to our knowledge 19 studies were published on the diversity and ecology of testate amoebae of New Zealand, reviewed in McKeown et al. (2021). A limited number of studies described new species for science, new records for New Zealand or, more recently, focused primarily on the community ecology of testate amoebae and their application in paleoecology (Bamforth, 2015; Charman, 1997; Deevey, 1955; Hoogenraad & de Groot, 1948; McGlone & Wilmshurst, 1999; McKeown et al., 2019; van Oye, 1956; Stout, 1984; Wilmshurst et al., 2002, 2003; Winterbourn & Brown, 1967). However, there has been no modern study on testate amoeba taxonomy that incorporates molecular, morphometrical and ecological data, which are required to accurately describe new species (Lara et al., 2020). As a consequence, it is most likely that many New Zealand species remain overlooked or undescribed.

Here we report *Nebela penardi* from *Sphagnum* peatlands in New Zealand for the first time since its description (Brehm, 1928), a highly conspicuous species that had been superficially described and completely overlooked since then. Based on Cytochrome Oxidase Subunit 1 (COI) data we determine its phylogenetic position within genus *Apodera* and discuss its potential as flagship species for both biogeographical and ecological surveys. Furthermore, as *Nebela penardi* is an invalid name and there is no known available synonym we formally rename it *Apodera angatakere* in collaboration with Te Taura Whiri I te Reo Māori (The Māori Language Commission).

Material and Methods

Sampling, single cell isolation, and morphometry

Samples of *Sphagnum*, other mosses (unidentified brown mosses from wetlands) and forest litter were collected from numerous forests, peatlands, and fens across New Zealand's North and South Islands and Campbell and Auckland Islands as well as Macquarie Island as part of a wider project on the diversity of testate amoebae in New Zealand and peri-Antarctic islands. Testate amoebae that could be assigned to *Apodera angatakere* were found in 11 samples (Table 1). Testate amoebae were isolated for further analyses from four samples (Figs S1–S8):

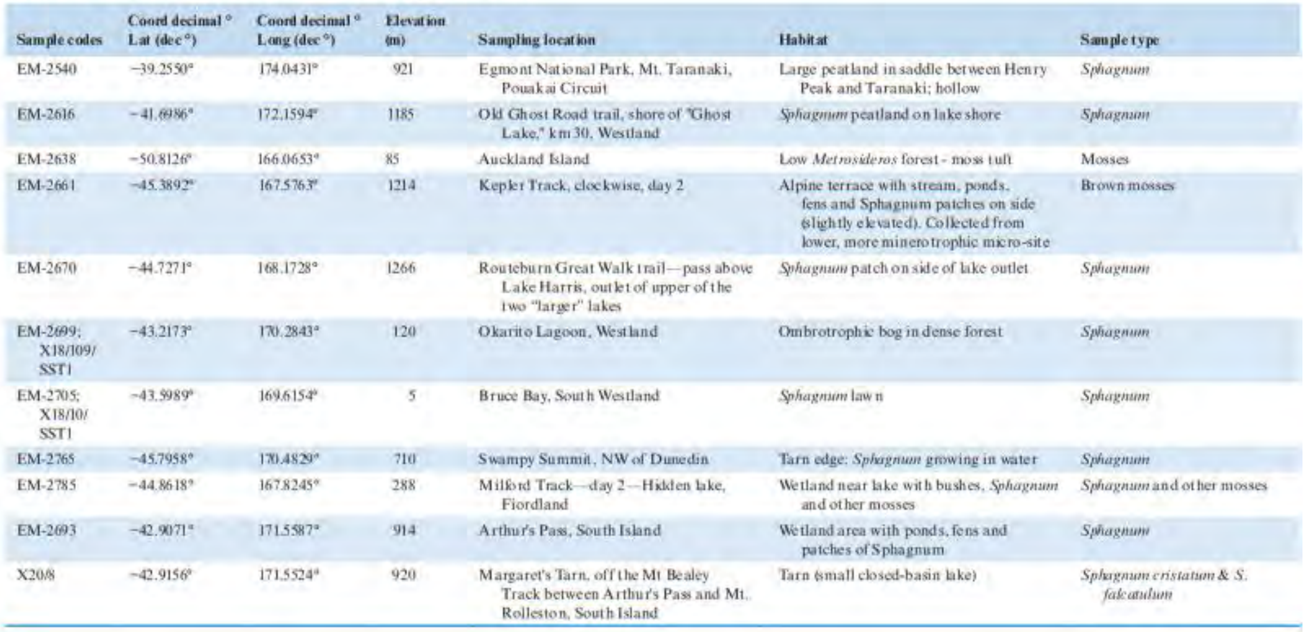
1. EM 2540: *Sphagnum* mosses from a hollow in Ahukawakawa Swamp, along the Pouakai crossing trail, on the saddle between Taranaki Maunga and Pouakai Hut, New Zealand's North Island. Coord: Lat −39.255058°, Long 174.043106°, Elevation: 921 m a.s.l.

2. EM 2543: Forest litter and humus from the Northern foothills of Taranaki Maunga, New Zealand's North Island. Coord. Lat −39.22505°, Long 174.122842°, Elevation: 478 m a.s.l.

3. EM 2935: *Sphagnum cristatum* and *S. falcatulum* from Margaret's Tarn, off the Mt Bealey Track between Arthur's Pass and Mt. Rolleston on the South Island. Coordinates: Lat −42.915655°, Long 171.552487°, Elevation: 920 m a.s.l.

4. EM 2764: *Sphagnum falcatulum* from the north shore of Macquarie Island, collected along Featherbed Track, between Handspike Corner and West Beach. Coordinates: Lat −54.49928200°, Long 158.90512300°, Elevation ~5 m a.s.l

TABLE 1 Samples collected in New Zealand containing *Apodera angatakere*



Sample codes	Coord decimal ° Lat (dec °)	Coord decimal ° Long (dec °)	Elevation (m)	Sampling location	Habitat	Sample type
EM-2540	−39.2550°	174.0431°	921	Egmont National Park, Mt. Taranaki, Pouakai Circuit	Large peatland in saddle between Henry Peak and Taranaki; hollow	*Sphagnum*
EM-2616	−41.6986°	172.1594°	1185	Old Ghost Road trail, shore of "Ghost Lake," km 30, Westland	*Sphagnum* peatland on lake shore	*Sphagnum*
EM-2638	−50.8126°	166.0653°	85	Auckland Island	Low *Metrosideros* forest - moss tuft	Mosses
EM-2661	−45.3892°	167.5763°	1214	Kepler Track, clockwise, day 2	Alpine terrace with stream, ponds, fens and Sphagnum patches on side (slightly elevated). Collected from lower, more minerotrophic micro-site	Brown mosses
EM-2670	−44.7271°	168.1728°	1266	Routeburn Great Walk trail—pass above Lake Harris, outlet of upper of the two "larger" lakes	*Sphagnum* patch on side of lake outlet	*Sphagnum*
EM-2699; X18/109/ SST1	−43.2173°	170.2843°	120	Okarito Lagoon, Westland	Ombrotrophic bog in dense forest	*Sphagnum*
EM-2705; X18/10/ SST1	−43.5989°	169.6154°	5	Bruce Bay, South Westland	*Sphagnum* lawn	*Sphagnum*
EM-2765	−45.7958°	170.4829°	710	Swampy Summit, NW of Dunedin	Tarn edge: *Sphagnum* growing in water	*Sphagnum*
EM-2785	−44.8618°	167.8245°	288	Milford Track—day 2—Hidden lake, Fiordland	Wetland near lake with bushes, *Sphagnum* and other mosses	*Sphagnum* and other mosses
EM-2693	−42.9071°	171.5587°	914	Arthur's Pass, South Island	Wetland area with ponds, fens and patches of Sphagnum	*Sphagnum*
X20/8	−42.9156°	171.5524°	920	Margaret's Tarn, off the Mt Bealey Track between Arthur's Pass and Mt. Rolleston, South Island	Tarn (small closed-basin lake)	*Sphagnum cristatum* & *S. falcatulum*

The samples were placed in closed plastic sampling bags. Once in the laboratory the samples were sub-sampled and material was placed in a container and shaken in tap water for 1–2 min and then filtered over a 200-µm mesh filter. The filtrate was observed under an inverted microscope and testate amoeba specimens that could be assigned to genus *Apodera* were isolated with a narrow diameter pipette and transferred to a new slide with distilled water to remove any unwanted material. Pictures were taken for each individual specimen used for DNA barcoding, which was then transferred to a PCR tube with 50 µl of guanidinium thiocyanate solution (Chomczynski & Sacchi, 1987). Additional living or dead individuals were used for scanning electron microscopy (SEM) imaging. These specimens were further cleaned in ethanol and transferred to a SEM stub on which a carbonate filter had been fixed. The samples were then sputter coated with gold. Images were taken with a Hitachi TM 3030 plus scanning electron microscope operating at 5–15 kV. SEM images were taken at the electron microscopy facility of Manaaki Whenua/Landcare Research, Lincoln, New Zealand.

Measurements were taken on light microscopy and SEM images for each observed specimen: test length, test width, aperture long axis, length of the neck, width at the base of the neck, maximum width of the neck, and length of the main (aboral) part of the test (Figure 1). Depth could only be measured accurately on four individuals resting on their side, but it could be estimated based on the height of the focus plane for individuals resting flat.

DNA extraction, PCR amplification, and DNA sequencing

DNA extraction was performed with a guanidinium thiocyanate solution following the protocol of Duckert et al. (2018) adapted from Chomczynski and Sacchi (1987). PCR of partial mitochondrial cytochrome oxidase I (COI) gene were processed using a nested PCR protocol. The first PCR was conducted with the eukaryote-general primers LCO1490 (GGT CAA CAA ATC ATA AAG ATA TTG G) and HCO2198 (TAA ACT TCA GGG TGA CCA AAA AAT CA) (Folmer et al., 1994) under the following conditions: Denaturation step at 95℃ for 5 min, then 45 cycles with a denaturation step at 95℃ for 15 s, an hybridization step at 43℃ for 15 s, an elongation step at 72°C for 1 min, and a final elongation at 72°C for 10 min. *Apodera* vas specimens EM2543-15, EM2543-22 and *A. angatakere* EM2540-14, EM2540-19 were then amplified with LCO1490 and ApocoxR (Kosakyan et al., 2012) under the following conditions: Denaturation step at 95°C for 5 min, then 45 cycles with a denaturation step at 95°C for 30 s, an hybridization step at 50°C for 30 s, an elongation step at 72°C for 1 min, and a final elongation at 72°C for 10 min. On the other hand, *A. angatakere* EM2540-11 and A. vas EM2764-2 were amplified using Arcelcox1F (Kosakyan et al., 2012) and Apo2F (a newly designed forward primer: TGG AAT TAG CAT ATC MGG AAT T) respectively, with HCO2198 under the same condition as the first PCR, with the only exception of an hybridization step of 45°C for the former taxa. Positive PCR products were directly purified using Milipore kit and sent for sequencing with an ABI3730XL DNA sequencer (Applied Biosystems) at Macrogen, Amsterdam NL. Sequences were deposited in GenBank with the accession numbers MZ615186–MZ615191. Light microscopy pictures of the corresponding cells are shown in Figure 2.

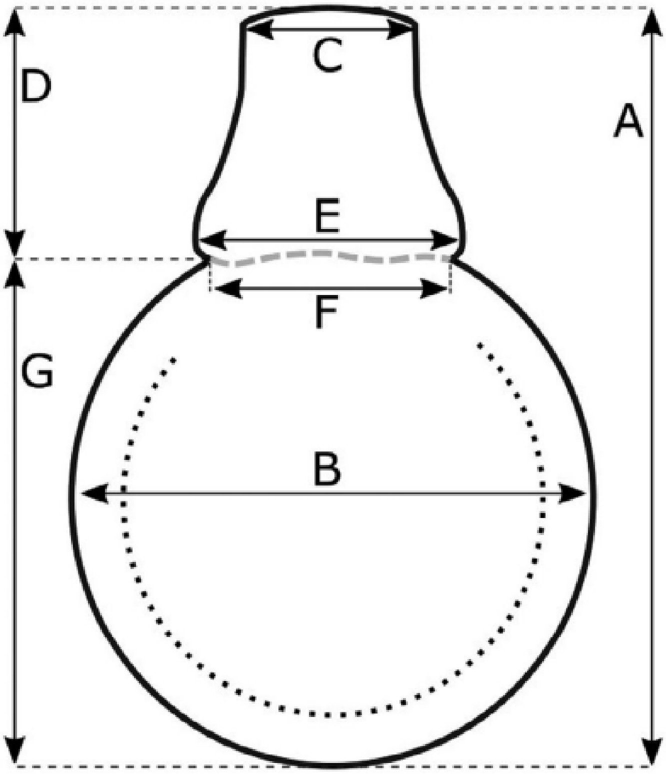


Figure 1 Schematic drawing of Apodera angatakere and codes of the measurements taken. (A) Length of the test; (B) width of test; (C) width of the pseudostome; (D) length of the neck; (E) maximal width of the neck; (F) width of the test at the constriction; (G) length of the test without the neck

Phylogenetic analysis

All sequences obtained were aligned manually using BioEdit (Hall, 1999) together with an exhaustive reference database composed of three previously published sequences of genus *Alocodera* and three of genus *Padaungiella* (Kosakyan et al., 2012) retrieved from the GenBank database (Table S2). The tree was rooted with 10 Hyalospheniformes sequences, corresponding to eight different genera, published in four previous studies (i.e., Duckert et al., 2018; Kosakyan et al., 2012, 2013; Singer et al., 2015). Phylogenetic reconstruction was conducted on the complete LCO-HCO fragment (i.e., 655 nucleotides) using the CIPRES Science Gateway V. 3.3 (Miller et al., 2010). A maximum likelihood phylogenetic tree was built using RAxML v.8.2.10 (Stamatakis, 2014) with the GTR + GAMMA model and automatic bootstrapping halt. We further evaluated node robustness using a Bayesian approach with MrBayes (Ronquist & Huelsenbeck, 2003) using the GTR + GAMMA model with default settings on two independent runs sampled every 100 generations.

The analysis was automatically stopped when convergence was reached after 260, 100 generations resulting in 5202 trees of which 25% were discarded as the burn-in.

Collaborating with Māori in the naming process

Naming species that are discovered in regions where indigenous people live should respect the cultural principles. According to the Waitangi tribunal Wai 262 report (Waitangi Tribunal Report Wai 11, 1986), Māori retain "kaitiakitanga" (guardianship) over "taonga" (treasured) species and the cultural relationship between kaitiaki (guardian) and taonga (treasured) species is entitled to reasonable protection. According to Veale et al. (2019), this implies that there is an imperative to conduct processes such as formally naming and describing species of cultural relevance to Māori in partnership with Māori. In agreement with this principle, as a new species name needed to be found, this was done in partnership with Te Taura Whiri I te Reo Māori (The Māori Language Commission). Our aim was to find a name that would be appropriate for the organism and ideally reflect the specific morphology: a flattened gourd with a constriction at the base of the neck and a hollow "keel." The choice of a name was an iterative process involving Ngahiwi Apanui from Te Taura Whiri I te Reo Māori and all authors.

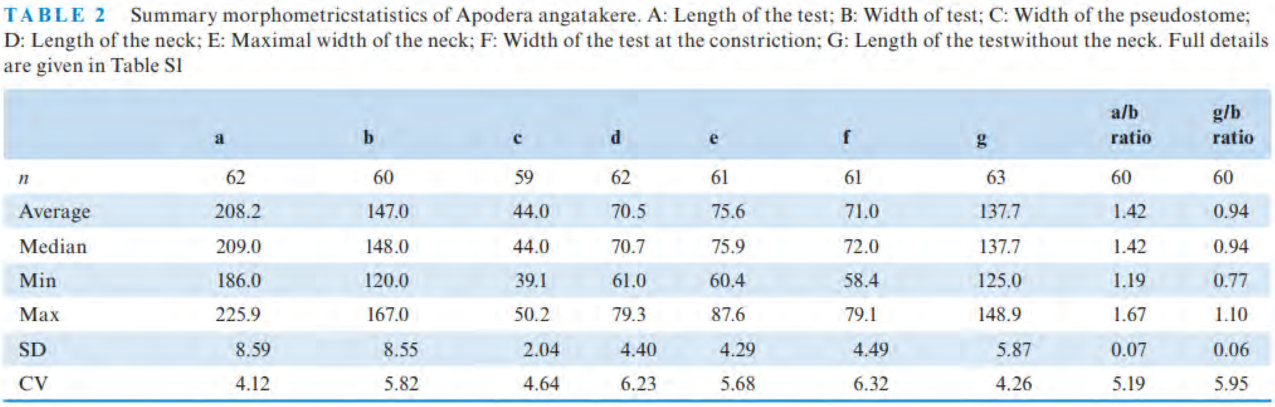


TABLE 2 Summary morphometricstatistics of Apodera angatakere. A: Length of the test; B: Width of test; C: Width of the pseudostome; D: Length of the neck; E: Maximal width of the neck; F: Width of the test at the constriction; G: Length of the testwithout the neck. Full details are given in Table S1

	a	b	c	d	e	f	g	a/b ratio	g/b ratio
n	62	60	59	62	61	61	63	60	60
Average	208.2	147.0	44.0	70.5	75.6	71.0	137.7	1.42	0.94
Median	209.0	148.0	44.0	70.7	75.9	72.0	137.7	1.42	0.94
Min	186.0	120.0	39.1	61.0	60.4	58.4	125.0	1.19	0.77
Max	225.9	167.0	50.2	79.3	87.6	79.1	148.9	1.67	1.10
SD	8.59	8.55	2.04	4.40	4.29	4.49	5.87	0.07	0.06
CV	4.12	5.82	4.64	6.23	5.68	6.32	4.26	5.19	5.95

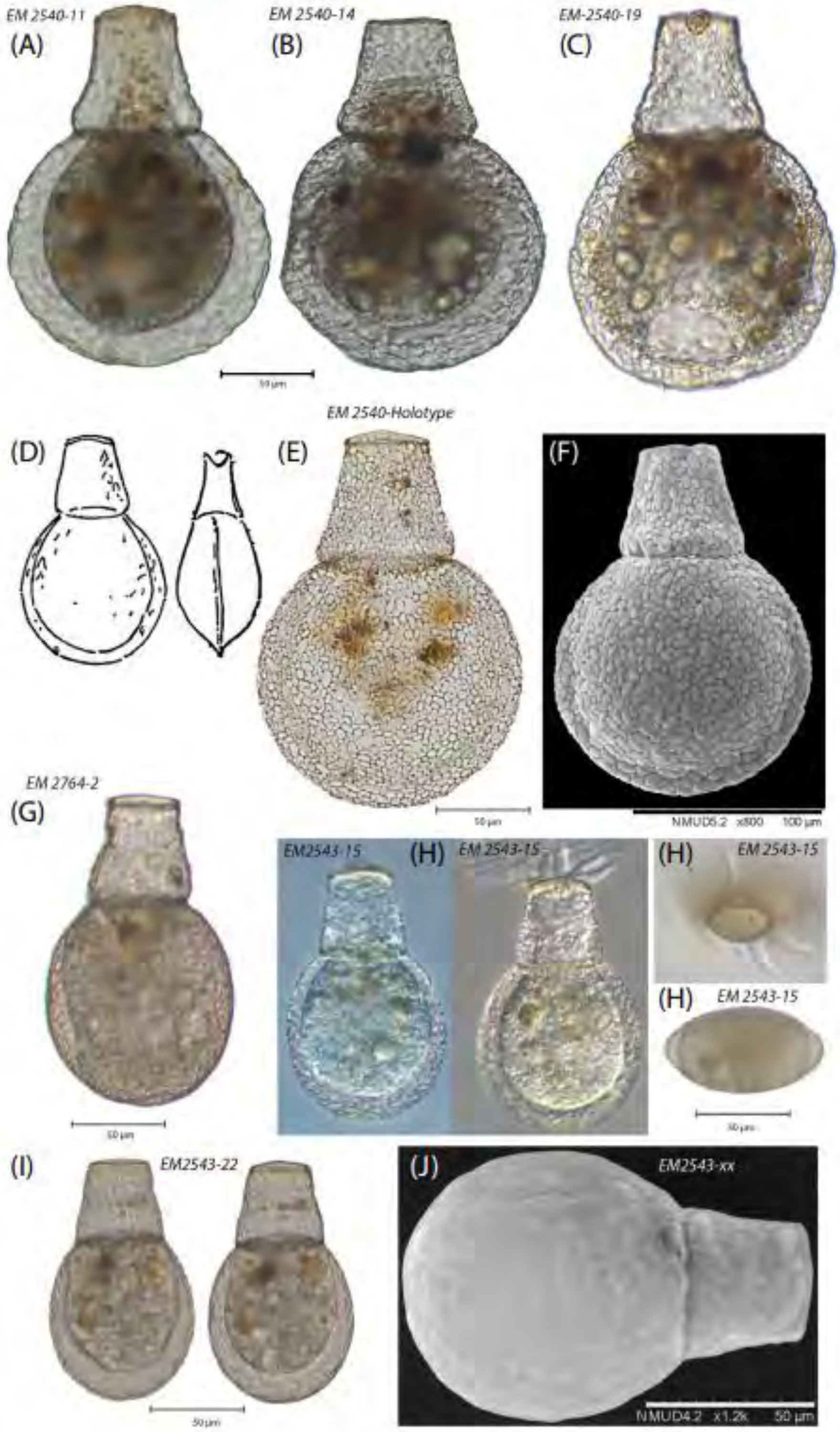


Figure 2 Top half: Apodera angatakere *comb. nov. (A–C, E and F), five specimens from Ahukawakawa swamp, Taranaki Maunga, New Zealand's North Island (sample EM-2540): (A–C) three barcoded individuals, (D) Brehm's original drawing of* Apodera angatakere *(described as* Nebela penardi*) from Margaret's Tarn, Arthur's Pass, New Zealand's South Island, (E and F), two individuals from sample EM-2540 (LM and SEM, respectively). E is the holotype. Note the presence of a ca. 10 µm wide keel. All specimens illustrated here as well as in Figures S2–S8 were used for morphometrical analyses (Figure 1). Scale bars (20, 50, or 100 µm) are shown for all specimens but were not provided in the original description. Bottom half: Apodera vas. (G) barcoded specimen from Macquarie Island (sample EM-2764), (H–J) three specimens from forest litter collected on the lower slopes of Taranaki Maunga, New Zealand's North Island (sample EM-2543). (H and I) Two barcoded specimen, (J) SEM of a third individual; note the absence of a keel. The codes of the barcoded specimens are the same as in the phylogenetic tree (Figure 3)*

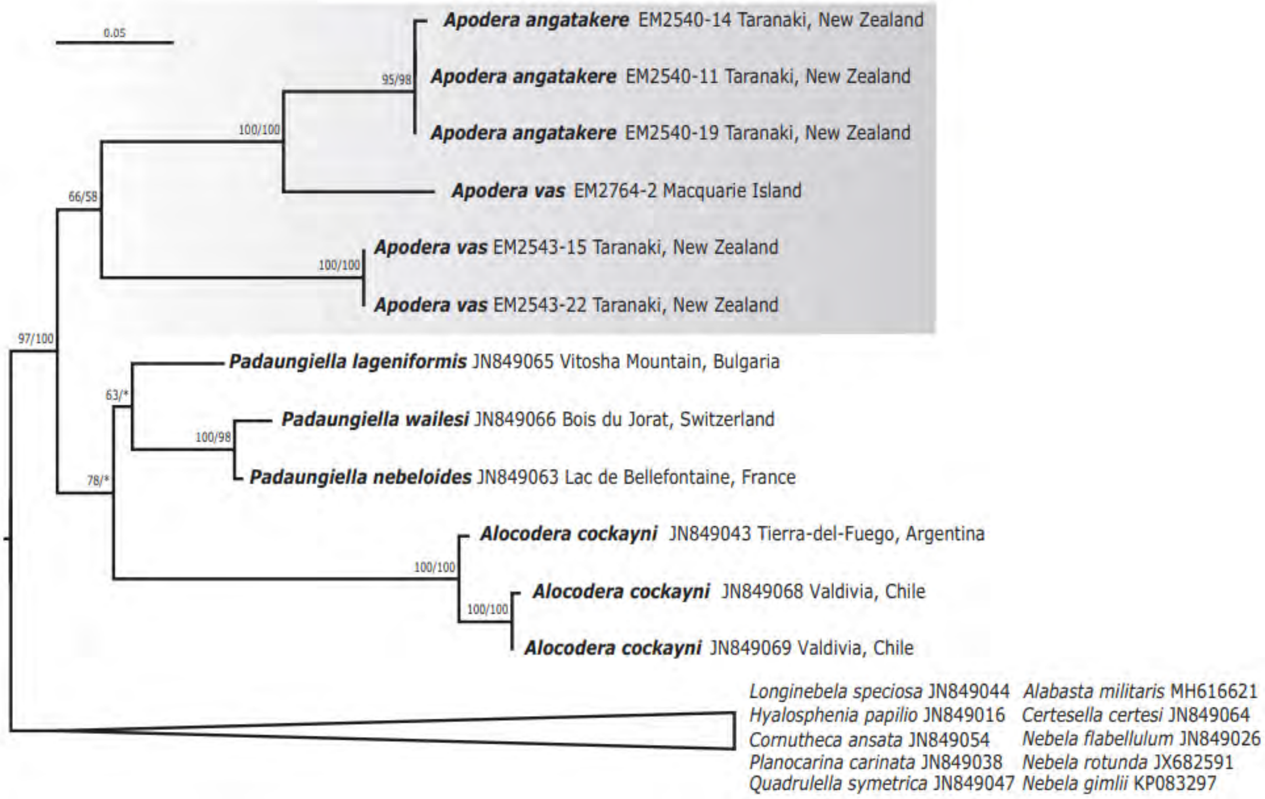


Figure 3 Maximum likelihood phylogenetic tree of the Hyalospheniformes with a focus on Apodera, Alocodera*, and* Padaungiella *based on COI gene sequences. Bootstrap values (bs) and Bayesian posterior probabilities (p.p. as percents) are indicated respectively between branches. COI sequences from genera other than* Apodera *were retrieved from GenBank*

Results and Discussion

Morphological characterization of the tests

We found two distinct morphotypes that could be assigned to genus *Apodera* based on their lageniform shell composed of recycled xenosomes, with a clear constriction that separates the neck from the body. These two morphotypes could easily be distinguished based on their size and the presence of a hollow keel on the larger morphotype (Figure 2). The smaller morphotype (mean length: 147 µm, data not shown) corresponds well to the description of *Apodera vas* (Certes) and our measurements fall in the middle of the range recorded for this taxon (Zapata & Fernandez, 2008). It must be noted that the high degree of variability in dimensions and shape reported for *Apodera vas* suggests the existence of a complex of distinct species (Penard, 1911; Zapata & Fernandez, 2008). Although, the size of the larger morphotype (mean length: 208 µm) (Table 2) falls within the range recorded for *Apodera vas*, it can be easily differentiated by the presence of a distinct hollow keel surrounding the main body, as in genus *Gibbocarina*. The margins of the neck range from almost straight to slightly concave with a bulge at the base. The margins of the neck are compressed, except in the basal bulge region, as an extension of the hollow keel. This is easily visible on SEM images but is less obvious, and possibly overlooked in light microscopy. This morphology matches well with the line drawing of the original description of *Nebela penardi* Brehm, 1928 (hereafter *Apodera angatakere*), which unfortunately lacked a scale or indication of size. The tests of *Apodera vas* were found in forest litter samples, brown mosses, and Sphagnum whereas tests of *Apodera angatakere* were almost exclusively found in Sphagnum samples collected in peatlands but also brown mosses in an alpine fen and mosses in low *Metrosideros* forest on Auckland Island.

Phylogenetic placement within Hyalospheniformes and paraphyly of *Apodera vas*

We obtained partial COI sequences of 655 nucleotides from three specimens of *Apodera vas* and three of *Apodera angatakere*. Sequences of genus *Apodera* form a moderately supported clade (bs: 66; pp: 0.58; Figure 3) and together with the genera *Alocodera* and *Padaungiella* they form a highly supported clade that is sister to the clade formed by all the other genera of Hyalospheniformes used to build the tree.

While sequences of *Apodera angatakere* form a highly supported monophyletic clade, A. vas appears to be paraphyletic with one sequence branching at the base of *A. angatakere* and two other sequences forming a distinct clade within *Apodera*. Interestingly the two specimens of A. vas branching together were found in the same sample from Taranaki Maunga, New Zealand's North Island, whereas the one that branches at the base of *A. angatakere* was found on Macquarie Island. This strongly supports the hypothesis that *Apodera vas* is currently a complex of distinct species, possibly with distinct distribution patterns.

Taxonomic status of *Apodera angatakere* (*Nebela penardi*)

Apodera angatakere was first briefly reported as *Nebela penardi* in a publication focusing mostly on copepods (Brehm, 1928). Only two drawings were shown (Figure 2D) and no indication of size or scale was provided. Brehm had sent a moss sample containing testate amoebae to Eugène Penard who described the finding as follows: “It is similar to *Nebela vas* Certes (now *Apodera vas*), but differs therefrom in that the shell possesses a broad, hollow, and characteristic keel, which is always present and might well be regarded as a sufficient character to distinguish a new species. I have not been able to identify it with any known species.” (Brehm, 1928). As a result, Brehm proposed to name the species *Nebela penardi*.

To our knowledge there is no further mention of this species in the scientific literature and the species has been overlooked since then. Notably, Deflandre did not mention it in his 1936 monograph of the genus *Nebela* (Deflandre, 1936). Moreover, at the time of its description, the name *Nebela penardi* Brehm, 1928 was preoccupied by *Nebela penardi* Heinis, 1914. It is therefore a junior homonym of this species, which implies that *Nebela penardi* Brehm was unavailable and thus invalid.

As there is no known synonym for this taxon, we therefore rename it in agreement with ICZN article 60.3, and as we transfer it to a new genus both a new generic name and a new species epithet are required. Furthermore, as a side note, Heinis (Heinis, 1914) used the name *Nebela penardi* to erroneously rename a taxon that, according to Deflandre (1936), corresponds to *Nebela martiali* (now *Certesella martiali*). *Nebela penardi* Heinis is therefore a junior synonym of *Certesella martiali*.

A very similar species was described under the name *Nebela kenyana* by Didier Chardez from mountain lakes in Kenya at over 4000 m a.s.l. (Chardez, 1982). This species has a similar size and overall morphology including a hollow keel surrounding the fundus of the test and a distinct neck with swollen sides. However, it lacks the characteristic constriction at the base of the neck which defines *Apodera* and, in that, it is more similar to genus *Padaungiella*.

Choice of a species name—an iterative process involving the Māori Language Commission

The choice of a name was an iterative process involving all authors in collaboration with Te Taura Whiri I te Reo Māori. We aimed to find a species name that would be appropriate for the organism and ideally reflect the specific morphology which could be described in general words as a flattened gourd with a constriction at the base of the neck and a hollow “keel.” We also aimed to represent the Mauri (life force) of this unique species that was named by Māori for them as kaitiaki (guardian) of Aotearoa (New Zealand).

The options considered were:

1. An amoeba with a hard shell. According to the Reed Dictionary of Modern Māori, the word “amoeba” is translated as “pūora hurikē” and a “hard shell” is translated as “anga.” Combining the two with the word “whai” meaning “in possession of” leads to the name “Pūora hurikē whaianga.”

2. Considering that a shelled amoeba is analogous to a miniature snail, the second option was “A snail with a keel-like shell.” Snail and keel being respectively translated as “ngata” and “takere,” this would then form “Ngata anga whaitakere.”

3. An amoeba with a keel-like hull: pūora anga whaitakere.

4. An amoeba with a keel: pūora whaitakere.

5. A shell with a keel: Anga whaitakere.

The fifth option was chosen both for the way it sounded and the fact that the name would describe well the morphology of the amoeba. However, in a final discussion, Mr. Ngahiwi Apanui from the Māori Language Commission suggested that the “whai” could be dropped to shorten the name. The final chosen name was then «Angatakere», to be pronounced æŋætɑkɛrɛ (AN-GAH-TAH-KEH-REH).

Implications for biodiversity estimates and conservation

It is estimated that 10% of the New Zealand mainland was covered by wetlands before human arrival around 780 cal. Yr BP (Ausseil et al., 2011; McGlone, 2009), and while testate amoebae were too small to be recognized by Māori until the introduction of the microscope, the wetland habitats that supported these organisms were highly valued by Māori for centuries, as mahinga kai/hauanga kai (food gathering areas), rongoā (gathering plants for medicinal use) and for material resources. Wetland extent was minimally affected by early Māori settlers, although extensive deforestation of dryland forest by burning transformed the landscape (Argiriadis et al., 2018; McWethy et al., 2010; Perry et al., 2014). Wetland loss rapidly accelerated after European arrival in the 1800s, largely for agricultural development, and is now only 10% of the original extent (Ausseil et al., 2011). It is estimated that the 250,000 ha of wetlands that remain are under increasing pressure from drainage, area loss, fragmentation, grazing, fire, pollution, and climate change (Meyer et al., 2013; Robertson et al., 2019). The fact that we can still find novel diversity or diversity that has been very poorly described in such habitats makes them even more precious and worthier of conservation.

Describing the still mostly unknown diversity of protists requires a major effort in basic taxonomy (Heger et al., 2014). The magnitude of land-use changes and natural habitat destruction occurring throughout the world and the now well-established existence of restricted geographical distribution patterns in free-living protists (Foissner, 2008) implies that a large proportion of protist diversity will likely disappear before it can be described, and thus the conservation of protists should indeed be a priority (Cotterill et al., 2008; Qin et al., 2016). *Apodera angatakere* is a highly conspicuous genus of testate amoeba and has to date only been found in New Zealand. Large species are more likely to have restricted geographical distribution as shown empirically for terrestrial and subaquatic testate amoebae in the southern temperate and Antarctic zones (Wilkinson, 1994), at the global scale (Wilkinson, 2001; Yang et al., 2010) and confirmed by an atmospheric circulation modeling study (Wilkinson et al., 2012). Given its large size it is likely an endemic taxon and as such could represent the first documented microbial species for which New Zealand has a conservation responsibility at the global scale. *Apodera angatakere* could therefore be considered as a flagship species for microbial biogeography and conservation.

To discover, or not to discover: that is the question

Columbus thought he had discovered a new world hitherto unknown to Europeans. But it was later established that the Vikings had already made this discovery before him. And of course, the sheer notion of this major discovery disregarded the fact that native populations had already colonized the Americas millennia before Europeans were even able to conceive of the idea of sailing across the ocean.

This history is mirrored in the story of *Apodera angatakere*: Just like Columbus one of us (EM) thought he had discovered a new species only to be brought to the attention by another one of us (SL) that it had been previously described in a publication on microcrustaceans which had escaped the attention of previous researchers studying testate amoebae in New Zealand. But just as the Vikings did not establish a permanent settlement in America, Brehm's discovery was lost to science, or almost so.

The microbial world remains unknown for most of the people but charismatic groups such as testate amoebae are useful as messengers of the invisible dimension of nature's wonders and our impact on the biosphere. The fact that *A. angatakere* is known only from New Zealand and is restricted to ecosystems that have been almost entirely destroyed since European colonization is a perfect illustration of the fact that many species are being lost before we even have a chance to describe them.

Taxonomic actions

Taxonomic summary:

Amorphea Adl et al. 2021

Amoebozoa Lühe 1913, sensu Cavalier-Smith 1998

Tubulinea Smirnov et al. 2005

Elardia Kang et al. 2017

Arcellinida Kent 1880

Difflugina Meisterfeld 2002, sensu Kosakyan et al., 2016

Hyalospheniformes Lahr et al., 2019

Hyalospheniidae (Schultze 1877) Kosakyan et Lara 2012

Apodera angatakere (Brehm, 1928) Mitchell, Blandenier & Duckert 2021

1928 *Nebela penardi* Brehm

Icon: Brehm, 1928 Fig. 52

Description

Test composed of two clearly distinct parts, a subcircular, oval, or ellipsoidal, compressed posterior part (body) and a neck. The two parts separated by a deep constriction around the entire base of the neck. Sides of the neck straight to slightly concave with a bulge at the base in broad view. The margins of the neck sometimes compressed. Body almost circular. Dimensions based on 63 specimens: 53 individuals from

Taranaki Maunga, North Island, four from the Old Ghost Road, South Island, and six from the Keppler Track, South Island: Length (min.–average–max.): 186–208– 226 µm, width: 120–148–167 µm, pseudostome: 39–44–50 µm. Circa 80 µm in breadth.

Etymology

In the Māori language “angatakere” can be translated to “a keeled shell,” referring to the conspicuous keel present on the outline of the test.

Neotype

Brehm did not preserve any specimen, and the original type material is only represented by two simple drawings without indication of size. Because small variations in the morphology of the test can be used to distinguish closely related species, only high magnification microphotographs can be used to accurately represent a species and reliably distinguish it from taxa yet to be described (Duckert et al., 2020; Kosakyan et al., 2016). For this reason, we designate the specimen in pictures Figure 2E as the neotype. As we were unable to find unfractured tests at the previous type locality (Margaret's tarn, Mt. Rolleston near Arthur's pass, New Zealand South Island) the neotype has been designated among a population from Taranaki Maunga, New Zealand's North Island. However, tests from the previous type locality and the ones from Taranaki Maunga were similar in all points. A permanent slide has been deposited at the Natural History Museum of Neuchâtel (Switzerland) with the ID 95-1.

New type locality

Ahukawakawa swamp, along the Pouakai crossing trail, on the saddle between Taranaki Maunga and Pouakai Hut, New Zealand's North Island. Coord: −39.255058°, 174.043106°, Elevation: 921 m a.s.l.

Geographical distribution

Known from New Zealand North and South Islands and Auckland Island. Likely also Campbell and Chatham Islands. We did not find it in Macquarie Island where all Apodera specimens lacked the characteristic hollow keel.

Habitat

Sphagnum and brown mosses in peatlands and alpine wetlands in New Zealand's North and South Islands, mosses in low *Metrosideros* forest (Auckland Island).

Remarks

We did not succeed in obtaining DNA sequences from material collected from the previous type locality (Margaret's tarn, Mt. Rolleston near Arthur's pass, New Zealand South Island) and found only fractured test unfit to be designated as the neotype, we thus chose a specimen from Taranaki Maunga, New Zealand's North Island as the neotype. Given that 13 distinct molecular clades were recorded within Hyalosphenia papilio, a common but smaller species commonly found in Holarctic Sphagnum peatlands (Heger et al., 2013; Singer et al., 2019), it is possible that several cryptic or pseudo-cryptic species exist within A. angatakere and that specimens from Margaret's tarn constitute a distinct species. If this were the case a new species would need to be described from Margaret's tarn, New Zealand South Island with its own type locality. The Auckland Island record is based only on microscopic observation (not illustrated) and is considered valid given the characteristic morphology. Nevertheless, it would need to be further confirmed by molecular data.

Three COI gene sequences of Apodera angatakere and Apodera vas (352–655 bp) were deposited in GenBank under the number MZ615186–MZ615188 and MZ615189–MZ615191, respectively.

ZooBank registration number

urn:lsid:zoobank.org:act:921D8CE1-EF0F-4839-B264-97ED438B5694.

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Chapter 4 - Description of *Phaeobola aeris* gen. nov., sp. nov (Rhizaria, Cercozoa, Euglyphida) Sheds Light on Euglyphida's Dark Matter (published)

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Abstract

The majority of Euglyphida species are characterised by shells with imbricated silica scales. Environmental surveys indicate a large unexplored diversity and recent efforts hinted at a certain diversity of yet undescribed, inconspicuous, scale-lacking Euglyphida. Here we describe *Phaeobola aeris* gen. nov., sp. nov. that shows a variety of morphological characters typical for the Euglyphida but lacks silica scales-instead, this species bears an agglutinated test. Neither its morphology nor phylogenetic placement allows its assignment to any currently described family. We erected the yet monospecific genus *Phaeobola* gen. nov., which with yet available data remain Euglyphida *incertae sedis*.

EUGLYPHIDA are an abundant order of testate amoebae in soil litter, mosses, and freshwater habitats, with some taxa being also found in brackish and marine environments (Heger et al. 2010; Todorov et al. 2009). They secrete a characteristic test (shell), which in most described species is reinforced by silica scales whose size, shape, and arrangement are taxonomically informative (Chatelain et al. 2013; Heger et al. 2010; Kosakyan et al. 2016). Phylogenetic analyses suggested a close relationship of several cercozoan taxa, that is Euglyphida, Thaumatomonadida, Spongomonadida, and others that are able to build silica scales, which were accordingly grouped within the class Imbricatea (Cavalier-Smith and Chao 2003; Scoble and Cavalier-Smith 2014). Scale shape, size, and arrangement vary within Imbricatea. Many Euglyphida bear single-tiered scales agglutinated to a shell with a yellowish to brownish organic cement, while most Thaumatomonadida bear interlocking two-tiered scales with much higher complexity and variation in shape (Dumack and Siemensma 2020; Dumack et al. 2018; Scoble and Cavalier-Smith 2014). The ancestor of Imbricatea is presumed to have possessed more or less oval single-tier scales, which subsequently diversified in shape (Scoble and Cavalier-Smith 2014). The recent discovery of *Kraken*, which bears such scales and branches weakly at the base of Imbricatea and its sister class Thecofilosea, supports this hypothesis (Cavalier-Smith et al. 2018; Dumack et al. 2016, 2017).

However, not all Imbricatea bear scales. Increasing reports of scale-lacking Imbricatea, which do not necessarily have to be closely related, indicate a frequent loss of scales in Imbricatea (Dumack et al. 2019; Howe et al. 2011; Scoble and Cavalier-Smith 2014; Shiratori et al. 2014). Although scales have been considered an important synapomorphy defining the Euglyphida (Meisterfeld 2002), novel findings of scaleless taxa in Euglyphida cast doubt on the validity on the taxonomical value of this trait. The Paulinellidae, for example, include two shelled but scaleless taxa, that is the small marine *Ovulinata parva* and an even smaller terrestrial *Micropyxidiella edaphonis* (Howe et al. 2011; Tarnawski and Lara 2015). These diminutive euglyphids (8–15 µm) have probably gone unnoticed until recently due to their inconspicuous appearance.

Findings of small inconspicuous taxa in an otherwise well-studied group raise the question about a hidden diversity of minute euglyphids that do not exhibit silica scales, especially since metabarcoding studies showed a wealth of Euglyphida-assigned environmental sequences that may represent new families (Lara et al. 2016). One of these sequences even became an indicator taxon of late decomposition stages when dating decay of corpses in a forensic study (Seppey et al. 2016). The identity of these “dark matter” euglyphids remains an open question, as most genera of Euglyphida still have not been sequenced, but can be well placed within known families based on their morphology (Kosakyan et al. 2016). It is well-proven phenomenon that taxonomists tend to describe large species earlier than smaller species (Gaston 1991), a relationship so general that it is used to estimate levels of undescribed arthropod diversity (Stork et al. 2015). A logical conclusion is that Euglyphida-assigned environmental sequences are likely composed of inconspicuous forms, probably small-sized and scaleless. If this hypothesis holds true, a large part of Euglyphid diversity could be composed of these cryptic forms (Lara et al. 2016).

With this study, we add a further element to this debate by describing *Phaeobola aeris,* a new small-sized euglyphid species (about 17 µm in diameter) that lacks silica scales. We discuss its phylogenetic position and the extent of euglyphid “dark matter” that may make these cercozoans the most diverse group of testate amoebae.

Materials and Methods

The amoebae were sampled in August 2018 from sediments of a quarry pond surrounded by reeds, tall grass, and deciduous trees in Cologne Pesch, Germany. The samples were repeatedly screened for filose amoebae with a light microscope (Nikon Eclipse TS100; Ph1; up to 400X). Single cells were isolated and individually transferred into a new well of a 24-well plate filled with Waris-H + Si (Mcfadden and Melkonian 1986) and a mixture of *Nitzschia communis* and *Characium* sp. as prey. Photographs were taken with a Nikon Eclipse 90i (DIC, up to 600X). Unfortunately, the culture died before the time of publication. For electron microscopy, individual cells were washed in distilled water and then in ethanol (70%). The cells were subsequently transferred onto SEM stubs and left overnight in a desiccator. Cells were coated with gold (ca. 8 nm thick film) using a Balzers SCD004 Sputter Coater and observed with a Hitachi S3000N microscope at a tension of 15 kV. For sequencing, single individuals were starved overnight. Approximately 1 µl medium containing a single cell was transferred into a PCR tube containing 4 µl ddH_2O. Subsequently, 4.6 µl PCR mixture was added, including 1.7 µl ddH_2O, 1 µl Thermo Scientific Dream Taq Green Buffer, 1 µl of 10 µM forward and reverse primers each, 0.2 µl 10 µM dNTPs, and 0.1 µl DreamTaq polymerase (Thermo Fisher Scientific, Dreieich, Germany). The SSU rDNA was amplified with the general eukaryotic primers EukA & EukB (Medlin et al. 1988). Using 1 µl of the first PCR as a template, semi-nested reamplifications were conducted with the primer pairs EukA + 963R_Cerco and S616F_Cercomix + EukB (Fiore-Donno et al. 2017). Eight µl of the PCR products were purified by adding 0.15 µl of exonuclease, 0.9 µl FastAP and 1.95 µl ddH_2O and heating the mixture for 30 min at 37 °C, and subsequently for 20 min at 85 °C. The Big dye Terminator Cycle sequencing

Kit (Thermo Fisher Scientific) and an ABI PRISM automatic sequencer were used for the sequencing. The sequence (1,510 bp length) was submitted to the NCBI database as accession number MW186810. The sequence was screened for close relatives via the BLASTn algorithm (V2.3.0) against the NCBI nucleotide database, which indicated a relationship to Euglyphida. Phylogenetic trees of the Euglyphida using several species of Thecofilosea and Sarcomonadea as outgroup (GenBank accession numbers: AF411276, AF411275, AF411265, DQ211597, AF411270, AY496046, AJ418794, DQ303924, AF411 283) were constructed using the RAxML v.8.2.10 (Stamatakis 2014) and MrBayes (Ronquist and Huelsenbeck 2003) as implemented on the CIPRES Portal (Miller et al. 2010). The RAxML analysis was conducted using The GTR + GAMMA model with default settings and 1,000 bootstraps. The MrBayes analysis was conducted using The GTR + GAMMA model with default settings on two independent runs sampled every 100 generations. The analysis was automatically stopped when convergence was reached after 1,065,000 generations resulting in 21,300 trees of which 25% were discarded as the burn-in.

Results and Discussion

Observations and phylogeny

The amoebae bore a roundish to oval test with 17.6 ± 3.4 µm in length and 15.9 ± 2.9 µm in width and an average mean length:width ratio of 1.1 ± 0.2 (*n* = 27). The test was colourless to amber-coloured and covered with organic cement with a rough surface that exhibited attached xenosomes (Fig. 1A, C). The ellipsoid nucleus (mean length: 7.3 ± 0.8 µm; mean width: 5.7 ± 0.8 µm; *n* = 15) with its central and spherical nucleolus (mean length: 3.1 ± 0.7 µm; mean width: 2.7 ± 0.6 µm) was located at the apical end of the cell (opposite to the aperture, Fig. 1D). Central to the cell, a layer of granules formed which surrounded the nucleus in a pattern typical for Euglyphida (Kosakyan et al. 2016). Close to the aperture, contractile and food vacuoles could be observed (Fig. 1D). The filose pseudopodia were used to creep over the surface. They were readily branching and sometimes anastomosing (Fig. 1B). The amoebae fed on both offered species of algae. No cysts were observed.

Phaeobola aeris branched robustly within the Euglyphida (bootstrap = 93%, posterior probability = 0.99), and could not be assigned to any of the already described families. Instead, it branched weakly (B = 29%, PP = 0.96%) together with unidentified environmental sequences retrieved from forest soil (Seppey et al. 2016) However, the phylogenetic position of *P. aeris* is still uncertain because of its long branch.

Taxonomic assignation of *Phaeobola aeris*

Phaeobola aeris exhibits both an unusually distinct morphology and distinct SSU rDNA sequence thus complicating its taxonomic placement. Based on the morphology of this testate amoeba (size, shape, pseudopodia morphology, and zonation of the cell body), it is quite clearly cercozoan and potentially may resemble a distinct lineage in Imbricatea or even its sister group Thecofilosea. Recently, Dumack and Siemensma (2020) discussed extensively differences in the organic cement of thecofiloseans and imbricateans. In brief, imbricatean species may appear often yellowish to brownish, while thecofilosean species are colourless. *Phaeobola aeris* shows a conspicuous gradient of shell colours ranging from colourless over yellowish to brownish specimens, exactly what can be expected for the organic cement of Imbricatea. Based on its morphological appearance, a closer relationship to the Euglyphia may be inferred (Siemensma and Dumack, 2020), and due to its size and shape, it fits well to the diversity of Euglyphida. However, it must be mentioned that *P. aeris* incorporates xenosomes into its shell, unlike any other described Euglyphida species, not even the scaleless genera *Micropyxidiella* and *Ovulinata* (Anderson et al. 1996; Dumack et al. 2018; Howe et al. 2011; Tarnawski and Lara 2015; Fig. 1C). Moreover, the shell of *P. aeris*, which is covered with rough organic cement, is unlike the smoothly covered shells of other Euglyphida (Fig. 1C).

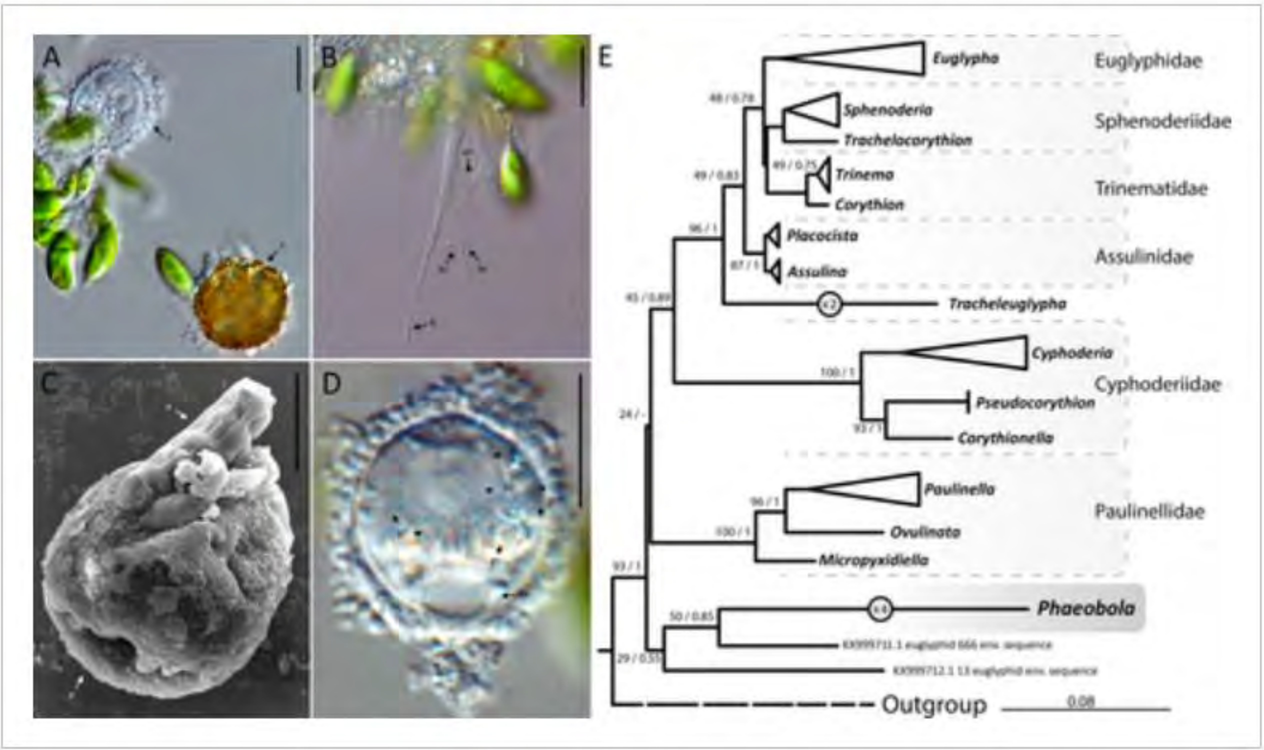

Figure 1 Morphological overview of Phaeobolaaeris (A–D) and phylogenetic placement (E). Scale bars indicate 10 µm; pictures A, B, and D were taken with DIC; picture C was taken with a scanning electron microscope. (A): two differently coloured individuals with xenosomes on the surface of their test, colour difference of individuals is shown, (B): branching and anastomosing network of pseudopodia, (C): surface features are shown, including a coated diatom as xenosome, (D): cellular features with zoning and granular layer, (E): Maximum likelihood phylogenetic tree of the 18S rDNA sequences available on GenBank with the consensus sequence of P. aeris. Bootstrap values and Bayesian posterior probabilities, respectively, are indicated along with the nodes. The minus indicates that this split was recovered as a polytomy with the parent node on the Bayesian phylogeny. As every genus appeared as monophyletic, their branches were collapsed. The branches leading to Tracheleuglypha and Phaeobola have been shortened for clarity. a = aperture; an = anastomosing; br = branching; cv = contractile vacuole; fi = filopodia; gr = granules; nu = nucleus; no = nucleolus; t = test; x = xenosome.

Phaeobola aeris branches robustly within the Euglyphida in our phylogenetic analysis based on SSU rDNA data (support 93% bootstraps, 1 posterior probability). However, it cannot be placed with confidence in any of the described families; indeed, while other Euglyphid families are defined by the complex shape and arrangement of siliceous scales (Chatelain et al., 2013; Lara et al., 2007), *P. aeris* does not possess such structures but has characteristics on its own, that is a shell of rough organic cement and xenosomes. The long branch it produces in SSU rRNA-based trees does not clarify its placement either, at least using a single-gene phylogeny. Based on its morphology, unique amongst known Euglyphida, and on its phylogenetic position, we justify the erection of the genus *Phaeobola* to accommodate *P. aeris*.

Euglyphida's dark matter?

Environmental DNA surveys revealed considerable numbers of Euglyphida-related sequences that could not yet be assigned to any known family (Bass and Cavalier-Smith 2004; Kosakyan et al. 2016; Seppey et al. 2016). It is likely that these organisms, like *P. aeris,* are small and inconspicuous. Lara et al. (2016) defined four environmental clades of Euglyphida in forest litter and mosses (65 OTUs in EEC1-4) that could not be directly assigned to any known group. *Phaeobola aeris* did not branch convincingly within any of them (not shown)—our phylogenetic analysis shows only a weak relationship with two environmental clones obtained in another study (Seppey et al. 2016). The long branch at the base of the *P*. *aeris* sequence in the SSU rDNA tree suggests that related sequences are difficult to retrieve from the environment with broad-spectrum eukaryotic primers. This indicates that in addition to a large fraction of Euglyphida that have been likely overlooked by protistologists due to their inconspicuousness, even more sequences probably escaped molecular detection because of their divergent SSU rDNA gene sequences. Altogether, this suggests the existence of a taxonomically diverse and hitherto unseen majority ("dark matter") of euglyphids, with *P*. *aeris* being the first identified representative.

Taxonomic Acts

Taxonomic summary:

Euglyphida Copeland, 1956.

Euglyphida *incertae sedis*

Phaeobola gen. nov

Diagnosis: Cells roundish to oval shaped. Shell colourless to amber-coloured, rough surface, few xenosomes included.

Etymology: *Phaeobola* (feminine)—derived from the Greek words phaios (=φαιός, brown) and obolos (=ὀϐολός, a coin in ancient Greece; referring to the shape of the organism); the created name was feminised since most shelled amoeba taxa are by tradition feminine. The specific epithet refers to the amber-coloured shell that reminds of a bronze ("aes" in latin) coin.

Type Species: *Phaeobola aeris* sp. nov.

Phaeobola aeris sp. nov.

Diagnosis: Cells exhibit characters as *Phaeobola*. Roundish to oval test, 17.6 ± 3.4 µm in length and 15.9 ± 2.9 µm in width, average mean length:width ratio of 1.1 ± 0.2 µm, ellipsoid nucleus, mean length: 7.3 ± 0.8 µm and width: 5.7 ± 0.8 µm; *n* = 15, central spherical nucleolus, mean length: 3.1 ± 0.7 µm; mean width: 2.7 ± 0.6 µm. Filopodia branching and anastomosing, sometimes reminding of a reticulose network.

Remarks: The genus is so far monotypic. Algivorous, probably bacterivorous as well; thus we consider it as omnivorous.

Type: Specimens depicted in Fig. 1 constitute the type, as illustrations can constitute a type in testate amoebae (Lara et al. 2020); in addition, a stub has been deposited at the Royal Botanical Garden of Madrid RJB-MA-Algae 11249.

Type locality: Quarry pond in Cologne Pesch, Germany; Coordinates: 50.995234, 6.866434

ZooBank registration number: urn:lsid:zoobank.org:pub:6B3F10E1-D014-412C-B8A3-1810AF87E45C

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Chapter 5 - *Hyalosphenia papilio paynei* subsp. nov. - a highly consipicuous, likely recently evolved and localized *Sphagnum* peatland testate amoeba

Running title: *Hyalosphenia papilio paynei* subsp. nov.

This work is preliminary, and the taxonomic acts presented here are not to be considered by the ICZN.

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Abstract

Most microbial biodiversity is undescribed, and the majority of species are morphologically indistinguishable. Notable exceptions are so-called flagship species which are highly conspicuous and can therefore be used to address biogeographical questions. Here we describe *Hyalosphenia papilio paynei*, an arcellinid testate amoeba (Amoebozoa;Arcellinida; Hyalospheniidae) from wet hollows in two *Sphagnum* peatlands, one in Wales and one in Ireland. Phylogenetic analysis based on Cytochrome C oxidase subunit I (COI) sequencing places it within the lineage A of the *H. papilio* complex, but it differs from all 13 known *H. papilio* genetic species by its very distinctive, wider than long, morphology. The fact that such a conspicuous taxon was never reported in hundreds of studies published on Holarctic *Sphagnum* peatlands since Leidy's description of *H. papilio* in 1874 suggests that this subspecies has recently evolved and has not yet dispersed beyond Britain and Ireland. The discovery of this new taxon calls for a more detailed analysis of the morphological, ecological and molecular diversity of the *H. papilio* species complex.

Key words: Testate amoebae, taxonomy, *Hyalosphenia*, dispersal limitations, microbial biogeography

Introduction

Testate amoebae are single-celled amoeboid organisms that build a test (shell). Because of their relatively small size and high abundance, it was presumed that they had high dispersal abilities resulting in an absence of biogeographic patterns and a low diversity. However, morphological and molecular evidence have revealed a high diversity, including cryptic diversity, and geographical distribution patterns within morphospecies in both euglyphids and arcellinids, the two major groups of testate amoebae (Heger et al. 2013, Singer et al. 2019, Lara et al. 2011). The best studied example to date is *Hyalosphenia papilio* (Arcellinida, Hyalospheniidae), a common testate amoeba from Holarctic *Sphagnum* peatlands. Mitochondrial COI barcoding revealed the existence of at least 13 lineages that could each represent a distinct but morphologically indistinguishable species (Heger et al. 2013, Singer et al. 2019). These genetic lineages show contrasted distribution patterns, some being endemic to certain regions while others are found across the Holarctic. It has been suggested that both differences in ecological tolerances (Heger et al. 2013) and dispersal limitations (Singer et al. 2019) have shaped the current distribution of those lineages but the exact drivers of this speciation are not known.

Here, based on morphology and DNA barcoding, we describe *Hyalosphenia papilio paynei*, a new subspecies that is phylogenetically nested within one of the previously documented lineages of *H. papilio* but is morphologically very distinct. Furthermore, in contrast to the Pan Holarctic lineage to which it is associated, *H. papilio paynei* has currently only been found in two peatlands, one in Wales and one in Ireland. We discuss its validity as a subspecies and argue that it possibly evolved due to dispersal limitation rather than environmental adaptation.

Methods

Study sites

Testate amoebae were extracted from *Sphagnum* mosses collected in lawn microforms for which water table depth data were available in the Cors Fochno peatland (Andrews et al. 2022), a raised estuarine *Sphagnum* bog on the coast of North Wales (coordinates 52.501944, -4.010833) which overlays tidal mud flats and formed during the mid-Holocene (Hughes and Schulz 2001), and in Annaghbeg peatland, a well-preserved raised *Sphagnum* bog in the centre of Ireland (coordinates 53.385278, -8.272500) that formed during the early Holocene (Meehan *et al*. 2019; Stastney and Black, 2020). As we did not find specimen of *H. papilio paynei* in the samples collected in Annaghbeg bog where it had been reported in hollow microforms, all analyses were based on specimens found in Cors Fochno. Specimens of *H. papilio paynei* were isolated from three out of 36 sampled sites and observed under an Olympus IX81 inverted microscope and measurements (length and width of the test, width of pseudostome) were taken on 87 individuals.

Molecular analyses and phylogenetic reconstruction

Living cells were isolated individually and DNA extraction was performed following the protocol of Duckert et al. (2018). The PCR amplifications were conducted following the protocol of Singer et al. (2019) using *Hyalosphenia*-specific primers to obtain partial mitochondrial COI gene sequences. A maximum-likelihood phylogenetic tree including the sequences gathered by Heger et al. (2013) and Singer et al. (2019) was built using IQTree2 (Minh et al., 2020) using the best-fit model and 2000 bootstraps.

Results

Morphology

Hyalosphenia papilio paynei has a highly conspicuous shape (Figure 1), being broadly ovate, almost triangular, about twice as wide as the typical *H. papilio* s.str. morphotype. We recorded the following measurements (Table 1, full table in Supplementary Material 1) based on 87 individuals: length: 87.5-132.5 µm (mean 108 µm), breadth: 101-160 µm (mean 127 µm), width of pseudostome: 27.5-65 µm (mean 47 µm). *Hyalosphenia papilio paynei* and *H. papilio* s.str. have distinct length-breadth ratios with no overlap: *H. papilio paynei* 0.72-1.12, mean 0.85; *H. papilio* s.str. 1.29-1.67 (Kosakyan et al. in press). Except for the difference in width, *H. papilio paynei* is very similar to *H. papilio* s.str.

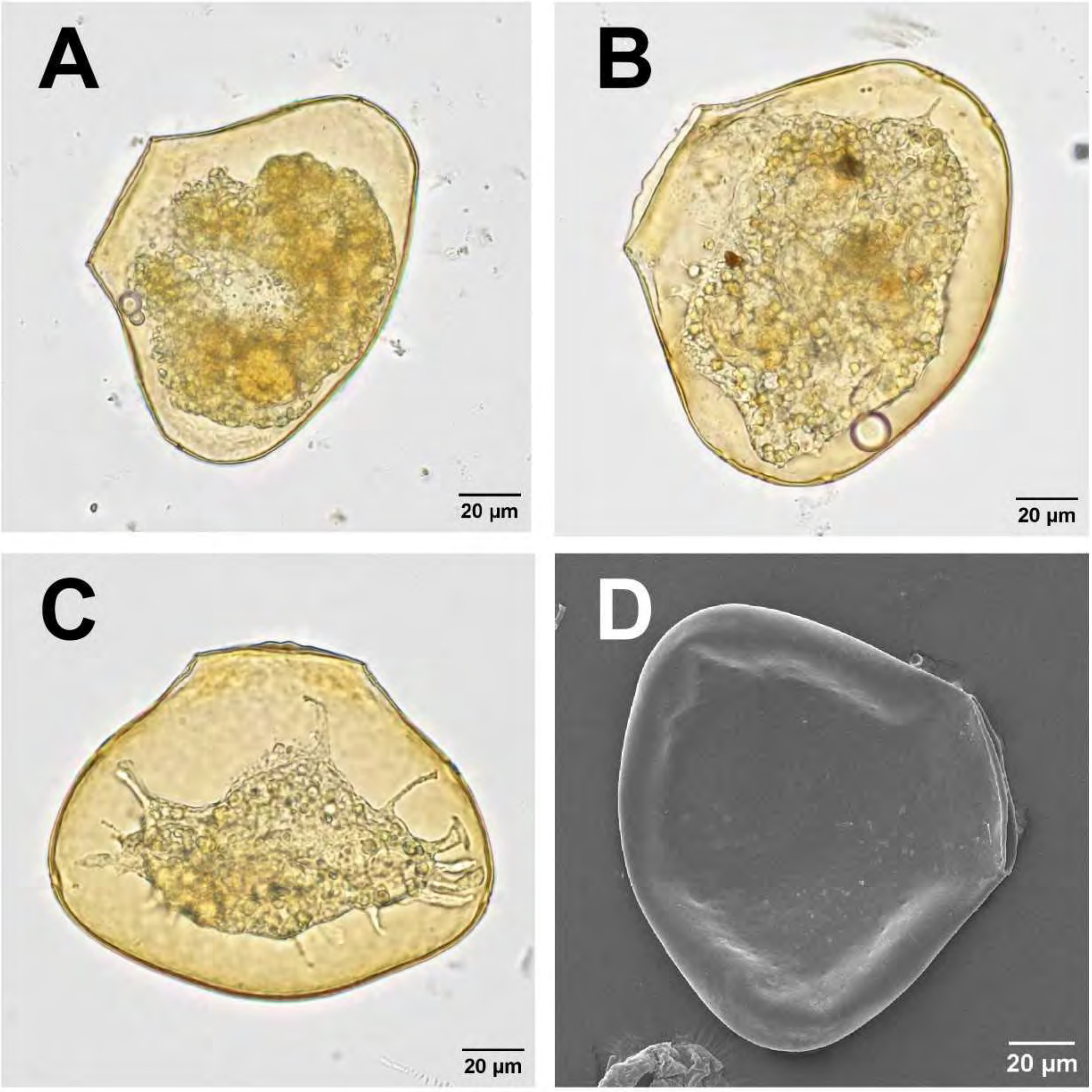

Figure 1. Pictures of four different specimens of Hyalosphenia papilio paynei*, A-C with Light Microscopy (LM) corresponding to barcoded cells 2a4, 2b4, 2b5 respectively and D with Scanning Electron Microscopy (SEM).*

Table 1. Summary of the measurements of 87 specimens of Hyalosphenia papilio paynei*, showing the mean, minimum, maximum, standard deviation and coefficient of variation of the length and width of the test, the width of the pseudostome, and the length-breadth ratio.*

	Mean	**Min**	**Max**	**SD**	**CV (%)**
Length	107.8	87.5	132.3	9.7	9
Breadth	127.0	100.7	160	12.3	9.7
Pseudostome Width	46.9	27.5	65	7.1	15
Length-Breadth Ratio	0.85	0.72	1.12	0.07	8.8

Ecology

In the Cors Fochno peatland, *H. papilio paynei* was found on three of the wettest sites, but we did not find any specimen of *H. papilio paynei* in our samples from Annaghbeg bog despite its presence there being confirmed by a microphotograph (Phil Stastney, pers. comm.) showing a specimen in all points similar to those in Figure 1. This taxon might thus be limited to the wettest parts of the peatland, which might not have been sampled in Annaghbeg. Interestingly, we found only few individuals of the classical *H. papilio* s.str. in our samples from Cors Fochno and none in Annaghbeg peatland despite it being widespread and usually abundant in Holarctic peatlands. We did not find intermediate forms between *H. papilio paynei* and the regular morphotype.

Molecular data and phylogenetic reconstruction

We obtained partial COI gene sequences ca. 450 nucleotides long from 13 individuals using single-cell DNA extractions. eight of these sequences were identical and the 5 others differed only by one nucleotide. In our phylogenetic tree (data not shown), all sequences of *H. papilio paynei* clustered in lineage A of *H. papilio* as defined by Heger et al. (2013) and Singer et al. (2019), and 10 out of 13 formed a monophyletic clade. Despite not appearing as monophyletic, due to this part of the tree being unresolved as some sequences differ by only one nucleotide, each retrieved sequence of *H. papilio paynei* differs from most (but not all) members of lineage A by the same mutation on a single position, which suggests the monophyly of this subspecies. A genetic marker with a faster mutation rate is thus needed to clearly evaluate the evolution of this taxon.

Discussion

The barcoding gap for the COI gene in the Hyalospheniidae family has been estimated to 4% (Singer et al. 2019). Although *H. papilio paynei* is genetically very similar to other members of lineage A of *H. papilio* s.str. and does not reach that 4% threshold, several lines of evidence suggest it should be considered as a valid taxon. The clear morphological differences, the absence of intermediate individuals between *H. papilio paynei* and *H. papilio* s.str. and the probable monophyly of *H. papilio paynei* all suggest that *H. papilio paynei* has, from a certain point, evolved independently from *H. papilio* s.str. which justifies considering it as a distinct subspecies.

The morphological differences between *H. papilio* s.str. and *H. papilio paynei* could be interpreted as an adaptation to local environmental conditions, or possibly a result of phenotypic plasticity. Morphological changes correlated to the intensity of environmental factors have been observed for *H. papilio* s.str. in experimental mesocosm studies (Mulot et al. 2017) or along ecological gradients (water table depth) in natural settings (Booth & Meyers 2010). And phenotypic plasticity has been demonstrated experimentally for other arcellinid testate amoeba genera (Wanner and Meisterfeld 1994, Porfirio-Sousa et al. 2017).

However, as we did not observe any intermediate forms between *H. papilio* s.str. and *H. papilio paynei*, and the shape of *H. papilio paynei* is unlikely to be an on/off response to ecological factors present only in Cors Fochno and Annaghbeg bogs, we rule out the hypothesis of phenotypic plasticity.

This leaves an open question: "why did we observe only so few *H. papilio* s.str. in Cors Fochno and none from Annaghbeg bogs?", as these two peatlands do not seem to differ ecologically from other peatlands in Britain and Ireland where *H. papilio* is commonly reported (Amesbury et al. 2016) or from the other Holarctic sites sampled in Heger's and Singer's studies. The fact that *H. papilio* s.str. was rare in our samples from Cors Fochno and absent from Annaghbeg peatland, despite having been found in abundance in several other peatlands in Wales and other regions of the British Isles and Ireland (Cash and Hopkinson 1909) suggests either of two things: 1) Hyalospheniid testate amoebae disperse frequently and several lineages of *H. papilio* s.str. could have reached either Cors Fochno or Annaghbeg peatland, however *H. papilio paynei* (or the ancestor of what will become *H. papilio paynei*) has displaced *H. papilio* s.str. or prevented subsequent colonization by competitive exclusion. 2) Successful dispersal events are rare, and since these peatlands formed, they were colonized by *H. papilio paynei* (or its ancestor) which was then genetically isolated from *H. papilio* s.str. which arrived later. As Heger et al. (2013) and Singer et al. (2019) found up to four lineages (each possibly a distinct species) of *H. papilio* s.str. in the same samples, exclusion by competition seems unlikely. Additionally, these studies showed that *H. papilio* s.str. is rather limited in its dispersal abilities, we thus favor the second hypothesis. A detailed exploration of peatlands in the region would be useful to clarify if this taxon is endemic to only two (relatively) distant sites, which would be truly remarkable or if, more likely, it occurs in other sites in Britain and Ireland. Furthermore, analyzing subfossils test from peat cores in both sites and peatlands in between where this taxon might have occurred, but was lost could reveal which of the two morphotypes was there first.

The case of *H. papilio paynei* is likely not unique and it is possible that a non-negligible fraction of the testate amoeba diversity is composed of hyper endemic species that diverged only recently. However, most of these might be morphologically indistinguishable from their parental lineage and formally describing these would not be useful. Such cryptic and pseudo-cryptic microspecies pose challenges for testate amoeba taxonomy but as shown in the case of *H. papilio* s.str. (Heger et al. 2013, Singer et al. 2019) and the *Nebela tincta* complex (Singer et al. 2018) they are nevertheless useful models for the study of microbial biogeography and ecology.

Conclusion

Testate amoebae are a relatively well-studied group of free-living microorganisms but nevertheless some highly conspicuous taxa remain to be discovered, as recently shown with the examples of *Arcella peruviana* (Reczuga et al. 2015), *Arcella gandalfi* (Féres et al. 2016), *Apodera angatakere* (Duckert et al. 2021) and *Certesella larai* (Bobrov et al. 2022). While these examples may not be very surprising given that they were found in relatively poorly studied regions, *Hyalosphenia papilio* is one of the best studied testate amoeba taxon, and it is common in northern *Sphagnum* peatlands, arguably the most intensively studied ecosystem for testate amoebae for decades. The odds of finding a new taxon, especially a large and conspicuous one, were therefore low. This finding should motivate anyone studying testate amoebae to look for unusual morphospecies and report them. *Hyalosphenia papilio paynei* is so conspicuous that it should be easy to establish its geographical distribution and ecology accurately and we therefore call anyone studying peatland testate amoebae to report this new taxon, and ideally collect material for DNA sequencing. This is clearly a golden age for protistology and microbial biogeography.

Taxonomic actions

Description of new subspecies: *Hyalosphenia papilio paynei* DUCKERT, GREEVES, BLANDENIER, PAYNE, MITCHELL

Taxonomic summary:

Arcellinida Kent 1880.

Hyalospheniidae (Schultze 1877) Kosakyan et Lara 2012.

Hyalosphenia (Stein 1857) Schultze 1877

Hyalosphenia papilio Leidy 1874

Hyalosphenia papilio paynei subsp. Nov.

Description: Test proteinaceous, rigid, diaphanous, yellowish, lacking idiosomes or xenosomes, compressed, slightly wider than long, broadly ovate in broad view, almost triangular, with a maximal width at about one quarter from the fundus with concave sides tapering towards the aperture, sometimes with a prominent fundus. Pseudostome slightly convex in broad view, no distinct neck. Lateral pores usually present at the maximal width. Algae endosymbionts (*Chlorella*) present in the cytoplasm. Measurements: length=87.5-132.5 µm (mean 108 µm); breadth=100.5-160 µm (mean 127 µm); Width of the pseudostome=27.5-65 µm (mean 47 µm).

Differential diagnosis: *Hyalosphenia papilio paynei* can be easily distinguished from other members of the *H. papilio* species complex by its length-breadth ratio: *H. papilio paynei* (0.72-1.16, mean 0.85), *H. papilio* (1.29-1.67).

Etymology: We dedicate this subspecies to our esteemed colleague Richard Payne who first reported this taxon and tragically died in a mountaineering accident in the Indian Himalaya in May 2019.

Type: We declare the specimen in Figure 1.A as the type.

Type locality: Cors Fochno peatland in Wales. Coordinates: 52.501944, -4.010833

The COI gene sequences of *H. papilio paynei* have been submitted to GenBank under the accession number:XXXX **Accession numbers will be added once the article is accepted**

All LSID actions will be added once the article is accepted

LSID number for the taxonomic action:XXXX

LSID number for this publication:XXXX

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Supplementary material 1. Full measurement table.

Specimen	Length	Breadth	Pseudostome width	Length-Breadth Ratio	Pore number
1	122.5	151.3	55.0	0.81	5
2	122.5	131.3	57.5	0.93	2
3	97.5	122.5	46.3	0.80	2
4	101.3	123.8	41.3	0.82	2
5	125.0	142.5	55.0	0.88	3
6	117.5	145.0	52.5	0.81	4
7	126.3	130.0	55.0	0.97	2
8	123.8	145.0	55.0	0.85	3
10	112.5	130.0	42.5	0.87	2
11	107.5	126.3	46.3	0.85	2
12	105.0	121.3	40.0	0.87	2
13	115.0	160.0	57.5	0.72	3
14	100.0	131.3	41.3	0.76	2
15	97.5	133.8	42.5	0.73	2
16	128.8	137.5	60.0	0.94	2
17	108.8	125.0	32.5	0.87	2
18	102.5	120.0	45.0	0.85	2
19	95.0	120.0	42.5	0.79	2
20	121.3	145.0	51.3	0.84	2
21	110.0	117.5	47.5	0.94	2
22	108.8	120.0	45.0	0.91	3
23	103.8	122.5	40.0	0.85	3
24	122.5	140.0	45.0	0.88	3
25	98.8	125.0	45.0	0.79	2
26	102.5	118.8	43.8	0.86	5
27	110.0	113.8	41.3	0.97	2
28	100.0	120.0	45.0	0.83	2
29	102.5	112.5	40.0	0.91	1
30	100.0	127.5	45.0	0.78	2
31	106.3	120.0	27.5	0.89	2
32	102.5	125.0	43.8	0.82	2
33	100.0	115.0	43.8	0.87	2
34	97.5	112.5	43.8	0.87	2
35	113.8	137.5	48.8	0.83	1
36	112.5	142.5	53.8	0.79	4
37	105.0	112.5	45.0	0.93	2
38	102.5	137.5	51.3	0.75	1
39	107.5	131.3	60.0	0.82	2
40	105.0	125.0	27.5	0.84	2
41	87.5	115.0	38.8	0.76	2
42	107.5	137.5	52.5	0.78	2
43	123.8	137.5	62.5	0.90	3
45	120.0	138.8	65.0	0.86	2
46	117.5	135.0	47.5	0.87	4
47	112.5	140.0	55.0	0.80	3
48	112.5	137.5	52.5	0.82	2
49	123.8	126.3	50.0	0.98	3

50	100.0	120.0	42.5	0.83	2
51	95.0	110.0	43.8	0.86	2
52	97.5	105.0	47.5	0.93	2
53	107.5	117.5	46.3	0.91	2
54	100.0	120.0	42.5	0.83	2
55	98.8	115.0	43.8	0.86	3
56	105.0	120.0	43.8	0.88	2
57	104.2	122.9	44.1	0.85	2
58	105.3	122.1	43.1	0.86	3
59	110.4	142.3	49.6	0.78	3
60	97.9	118.0	42.8	0.83	2
61	94.8	129.4	46.4	0.73	3
62	107.5	114.4	40.4	0.94	2
63	92.7	102.1	37.7	0.91	5
64	114.8	133.7	48.1	0.86	3
65	98.5	124.0	42.9	0.79	2
66	101.3	116.8	43.9	0.87	2
67	94.0	114.9	40.5	0.82	3
68	103.1	122.8	45.7	0.84	3
69	97.6	109.0	36.9	0.90	2
70	112.3	100.7	43.5	1.12	2
71	115.0	144.2	50.6	0.80	7
72	113.5	144.3	54.9	0.79	4
73	113.7	124.4	44.5	0.91	2
74	114.3	103.4	41.2	1.11	2
75	113.9	148.1	50.0	0.77	2
76	96.3	125.6	40.0	0.77	2
77	94.5	114.3	40.4	0.83	3
78	124.4	124.5	59.6	1.00	2
79	95.6	112.9	42.6	0.85	2
80	116.0	139.5	52.4	0.83	3
81	116.3	137.6	55.5	0.85	4
82	112.9	145.0	58.4	0.78	2
83	108.4	114.6	39.8	0.95	2
84	106.3	141.5	51.9	0.75	2
85	132.3	132.4	52.9	1.00	5
86	105.7	138.7	52.2	0.76	2
87	102.3	136.3	52.0	0.75	2
88	96.3	120.4	46.0	0.80	3
89	112.0	133.4	50.3	0.84	2

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Chapter 6 – *Certesella larai* (Amoebozoa: Arcellinida: Hyalospheniformes) a new soil testate amoeba species from the Dominican Republic and Chile challenges the definition of genera *Certesella* and *Porosia* (Published)

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Testate amoebae, Hyalospheniidae, genus *Porosia*, genus *Certesella*, forest litter, peat bog

Abstract

Microbial diversity is known to be huge but remains only partly documented. Testate amoebae are a relatively well studied group of free-living protists that build morphologically characteristic shells on which the taxonomy of this group is mainly based. Among testate amoebae, the Hyalospheniformes are especially well documented and are a model group for microbial biogeography. Here we describe a new species of genus *Certesella* from *Sphagnum* mosses and forest litter samples collected in the Dominican Republic and Chile. We name this species *Certesella larai* to honour our esteemed colleague Enrique Lara. This species bears two large pores connected by tubes, which are characteristic of the genera *Porosia* and *Certesella*. The new species fits best in the latter genus given the presence of a distinct, sometimes denticulated, neck with a bulge that is absent in genus *Porosia* but occurs in *Certesella*. Its elongated shape (length/breadth ratio 2.03–2.58) only overlaps with *C. certesi*, which is distinguishable by the presence of a distinct longitudinal groove in the neck region that is absent from the new species. Interestingly most specimens lacked the internal teeth which are usually present in members of genus *Certesella*. As such, while the morphology of *C. larai* strongly suggests a belonging to genus *Certesella*, molecular analyses are still required to confirm its phylogenetic position, clarify the relationships among all species in genera *Porosia* and *Certesella* and the definition and validity of these two genera. The presence of this new species in two rather distant regions – Caribbean and central Chile – suggests it is likely widespread, but possibly rare. However, this perception may be due to a sampling bias in favour of *Sphagnum* mosses and wet and organic-rich habitats. This illustrates the need for a more systematic sampling effort to document the full and mostly unknown taxonomic diversity of soil protists.

Introduction

Testate amoebae are a group of free-living protists commonly found in terrestrial and aquatic habitats. They build characteristic shells (tests) whose morphology and dimensions allow species identification. These tests are well preserved in dry soil, moss samples, lake sediments, peat and permafrost, making them useful proxies in palaeoenvironmental studies (Charman 2001; Ilyashuk et al. 2006; Patterson et al. 2012; Swindles et al. 2019). Testate amoebae are also increasingly used as model organisms for microbial biogeography and several genera of hyalospheniid testate amoebae (e.g., genera *Apodera, Certesella, Alocodera*) are regularly cited as examples of non-cosmopolitan distribution in support for the vicariance hypothesis (Heger et al. 2011b; Smith et al. 2008).

About 2000–3000 testate amoeba taxa have been described (Meisterfeld 2002a, b). However, it is currently understood that the vast majority should be treated as complexes of distinct species and that their global diversity is likely much higher. Despite the wealth of chorological data on testate amoebae, many knowledge gaps exist including basic taxonomy and distribution of taxa. This is well illustrated by the few extensive reviews for some regions (Beyens et al. 2016; Beyens et al. 1995; Bobrov et al. 2013; Fernández et al. 2016; Fernández et al. 2015; Qin et al. 2011). Indeed, surveys of testate amoebae in under-studied regions regularly lead to the description of new taxa, which are often highly conspicuous (Bobrov et al. 2015; Féres et al. 2016; McKeown et al. 2021; Reczuga et al. 2015).

In the context of on-going studies on the diversity and geographical distribution of the Circum-Australian group of testate amoebae as well as their morphological and morphometric variability, here we report the finding of a new species in genus *Certesella* from forest litter and peatland samples collected in the Dominican Republic and Chile. This species possesses traits of genera *Certesella* and *Porosia* and call for further studies to clarify the validity of these genera.

Materials and Methods

Three samples in which the new species was found were collected in the Dominican Republic and Chile (Table 1). Testate amoebae were extracted from filtered litter or moss samples by shaking the sample in water and then filtering it over a ca. 250 µm mesh. The specimens of interest were isolated with a narrow pipette. The cells were observed and imaged using an Axioplan 2 (Carl Zeiss) light microscope under 200× and 400× magnifications, an inverted DIC microscope Olympus IX81 under 400–1000× magnifications, and a scanning electron microscope Jeol 6060 at a voltage of 20 kV for a detailed study of the structure of the shells.

Nine measurements were taken for shell characters and the length to width ratio calculated (Fig. 1). The average, standard deviation, standard error and coefficient of variation of all measurements were calculated for one population from Chile and one from the Dominican Republic and for the two populations combined. These measurements were compared to the morphometrical measurements reported for known species in genera *Porosia* (Bobrov et al. 2015) and *Certesella*.

Results

Companion species and ecology

The communities of testate amoebae from two different geographical regions in which the new *Certesella* species was found, despite differences in species composition, are characterised by the presence of sphagnobiont and hygro-hydrophilic species from the genera *Difflugia, Pontigulasia, Nebela, Argynnia, Heleopera, Hyalosphenia,* and *Padaungiella* indicating moist to wet conditions (Table 2).

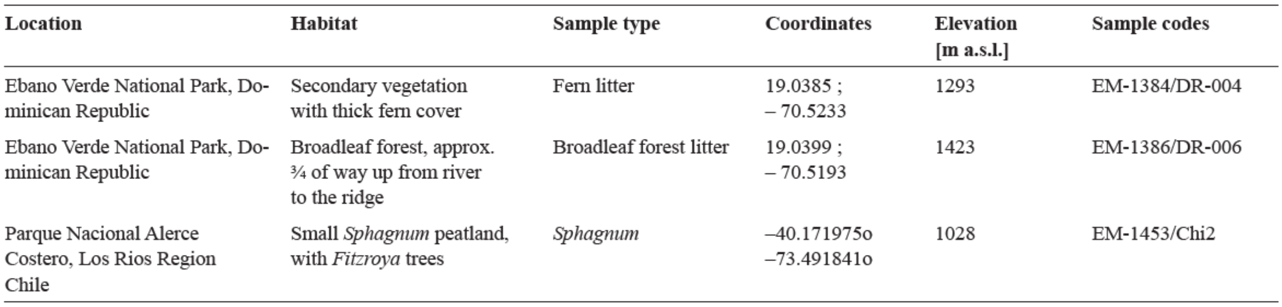
Taxonomic Description

Certesella larai sp. nov. (Fig. 1–7, Sup. Fig. 1; Table 3)

Description: Shell elongated pear-shaped (Figs. 1,2,4 & Sup. Fig. 1), compressed with two large, rounded to irregular-elongated elliptical pores clearly visible in broad view, connected by tubes. No visible lateral pore. Punctuations and inner teeth on the neck usually absent but visible in some specimens (Fig. 3). In broad view shell outline oval elongated at the fundus (aboral end of the shell), then narrowing slightly until a bulge near the area of the two large pores where the sides are approximately parallel, and narrowing again at the base of the neck. Neck relatively short with a slight bulge and then again approximately parallel near the aperture (Figs. 1,2 & 4). Shell colourless, covered with oval plates of different sizes and shapes (Fig. 4), most likely recycled idiosomes from euglyphid testate amoebae. Pores surrounded by smaller recycled idiosomes than the main body (Fig. 4). Pseudostome rim smooth and somewhat wavy, lip 2–2.5 µm thick (Figs. 2–4).

Measurements: Shell length: 135.8–153.4 µm, shell breadth: 56.8–68.2 µm, shell depth ca. 50 µm, aperture maximum dimension: 24.0–29.8 µm, distance from the fundus to the pores: 73.0–92.3 µm, distance from the fundus to the base (narrowest point) of the neck: 104.6– 125.0, distance between the pores: 22.8–34.1 µm, width of the neck at its narrowest point – 18.5–29.5 µm, pore length – 5.1–12.8 µm, pore width – 3.0–6.4 µm, shell length/breadth ratio 2.03–2.58 (Table 3). The variability of morphological characteristics is generally low (e.g., <6% for characters 1–6 Table 3), and only somewhat higher for the smallest dimensions for which the accuracy of measurements is lower.

Table 1. Description of the sampling locations.



Location	Habitat	Sample type	Coordinates	Elevation [m a.s.l.]	Sample codes
Ebano Verde National Park, Dominican Republic	Secondary vegetation with thick fern cover	Fern litter	19.0385 ; – 70.5233	1293	EM-1384/DR-004
Ebano Verde National Park, Dominican Republic	Broadleaf forest, approx. ¾ of way up from river to the ridge	Broadleaf forest litter	19.0399 ; – 70.5193	1423	EM-1386/DR-006
Parque Nacional Alerce Costero, Los Rios Region Chile	Small *Sphagnum* peatland, with *Fitzroya* trees	*Sphagnum*	–40.171975o –73.491841o	1028	EM-1453/Chi2

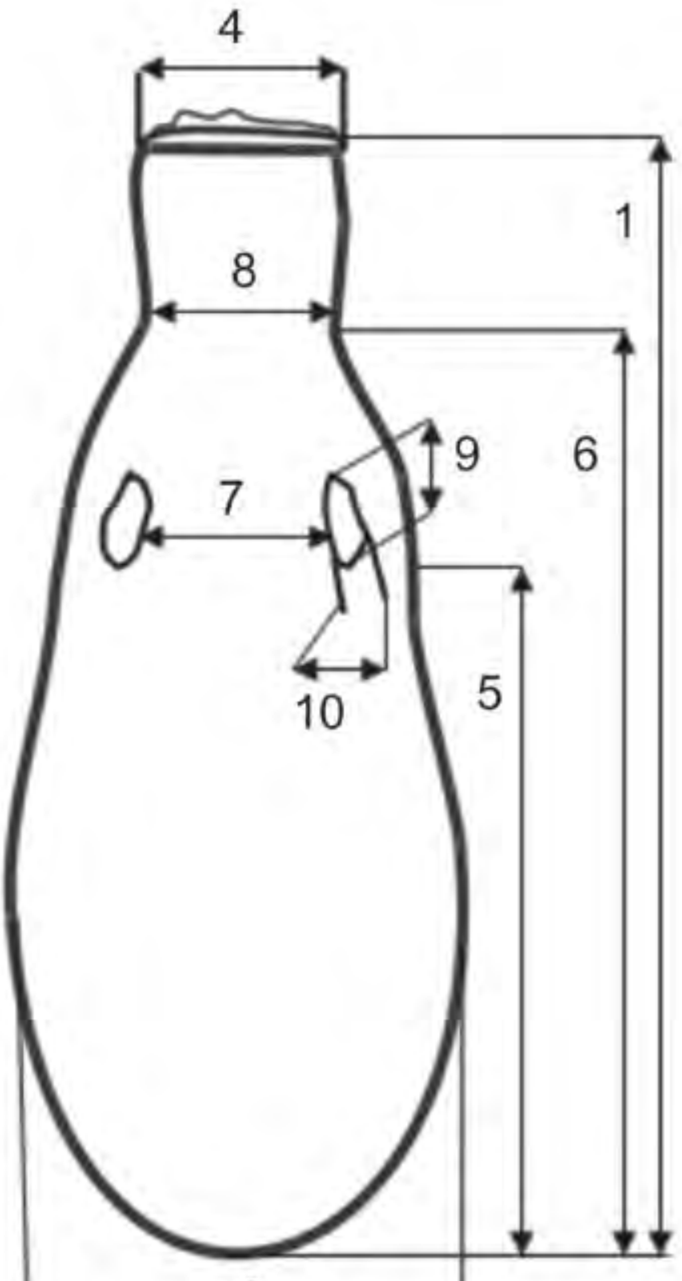


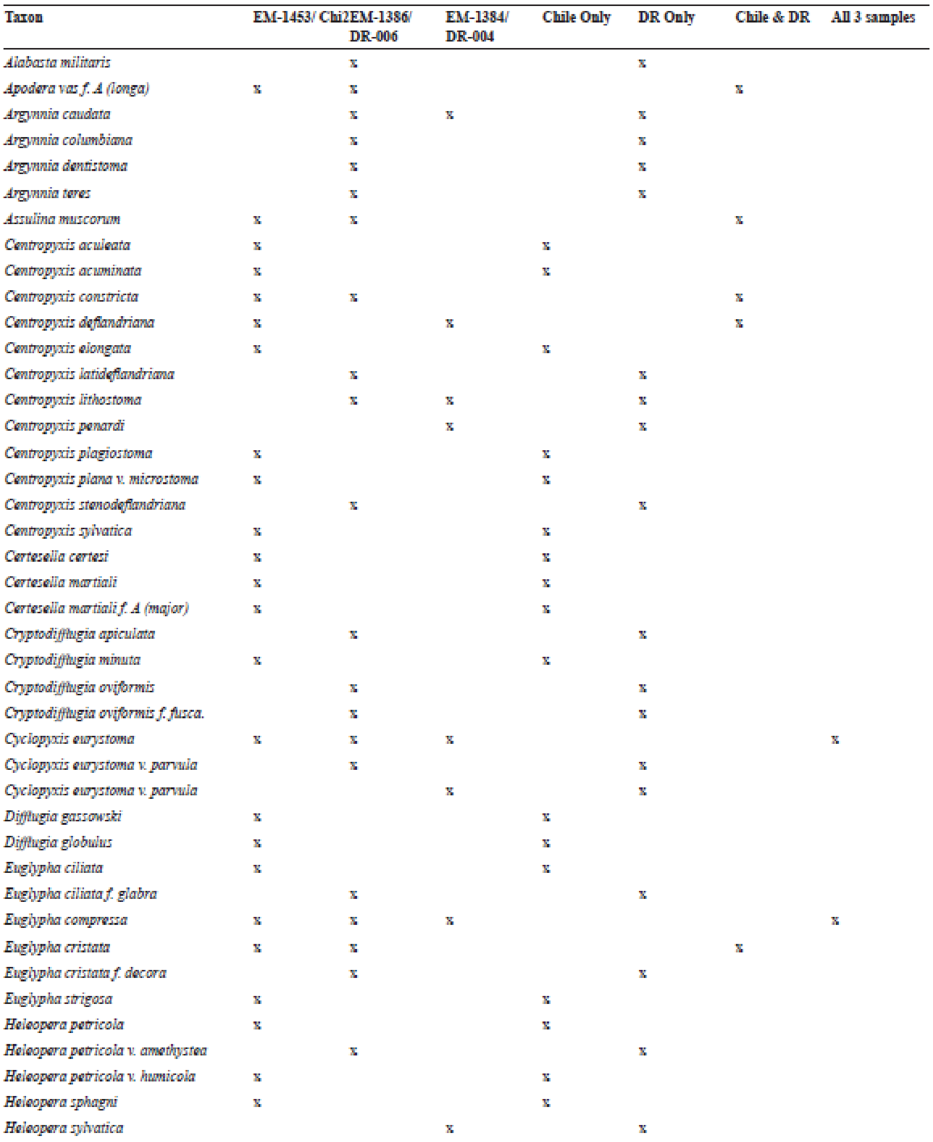
Fig. 1. General shape of Certesella larai *n.sp. and indication of the morphometrical measurements. 1 – shell length, 2 – shell breadth, 3 – shell length / breadth ratio (not illustrated), 4 – aperture (long axis), 5 – distance from fundus to the pores, 6 – distance from fundus to base of neck, 7 – distance between the pores, 8 – width of the neck at narrowest point, 9 – pore length, 10 – pore width.*

Type locality and habitat: Parque Nacional Alerce Costero, Los Ríos Region, Chile, Small *Sphagnum* peatland, with *Fitzroya* trees. In *Sphagnum*. Coordinates: –40.171975°; –73.491841°, elevation 1028 m. a.s.l., EM-1453 / Chi2. Locality of the paratype specimen: Ebano Verde National Park, Dominican Republic. Broadleaf forest litter Forest approximatively three quarters of way up from the river to the ridge. Elevation 1423 m. above sea level. Coordinates: 19.0399°; –70.5193°.

Type specimen: Type: Natural History Museum of Neuchâtel, Rue des Terreaux 14, 2000 Neuchâtel, Switzerland, slide No. 95–2. Paratype: Laboratory of Soil Bioindication, Department of Soil Geography, Faculty of Soil Science, Lomonosov Moscow State University, slide No. 4–2020.

Etymology: The species name was chosen to honour our esteemed colleague Enrique Lara, as a recognition for his major contribution to the molecular taxonomy and phylogeny of testate amoebae. We believe he may be amused by the fact that we name in his honour a species which is not straightforward to classify based solely on morphological data and clearly calls for molecular phylogenetic analyses … to which he has so greatly contributed.

Table 2. Occurrence of testate amoeba taxa associated to *Certesella larai* in three samples from Chile and the Dominican Republic.



Taxon	EM-1453/ Chi2	EM-1386/ DR-006	EM-1384/ DR-004	Chile Only	DR Only	Chile & DR	All 3 samples
Alabasta militaris		x			x		
Apodera vas f. A (longa)	x	x				x	
Argynnia caudata		x	x		x		
Argynnia columbiana		x			x		
Argynnia dentistoma		x			x		
Argynnia teres		x			x		
Assulina muscorum	x	x				x	
Centropyxis aculeata	x			x			
Centropyxis acuminata	x			x			
Centropyxis constricta	x	x				x	
Centropyxis deflandriana	x		x			x	
Centropyxis elongata	x			x			
Centropyxis latideflandriana		x			x		
Centropyxis lithostoma		x	x		x		
Centropyxis penardi			x		x		
Centropyxis plagiostoma	x			x			
Centropyxis plana v. microstoma	x			x			
Centropyxis stenodeflandriana		x			x		
Centropyxis sylvatica	x			x			
Certesella certesi	x			x			
Certesella martiali	x			x			
Certesella martiali f. A (major)	x			x			
Cryptodifflugia apiculata		x			x		
Cryptodifflugia minuta	x			x			
Cryptodifflugia oviformis		x			x		
Cryptodifflugia oviformis f. fusca.		x			x		
Cyclopyxis eurystoma	x	x	x				x
Cyclopyxis eurystoma v. parvula		x			x		
Cyclopyxis eurystoma v. parvula			x		x		
Difflugia gassowski	x			x			
Difflugia globulus	x			x			
Euglypha ciliata	x			x			
Euglypha ciliata f. glabra		x			x		
Euglypha compressa	x	x	x				x
Euglypha cristata	x	x				x	
Euglypha cristata f. decora		x			x		
Euglypha strigosa	x			x			
Heleopera petricola	x			x			
Heleopera petricola v. amethystea		x			x		
Heleopera petricola v. humicola	x			x			
Heleopera sphagni	x			x			
Heleopera sylvatica			x		x		

Hyalosphenia minuta		x			x		
Hyalosphenia subflava			x		x		
Hyalosphenia subflava f. A (major)		x			x		
Hyalosphenia undans		x			x		
Lesquereusia modesta	x			x			
Nebela barbata	x			x			
Nebela collaris		x			x		
Nebela parvula		x			x		
Padaungiella tubulata	x	x	x			x	
Physochila tenella	x			x			
Plagiopyxis labiata		x			x		
Pontigulasia spectabilis	x			x			
Pseudodifflugia gracilis v. terricola		x			x		
Schoenbornia humicola	x	x	x				x
Sphenoderia fissirostris	x	x	x				x
Trigonopyxis arcula	x	x				x	
Trigonopyxis microstoma	x			x			
Trinema complanatum	x		x			x	
Trinema lineare	x	x	x			x	
Trinema lineare v truncatum	x			x			
Trinema lineare v. minuscula		x			x		
Trinema lineare v. terricola	x			x			
Valkanovia elegans	x			x			
Total number of taxa	38	34	14	25	27	9	4

Fig. 2. Light microscopy images of Certesella larai *n.sp. from Parque Nacional Alerce Costero, Los Ríos Region, Chile. Left: DIC image of the type specimen deposited at the Natural History Museum of Neuchâtel (slide 95–2). Centre and right: brightfield images of specimens from the same sample. Scale bar 20 µm.*

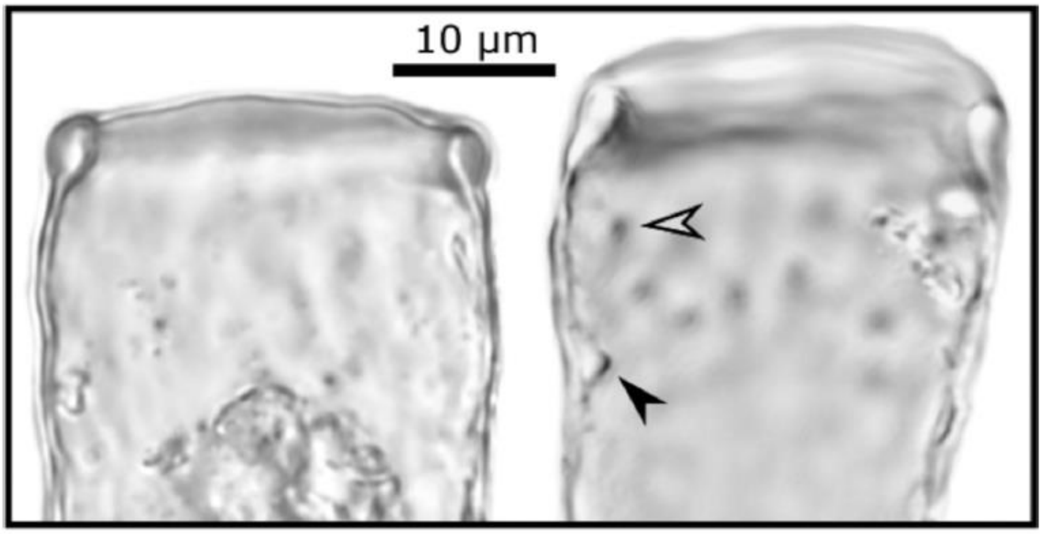


Fig. 3. Light microscopy images of Certesella larai *n.sp. from Parque Nacional Alerce Costero, Los Ríos Region, Chile, showing the detail of the pseudostome. The right image shows internal teeth fully visible on the flank of the neck (solid arrow and visible only in transparency (empty arrow). Such structures are only visible in ca. 10% of the specimens. Scale bar 10 µm.*

Comparison between the two populations: The two populations largely overlap in length and width (Fig. 5) and do not differ either in general shape (Sup. Fig. 1) and other characters (not illustrated).

Related species: There is no possible confusion with *C. australis* and *C. murrayi* as their shape is very different (Figs. 6 & 7). The two most similar species are *C. certesi* and *C. martiali*. The shell of the new species is more elongated (length/width ratio: 2.03–2.58) than *Certesella martiali* (1.83-1.90). The length/width ratio overlaps with C. *certesi* (range 1.75–2.92 based on the original description and several subsequent studies (Certes 1889; Deflandre 1936; Heinis 1914; Penard 1911). The contrast is even clearer with the two known species of the genus *Porosia – P. bigibbosa* and *P. paracarinata* (Table 4). Indeed, the dimensions of the new species do not overlap with any known species of genera *Certesella* and *Porosia* in a biplot of length vs. width (Fig. 7). The somewhat irregular, wavy pseudostome lip of *Certesella larai* may be specific to this species; but this should be assessed by a thorough comparative morphological study.

The outline of *Certesella larai* is similar to other species in genus *Certesella* (Figs. 6 & 7). However, most specimens lack the inner teeth on the neck that are considered as one of the characteristic features of this genus. While we consider it unlikely that the lack of internal teeth is an effect of phenotypic plasticity as observed for shell size in *Hyalosphenia papilio* (Mulot et al. 2017), we evaluate that the new species fits best in genus *Certesella*.

Zoobank registration number:

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Discussion

Taxonomy

Certesella larai clearly differs in shape and size from all known species of genera *Certesella* and *Porosia* and although we currently lack molecular data, we are therefore confident that this represents a new species. However, the taxonomic position of this new species is unclear from morphology alone and indeed we shifted back and forth between placing this new species into genera *Certesella* or *Porosia*.

The presence of internal teeth on the neck in *Certesella* is considered a major criterion to distinguish it from genus *Porosia* (Tsyganov et al. 2016). Most specimens did not have internal teeth on the neck, which would place this species into genus *Porosia*. However, some individuals (< 10%) did present minute denticulations that, in addition to the general shape and especially the presence of a well-developed neck allowed assigning this species to genus *Certesella* (Fig. 3). Those denticulations, which are often only visible as dark dots, are difficult to observe without high magnification and are clearly absent from most specimens we observed. However, the fact that most specimens of *C. larai* do not present this trait questions the validity of this criterion and blurs the limit between the two genera, as it implies that species closer to *Certesella* than *Porosia* could completely lack internal denticulations.

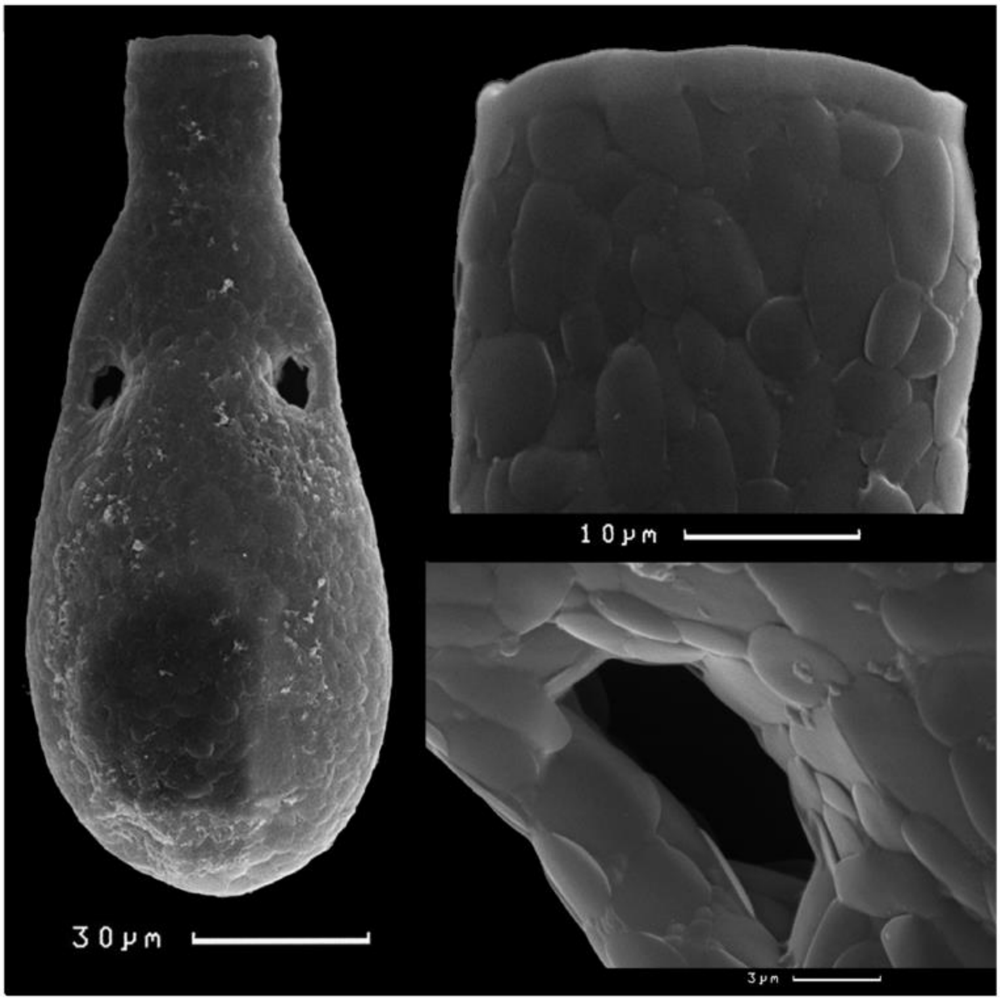
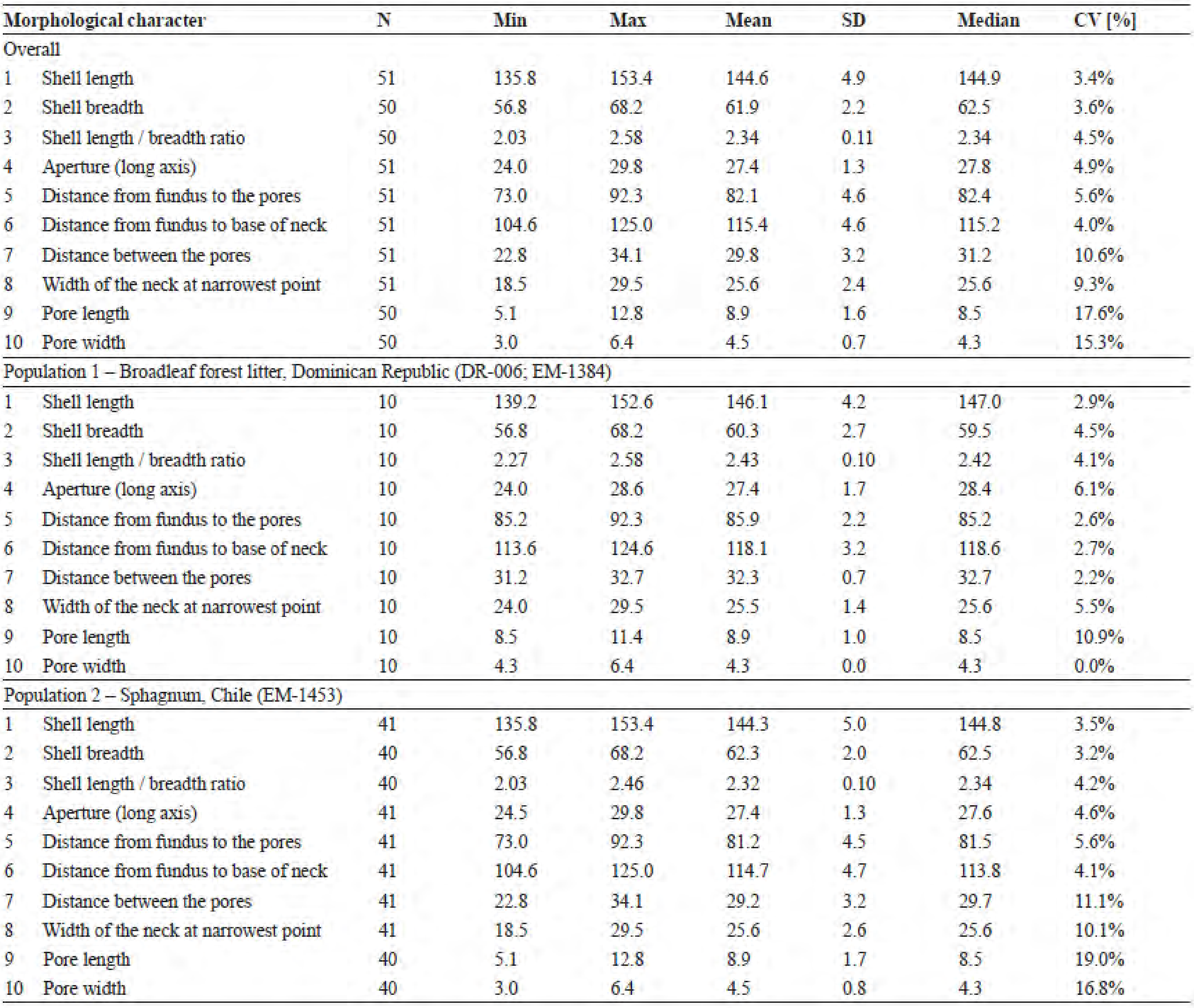


Fig. 4. Certesella larai *n.sp. scanning electron microscopy images showing the overall shape, the chitinous rim of the pseudostome, the detail of an elongated pore and the plates (presumably recycled from euglyphid testate amoebae) covering the shell.*

Among *Certesella*, the overall shape of *C. larai* is most similar to *C. certesi*, and this is an additional reason to place it in the genus *Certesella*. But *C. larai* lacks the characteristic longitudinal groove of *C. certesi*. It can be added that the range of illustrated morphotypes for *Certesella certesi* suggests this taxon corresponds to a species complex unified by this characteristic longitudinal groove rather than a single species.

The phylogenetic relationship of *Certesella* and *Porosia* remains unclear and currently only *Certesella martiali* has been characterized using DNA barcoding (Kosakyan et al. 2012). Thus, *Certesella* and *Porosia* should be high priority targets for future taxonomic efforts using molecular tools. Furthermore, as *Certesella* has only been found South of the Cancer Tropic while *Porosia* is cosmopolitan, a phylogenetic reconstruction including those two genera would be welcome to clarify the biogeographical history of arcellinid testate amoebae. The outcome may show that *Porosia* is the ancestral type and that *Certesella* is a younger clade, possibly nested within *Porosia* that originated in Gondwana after the breakup of Pangea, which would be consistent with an inferred origin of hyalosphenids at ca. 370 Mya (Lahr et al. 2015).

Table 3. Biometrical measurement (in mm) of *Certesella larai* from two populations from the Domincan Republic and Chile.



Morphological character	N	Min	Max	Mean	SD	Median	CV [%]
Overall							
1 Shell length	51	135.8	153.4	144.6	4.9	144.9	3.4%
2 Shell breadth	50	56.8	68.2	61.9	2.2	62.5	3.6%
3 Shell length / breadth ratio	50	2.03	2.58	2.34	0.11	2.34	4.5%
4 Aperture (long axis)	51	24.0	29.8	27.4	1.3	27.8	4.9%
5 Distance from fundus to the pores	51	73.0	92.3	82.1	4.6	82.4	5.6%
6 Distance from fundus to base of neck	51	104.6	125.0	115.4	4.6	115.2	4.0%
7 Distance between the pores	51	22.8	34.1	29.8	3.2	31.2	10.6%
8 Width of the neck at narrowest point	51	18.5	29.5	25.6	2.4	25.6	9.3%
9 Pore length	50	5.1	12.8	8.9	1.6	8.5	17.6%
10 Pore width	50	3.0	6.4	4.5	0.7	4.3	15.3%
Population 1 – Broadleaf forest litter, Dominican Republic (DR-006; EM-1384)							
1 Shell length	10	139.2	152.6	146.1	4.2	147.0	2.9%
2 Shell breadth	10	56.8	68.2	60.3	2.7	59.5	4.5%
3 Shell length / breadth ratio	10	2.27	2.58	2.43	0.10	2.42	4.1%
4 Aperture (long axis)	10	24.0	28.6	27.4	1.7	28.4	6.1%
5 Distance from fundus to the pores	10	85.2	92.3	85.9	2.2	85.2	2.6%
6 Distance from fundus to base of neck	10	113.6	124.6	118.1	3.2	118.6	2.7%
7 Distance between the pores	10	31.2	32.7	32.3	0.7	32.7	2.2%
8 Width of the neck at narrowest point	10	24.0	29.5	25.5	1.4	25.6	5.5%
9 Pore length	10	8.5	11.4	8.9	1.0	8.5	10.9%
10 Pore width	10	4.3	6.4	4.3	0.0	4.3	0.0%
Population 2 – Sphagnum, Chile (EM-1453)							
1 Shell length	41	135.8	153.4	144.3	5.0	144.8	3.5%
2 Shell breadth	40	56.8	68.2	62.3	2.0	62.5	3.2%
3 Shell length / breadth ratio	40	2.03	2.46	2.32	0.10	2.34	4.2%
4 Aperture (long axis)	41	24.5	29.8	27.4	1.3	27.6	4.6%
5 Distance from fundus to the pores	41	73.0	92.3	81.2	4.5	81.5	5.6%
6 Distance from fundus to base of neck	41	104.6	125.0	114.7	4.7	113.8	4.1%
7 Distance between the pores	41	22.8	34.1	29.2	3.2	29.7	11.1%
8 Width of the neck at narrowest point	41	18.5	29.5	25.6	2.6	25.6	10.1%
9 Pore length	40	5.1	12.8	8.9	1.7	8.5	19.0%
10 Pore width	40	3.0	6.4	4.5	0.8	4.3	16.8%

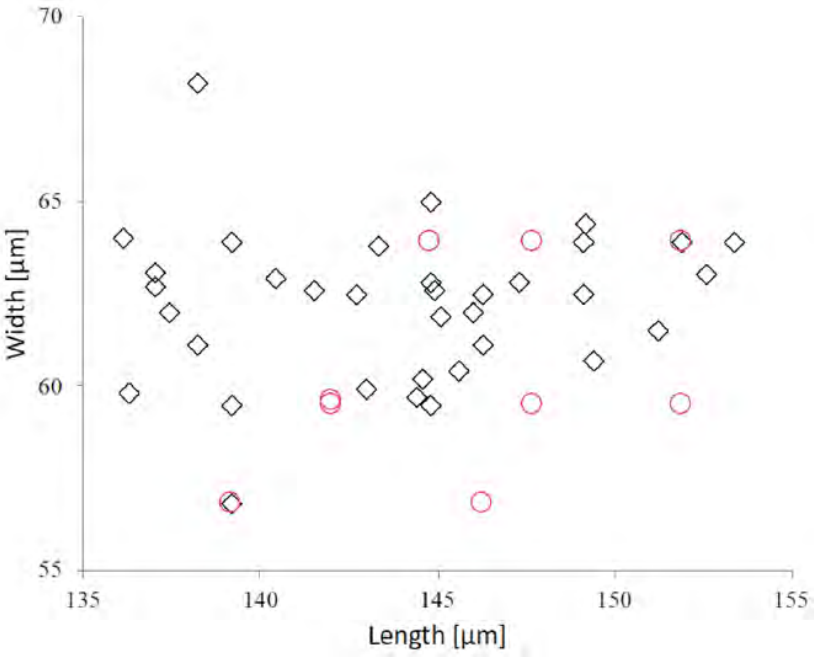


Fig. 5. Biplot of length vs. width of two populations of Certesella larai *n.sp. from Dominican Republic and Chile.*

Ecology

Given the fact that *Certesella larai* was rare in the three samples, its specific habitat as well as companion species may not necessarily reflect its ecological optimum. *Certesella certesi*, *C. martiali* and *C. australis* were all described from *Sphagnum* peatlands in South America (Certes 1889; Vucetich 1973; Wailes 1913). In Mexico, Bobrov et al. found *Certesella certesi* only in mountain cloud forests (Bobrov et al. 2013), while in New Zealand Bamforth found it only in lowland podocarp forests (Bamforth 2015). *Certesella martiali* was reported from mosses in Guatemala (Laminger 1973). Heger et al. observed *Certesella* sp. in mosses in several locations across Central America (Heger et al. 2011a). *Porosia bigibbosa* is a rare testate amoeba species in peatlands but is quite common in beech forests in Bulgaria (Todorov 2002) and a new species, *P. paracarinata* was also found in forests in Japan (Bobrov et al. 2015). The discovery of *Certesella larai* suggests that the genus *Certesella* is also not restricted to peatlands. Thus, while hyalospheniids in general are most diverse in acidic and nutrient-poor habitats such as *Sphagnum* peatlands, this is not true for genus *Porosia* and possibly not either for *Certesella*.

Table 4. Summary measurements reported for *Certesella* and *Porosia* species. Details of measurements for populations and individual publications are given in Supplementary Table 2.

Species	Length	Breadth	L/B	Aperture	Reference
Certesella larai sp. nov.	138.2–153.4	56.8–63.9	2.18–2.45	25.6–29.8	Present work
Certesella certesi	80–157	70–90	1.82–2.55		Certes, 1889, Penard, 1911, Heinis, 1914, Deflandre, 1936
Certesella martiali	147–238	77–130	1.55–1.83		Certes, 1889, Penard, 1911, Deflandre, 1936
Certesella australis	199.5–277.5	119–136.5	1.5–1.8		Vucetich, 1973
Certesella murrayi	120–136	95–100			Wailes, 1913
Porosia bigibbosa	128–178	83–123		34–51	Penard, 1890, Wailes & Penard, 1911, Cash et al., 1919, Deflandre, 1936, Ogden & Hedley, 1980, Hoogenraad & de Groot, 1940, Todorov, 2002 and Luketa, 2016
Porosia paracarinata	202–236	142–157		56–67	Bobrov & Kosakyan, 2015

As the ecology and diversity of testate amoebae have been much more intensively studied in peatlands than in forests the ecology of some species may not yet be fully understood. The fact that this new species was found in two very distant regions but was rare in the three samples and absent from ca. 100 other neotropical samples may reflect a sampling bias in favour of *Sphagnum* or other habitats which are a priori perceived as more favourable for testate amoebae. This can potentially cause interesting species to be overlooked. Another recent example is the discovery of a new *Quadrulella* species in a semi-desert environment in Mexico (Pérez-Juárez et al. 2017), which contrasts with the wetland habitats where species of this genus are usually found. The discovery of these new species illustrates that when under-sampled habitats are studied some surprising discoveries can be made. This should be a motivation for protistologists to continue exploring a broad range of habitats.

Fig. 6. Comparative overview of the morphology of all known species of genera Certesella *and* Porosia *species. A:* Certesella larai *n. sp., B:* C. certesi*, C:* C. australis*, D:* C. murrayi*, E:* C. martiali*, F:* Porosia paracarinata*, G:* P. bigibbbosa*. Images are not to scale as some early images lacked a scale.*

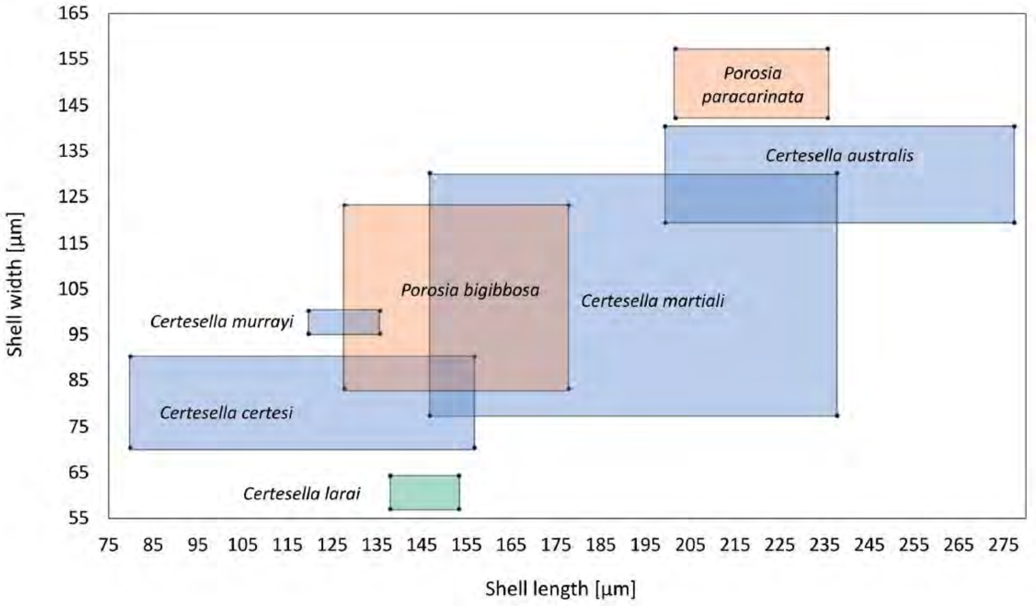


Fig. 7. Maximum range of shell length and width of the three known Certesella *and* Porosia *species showing a lack of overlap between* C. larai *n.sp. and all other species from the two genera in this two-dimensional space. Each species is illustrated (images not exactly to scale).*

Acknowledgements

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Chapter 7 - Comment on "Amoebae Assemble Synthetic Spherical Particles To Form Reproducible Constructs" (Published)

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Recently, Bian[1] published in Langmuir a study on the inclusion of synthetic particles in the shell of testate amoebae. Throughout their paper, Bian and colleagues refer to the presented testate amoebae as *Difflugia globulosa*, whereas the pictures clearly show specimens of the genera *Centropyxis* and *Netzelia*. While this does not change the findings of this article and their significance, it does perpetuate a history of misidentification that has been hindering the study of the wonderful but often mischaracterized testate amoebae, which justifies a corrigendum. Furthermore, correcting this imprecision would require minimum effort as it would only imply replacing the occurrences of "*Difflugia globulosa*" with "*Centropyxis* sp.", removing or changing Figure 1e and modifying the few lines on the historical background of *Difflugia* accordingly.

While the genera *Difflugia*, *Netzelia*, and *Centropyxis* are all characterized by their ability to agglutinate mineral particles to build their test, they can be differentiated by the position and shape of the aperture. The genera *Difflugia* and *Netzelia* have been defined as having either a terminal aperture in the case of the elongated species or a central aperture in the case of the spherical species (such as *Difflugia globulosa*). On the contrary, the genus *Centropyxis* is defined as having a ventral to subapical aperture that is off-center, and most of the species present a flattened ventral surface. Finally, species of the genus *Netzelia* can be easily distinguished from *Difflugia* by their lobed aperture with a thick organic lip.

Following these criteria, it appears that every specimen of testate amoebae shown in the pictures except one are to be assigned to the genus *Centropyxis* because of their flat ventral surface and eccentric aperture. Figure 1e is, however, to be assigned to the genus *Netzelia* given its lobed aperture.

Unfortunately, a major cause of misidentification is the scarcity of reliable identification keys for the testate amoebae. We can nonetheless mention Todorov,[2] Tsyganov,[3] and the Microworld Web site (Siemensma;[4] URL: arcella.nl), one of the most comprehensive and up-to-date sources for the taxonomy of testate amoebae.

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Chapter 8 - A revised classification of the testate amoeba family Euglyphidae based on scale morphology and DNA barcoding

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This work is preliminary, and the taxonomic acts presented here are not to be considered by the ICZN.

Abstract

The family Euglyphidae, characterized by one or several rows of conspicuous serrated scales surrounding the aperture, is one the most iconic groups of testate amoebae. However, the knowledge of their taxonomy is still very incomplete, which prevents them from being used as ecological proxies despite their presence and abundance in most terrestrial or freshwater habitats. Furthermore, despite evidence that the current taxonomic framework is inadequate, this group has not been critically reviewed since Decloitre's monograph in 1962. As such, the taxonomic literature of Euglyphidae is a taxonomic and nomenclatural mess. Here we re-evaluate the systematics of the group, and propose a new classification based on scanning electron microscopy, DNA barcoding and a critical review of the literature. We describe 23 new species and invalidate most taxa that were described before the onset of scanning electron microscopy as a taxonomy tool. Finally, we define sections to act as a simplified taxonomic framework to study and report observations of Euglyphidae based on light microscopy as generally done by (paleo)ecologists

Introduction

The Cercozoa are an immensely abundant and species rich clade of protists composed of disparate organisms that have been reunited only on the base of molecular phylogenetics (Cavalier Smith & Chao 2003). This group includes amoeboflagellates, heliozoan-like forms, plant, animal and protist parasites, and testate amoebae, amoeboid organisms bearing a self-secreted shell. Among these, the Euglyphida are probably the best known with their tests reinforced with self-secreted silica scales. The family Euglyphidae, arguably the most iconic subclade is characterized by one or several rows of conspicuous serrated scales surrounding the aperture. These organisms are present in virtually all freshwater and soil habitats where they are often abundant. They most certainly play major ecological and biogeochemical roles in the regulation of bacterial populations and the turnover of silica (Aoki *et al*. 2007, Wilkinson and Mitchell 2010). Like other Euglyphida, they have geographically limited distributions, and are most diverse in the tropics (Lara *et al*. 2016) where some remarkably large and conspicuous species can be found (Gomes e Souza 2022). They are often key species in biomonitoring studies centred on peatlands where the accuracy of species identification is considered key for environmental diagnosis (Bobrov *et al*. 1999).

Like other testate amoeba clades, the first Euglyphidae species were described based on the general test outline. Some 120 species and subspecies of *Euglypha* and *Scutiglypha*, described mostly between the late 19th and the mid-20th centuries were reviewed by Decloitre who also added many new descriptions (Decloitre 1962, 1976, 1979, 1982, 1986). However, with the application of scanning electronic microscopy, it appeared clearly that the shape, size and disposition of scales were taxonomically relevant criteria. Hedley *et al*. (1974), Coûteaux *et al*. (1979) and Ogden (1980) demonstrated the necessity of scanning electron microscopy (SEM) for accurate species identification with emphasis on the structure of the apical scales surrounding the aperture. DNA-based phylogenetic reconstruction confirmed that the fine structure of scales was much more reliable than the general outline of the test for species identification, as morphotypes based on the overall morphology often corresponded to polyphyletic entities (Wylezich *et al*. 2002).

Currently, the Euglyphidae include only two genera, *Euglypha* Dujardin 1841 and *Scutiglypha* Foissner and Schiller 2001, the latter being distinguished by the presence of scutiform scales (Foissner and Schiller 2001). Unfortunately, many of these descriptions are based only on the general test outline as observed in light microscopy and lack ultrastructural details of scale structure that would only be visible by SEM. Additionally, the absence of type material prevents re-examining most taxa, as is also the case for other testate amoebae (Duckert *et al.* 2020, Lara *et al.* 2020). Relatively little has been done on the taxonomy of the Euglyphidae since the pioneering electron microscopy work of Coûteaux, Ogden and Hedley during the 70's, and only a few morphospecies have been re-examined or described using modern tools (i.e., SEM and DNA sequencing). As a result, the current state of taxonomy of the family Euglyphidae is not satisfactory and this limits the potential for studies on their evolution, ecology and biogeography that require sound taxonomy.

Here we re-evaluate the systematics of the group, and propose a new classification based on an integrative taxonomic approach. We present a revision of the family Euglyphidae based on a review of the literature and the characterization of 41 cultures and isolates and the description of 23 new species. We identify synapomorphies at different taxonomic levels based on molecular and morphological examination allowing to designate two subgenera within *Euglypha* (*Euglypha* and *Calliglypha*) based on the morphology of the oral scales and to define several sections and invalidate the genus *Scutiglypha*. Furthermore, we invalidate most taxa that were not described using scanning electron microscopy, as their original or subsequent description are too vague to allow any identification. These older species should be considered as broad morphotypes, at most, but not real taxa. To avoid any confusion between the names of validated and invalidated taxa, the latter will be mentioned in ways that highlight the fact that we refer to broad morphotypes. As an example, rather than writing that a specimen can be assigned to the prevailing interpretation of *E. rotunda*, an invalidated taxon that should be regarded as an artificial aggregate of unrelated species, we refer to it as a "*rotunda*-like" specimen. We hope that this revision sets the basis for future work on the taxonomy of Euglyphidae as well as providing a useful identification basis for ecologists.

Material and methods

Amoebae isolation and observation

Euglypha specimens were isolated from mosses, topsoil, aquatic vegetation or freshwater sediments from 23 locations worldwide (Table 1). Samples were filtered through a 200 µm mesh to remove coarse particles and individual testate amoebae were manually isolated for cultivation or direct analyses under an inverted microscope using a narrow pipette.

Cultures

We tried to establish cultures for every isolate that we here characterize to avoid mixing different species. However, cultivation did not always succeed, and some strains were characterized using only wild specimens (data not shown). Cultures were established by pooling together one to six cells with the same morphology in a flask containing 10 ml of culture medium. Cultures were maintained for several months by transferring 3 ml of the culture medium containing the cells into a new flask filled with new culture medium. The culture medium composition for 1 l was: 1 l Volvic© bottled water, 20 mg KNO3, 10 mg KH2PO4, 7.3 mg Na2SiO3, 3 mg Na-EDTA and 7.5 ml of soil extract. The pH was adjusted between 6 and 7 with KOH or HCl, and *Escherichia coli* or *Saccharomyces cerevisae* were added as food source. If a culture was suspected to contain more than one species (see Results – Diagnostic traits), the data related to this culture were discarded.

Morphological observation and measurements

All isolates were morphologically characterized using high resolution optic microscopy and scanning electron microscopy, with a particular attention given to the fine structure of the parietal and oral scales. For each strain, we measured the test length and width, the aperture width, and the length and width of the oral and parietal scales of at least 10 specimens. The dimensions of the test and the aperture were measured under an inverted light microscope, while the dimensions of the scales were measured on both light and scanning electron micro-photographs. Several protocols and different scanning electron microscopes were used, but the method that yielded the best results was the following. For each strain, several tests were washed successively in a drop of demineralized water, a drop of 70% ethanol, and a last drop of demineralized water before being mounted on a stub. The stubs were then coated with gold by argon sputtering, and the stubs were examined using a Tescan Mira LMU SEM operated at 20 kV. Documented representative specimens were kept on stubs as voucher specimens, however due to this work being based on data gathered for more than 20 years, some of these specimens have been lost over time.

DNA extraction, amplification, and sequencing

The DNA was extracted using a thiocyanate guanidium protocol adapted after Chomczynski and Sacchi (1987) as described in Duckert et al. (2018). The 18S rRNA gene was amplified using different PCR protocols, and we present here only the nested PCR protocol using clade-specific primers that yielded the best results. For every strain, a first PCR was carried-out using Cercozoa-specific (cerco1F - ACA TAT GCT TGT CTC AAA GAC TAA G) and eukaryote general (1498R - CAC CTA CGG AAA CCT TGT TA) (Lopez-Garcia *et al*. 2003) primers with a first denaturation step at 95°C for 2 minutes, followed by 45 cycles of 95°C for 30 seconds, 59°C for 30 seconds and 72°C for 1:45 minutes, and a final elongation at 72°C for 5 minutes. For strains of subgenus *Euglypha*, a second PCR was done using a eukaryote general (EK82F – GAA ACT GCG AAT GGC TC) (Lopez-Garcia *et al*. 2001) and subgenus *Euglypha*-specific (subEugly – AGA TCG TCA AGT TTG TYC) primers with a first denaturation step at 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds, 54°C for 15 seconds and 72°C for 1:45 minutes, and a final elongation at 72°C for 5 minutes. For strains of subgenus *Calliglypha*, a second PCR was done using a Euglyphida-specific (EuglySSU2F – GCG TAC AGC TCA TTA TAT CAG CA) (Lara *et al*. 2016) and subgenus *Calliglypha*-specific (subCally – AGA TGA TCA AGT TTG GTT) primers with a first denaturation step at 95°C for 2 minutes, followed by 25 cycles of 95°C for 15 seconds, 54°C for 30 seconds and 72°C for 1:45 minutes, and a final elongation at 72°C for 5 minutes. The amplicons were purified using a Milipore kit according to the instructions of the manufacturer and sent for sequencing with an ABI3730XL DNA sequencer (Applied Biosystems) at Macrogen, Amsterdam NL.

Phylogenetic reconstruction

We obtained partial 18S rRNA gene sequences ranging from 1200 to 1800 nucleotides from 32 strains. The sequences were first automatically aligned with all *Euglypha* sequences available on GenBank (Bhattacharia *et al.* 1995, Wylezich *et al*. 2002, Lara *et al.* 2007, Tsyganov *et al.* 2017) using the software MAFFT (Katoh and Standley 2013) as implemented on the CIPRES portal (Miller *et al.* 2010) and then manually edited using the software AliView. We constructed a maximum likelihood phylogenetic tree using the RAxML v.8.2.10 (Stamatakis *et al.* 2014) as implemented on the CIPRES portal with the GTR + GAMMA model and 1000 bootstraps. The tree was first rooted using the genus *Trinema* as the outgroup, but we present the phylogenetic tree without any outgroup as it sometimes resulted in the outgroup being placed within the genus *Euglypha* (see discussion). We then generated Bayesian posterior probability by using the same alignment in MrBayes v. 3.2.7a (Ronquist and Huelsenbeck 2003). This analysis was run with parameters GTR

+ GAMMA on two independent MCMC runs for 300’000 generations sampled every 100 generations. This resulted in 6’002 trees, of which 25% were discarded as the burn-in, that were used to generate posterior probabilities after convergence of the two runs was checked.

Results

Our results consist in the morphological characterization using light- and scanning electron microscopy of 41 cultures and wild populations, the phylogenetic reconstruction of the genus *Euglypha* based on SSU rRNA sequences gathered from GenBank and obtained during this work, the formal description of 23 new species, a taxonomic history of this genus, a new classification of the genus *Euglypha*, and a comprehensive lists of taxon names associated to *Euglypha* based on a review of the taxonomic literature (Supplementary table 1). We furthermore invalidate most taxa that were described without the use of scanning electron microscopy, as their original description is not accurate enough to allow any identification.

History of diagnostic traits used to define the genus *Euglypha* and its taxa

Dujardin (1841) formally erected the genus *Euglypha* to accommodate two new species*, E. tuberculata* and *E. alveolata*, their unifying traits being their serrated aperture and, more importantly, the regular imprints covering the test which would give its name to the genus (in Greek: “*eu-*” = well; “*glypha*” = marked). These fine imprints were identified as overlapping scales covering the test since at least 1856 (Carter 1856). Later, Leidy recognized 1879 the serrated oral scales as the synapomorphy for the genus that distinguishes *Euglypha* from other similar euglyphids with an axial aperture such as *Assulina* and *Placocista* (Leidy 1879).

Posterior works recognized the fine morphology of the scales, and particularly the oral scales, as useful traits for species delimitation with Cash, Hopkinson and Wailes (1919) eventually writing “[...] it is possible to identify nearly every species if only a single aperture scale is available”. However, this seems to have not been taken into consideration by most later authors as Couteaux (1979), using scanning electron microscopy to assess the fine structure of the oral and parietal scales, also concluded that the morphology of the oral scales might be the most important diagnostic trait but that it was still only rarely present in *Euglypha* descriptions.

The importance of the scale fine morphology over the overall test shape to distinguish between closely related species was later confirmed with phylogenetic reconstruction when Wylezich et al. (2002) isolated and barcoded several strains that could be assigned to the morphospecies *E. rotunda*. As those strains presented consistent differences in the shape of their scales and did not form a monophyletic clade, this prompted to consider that even slight variation in the shape of the scales could indicate distinct species.

History of *Euglypha* systematics

The first taxonomic overview of the genus *Euglypha* was published by Cash, Hopkinson & Wailes (1915) in their compilation of the freshwater Rhizopoda and Heliozoa of the British Isles in which they present 25 taxa and propose a first classification based on the types of spines and the compression of the test. They divide the genus into two “divisions”, with the first division including taxa with an uncompressed test and ensiform spines, if any, while the second division included taxa with a compressed test and acicular spines, if any. The second division is further separated into two sections characterized by the shape of the aperture, which can be circular or elliptic. In the first monograph of the genus *Euglypha*, Decloitre (1962) furthermore recognizes 57 taxa distributed into groups also based on the nature of the spines and the compression of the test that roughly corresponded to the categories defined by Cash, Hopkinson & Wailes (1915). This monograph was updated in 1976, 1979, 1982 and 1986 to include 36, 6, 2 and 4 newly formally described taxa, respectively, for a total of 105 mostly subspecific and infrasubspecific taxa.

The shape of the parietal scales as a classification tool was used for the first time by Foissner and Schiller (2001) who split the genus *Euglypha* and erected genus *Scutiglypha* to accommodate species with crenate or scutiform parietal scales. This corresponds roughly to the divisions proposed by Cash, Hopkinson and Wailes (1915), as scutiform scales are always associated with uncompressed tests. In the first phylogenetic reconstruction of the genus, Wylezich *et al*. (2002) confirmed that the compressed and uncompressed species formed two distinct monophyletic clades. Furthermore, uncompressed species always presented flat oral scales while compressed species had gibbous oral scales with a central bump, confirming that the morphology of the scales could be clade specific. It must be noted that, in this work, the strains *E. filifera* CR (AJ418785) and *E. tuberculata* (AJ418787) were mis-annotated and the two should be swapped in the original and subsequent publications reusing these data. Lara *et al*. (2007) later added SSU rRNA sequences of three larger *Euglypha* with acicular spines which formed a highly supported monophyletic group. This revealed three clades within *Euglypha*: 1) one formed by *tuberculata*- and *acanthophora*-like species with an uncompressed test, flat oral scales and ensiform spines, when present, and includes genus *Scutiglypha*, 2) one composed of *ciliata*-like species with gibbous oral scales and a large compressed test always armed with acicular spines and, 3) a last group of *rotunda*-like and *filifera*-like species with gibbous oral scales and a compressed test with acicular spines, if any. Tsyganov *et al.* (2017) added two sequences assigned to the morphotypes *E. bryophila* and *E. cristata*. However, these results are in contrast with previously published data and seem also at odds with our classification based on combined morphological and molecular data: in their phylogenetic tree the *cristata*-like specimens branched not with the *bryophila*-like specimens, which share a similar morphology and spines, but with a specimen assigned to the morphotype *E. tuberculata*. We included the sequence of these *cristata*-like specimens in our study, but nevertheless mention these results in the section about the respective species. These results clearly call for more studies on these taxa to clarify this puzzle.

Morphological characteristics

The overall morphology of the tests range from lanceolate with straight sides tapering towards the aperture to ovoid. The test can be either compressed (subgenus *Calliglypha*), or circular in cross-section (subgenus *Euglypha*). The shape of the aperture ranges from circular (subgenera *Callyglypha* and *Euglypha*) to elliptic (subgenus *Callyglypha*).

The aperture is surrounded by serrated scales (Figure 1), here after referred to as oral scales, that can be either flat with serration on the oral margin as in subgenus *Euglypha*, or gibbous with a central knob and serration originating below the oral margin as in subgenus *Calliglypha*. Strains from subgenus *Calliglypha* always present a single row of identical oral scales (except on dikaryons, binucleated life stages occurring rarely in cultures which we did not document, or abnormal specimens), while strains from subgenus *Euglypha* can present more than one row. In this case, these oral scales can differ in shape and size from the one directly surrounding the aperture, and the additional rows are often incomplete. The oral scales of strains from subgenus *Calliglypha* mostly differ in their dimensions and the shape of their aboral margin, however some strains do present serrations that are significantly distinct in their position, number, or size. Conversely, the oral scales of strains from subgenus *Euglypha* share a similar overall morphology and we could not distinguish them reliably. The morphology of the oral scales can vary in the same strain and sometimes even on the same species, the aboral margin of some oral scales having a more concave outline. Also, the shape and number of denticulations can vary as the smaller, outermost denticulations can sometimes be absent or not fully formed.

The main part of the test is covered by non denticulated scales (hereafter referred to as parietal scales) that can be circular, elliptic, polygonal (e.g., rectangular, rhomboidal or hexagonal), scutiform or crenate. In most strains the shape and dimensions of the parietal scales varies depending on their location on the test, with the first row of parietal scales located directly next to the oral scales being often slenderer and smaller than the one located on the middle of the test, while the scales closer to the fundus are more elliptic. However, some specimens can present first-row and mid-test parietal scales with similar morphologies. Some specimens can also present parietal scales that are rounder and less characteristic than scales of other specimens of the same strain. In some of the larger and spinous strains, some specimens can present parietal scales that have a slight indentation on their aboral margin.

Acanthophora-, *ciliata*-, *bryophila*- and *filifera*-like strains present spines of three different types. *Acanthophora*-like strains presents large spines that are elongated parietal scales (hereafter referred to as ensiform spines) located closer to the fundus of the test, while *ciliata*- and *filifera*-like strains have thin spines (hereafter referred to as acicular spines) that are imbricated between the parietal scales, most often on or near the margin of the test. *Bryophila*-like strains have flat, ribbon-like spines imbricated between the parietal scales and arranged in a tuft on the fundus. The number of spines can vary between specimens of the same strain, especially in *acanthophora*-like strains in which case we observed some specimens without spines, albeit rarely.

Our strains present nuclei of three types: vesicular (with a single well-defined nucleolus), ovular (with several small nucleoli) and granular (nucleus homogenous with no nucleolus visible). Strains from subgenus *Euglypha* have only granular nuclei while strains from subgenus *Calliglypha* presented all three types.

To our knowledge there are no significant difference in the morphology of the pseudopods between strains. However, this has not been studied in as much detail as the morphology of the test and to a lesser extent other characters such as nucleus morphology.

In our observations based on both cultures and wild populations, the highest coefficient of variation (CV) for test length was 9.3%, and except for three outliers all strains had a CV below 7%, with the mean CV at 5.3%. The CV for the breadth of the test was similar, and all but one strains had a test breadth CV of 10% or less, the remaining strains showing a CV of 13%. The width of the pseudostome was more variable, with CVs reaching up to 16%. However, this could be in part due to the difficulty to precisely measure the limits of the pseudostome on optic microscopic pictures. In some strains from subgenus *Calliglypha* both circular and ellipsoid pseudostomes could be observed in the same strains. However, in such cases, one type of aperture was always more common than the other.

Phylogeny

A phylogenetic reconstruction of all SSU rRNA sequences of *Euglypha* specimens available on GenBank plus the ones acquired during this study is presented in Figure 2. The genus *Euglypha* can be divided into two highly distinct and fully supported phylogenetic groups with a clear synapomorphy that we here erect as the new subgenera *Euglypha* and *Calliglypha*. Subgenus *Euglypha*, represented by *tuberculata-, scutigera*- and *acanthophora*-like strains, includes strains with flat oral scales with serrations originating on the margin, while subgenus *Calliglypha* is composed of strains with oral scales with a central bump and denticulations originating below the margin, and includes *bryophyla*-, *ciliata*-, *filifera*- and *rotunda*-like strains. The defined subgenera, clades and subclades mentioned hereafter are well supported in our phylogenetic analysis, however the relations between closely related strains are not supported, very likely due to a lack of taxonomic resolution of the SSU rRNA marker.

Strains of subgenus *Euglypha* branched together into three groups which present increasing complexity in their parietal scales in relation to their phylogenetic distance from the root of the tree. Our *tuberculata*-like strain, with subcircular spineless scales, is placed at the very base of the subgenus, while all strains that presented mucronated parietal scales and ensiform spines branched into a genetically distant but fully supported clade, with the spineless strains with rounded scutiform parietal scales placed in between.

Except for two *bryophila*-like and one *rotunda*-like strains, all strains in subgenus *Calliglypha* were placed within three well-supported and mostly morphologically consistent clades. Clade *Calliglypha* A is composed of *ciliata*-like strains that are larger and wider than the other clades. All strains of this clades present acicular spines inserted between parietal scales on their body or margin. Clade *Calliglypha* B is composed of *rotunda*- and *filifera*-like strains. This clade can be divided further into two well-supported subclades. One subclade is composed of the *filifera*-like strains that are elongated and present acicular spines inserted between parietal scales on their margin, plus one elongated spineless strain branching at its base, and the other subclade is constituted of small and morphologically similar *rotunda*-like strains with elliptic parietal scales. Clade *Calliglypha* C is constituted of *rotunda*-like strains with rhomboidal or rectangular parietal scales.

Species delineation

Species delineation was based on strains from which we could obtain DNA sequences. Among those strains, morphological differences between strains were accompanied, in all cases but for one, by genetic discrepancies in the variable regions of the SSU rRNA marker; even slight discrepancies in the morphology of the oral and parietal scales were accompanied by at least one nucleotide of difference. We considered as distinct species all strains that could be reliably differentiated based on their overall shape or the morphology of their idiosomes (scales and spines). Furthermore, two species, *Euglypha regipurpurei* and *Euglypha AcUniNe*, were morphologically too different to be considered as a single species despite sharing an identical sequence, suggesting that the SSU rRNA gene might not be evolving fast enough to distinguish closely related species. As morphologically distinct species can have an identical SSU, we also consider that a single nucleotide difference suffices to distinguish two species, even in the absence of morphological differences. Such cases correspond to cryptic species. Based on these criteria, 39 of our 40 strains can be considered as distinct species, however we do not formally describe all these strains as we did not have enough data for some.

Diagnostic traits

Most of our species can be differentiated based on the dimension of their test or the morphology, dimensions, and disposition of their idiosomes, notably the degree of overlap between scales. Examples of these diagnostic traits are presented in the species diagnoses, especially in the section that describes how to distinguish as species from a similar looking taxon. While a single diagnostic trait was already enough to differentiate our species, in most cases two species differed by more than one morphological trait. However, due to the natural variation and the inconspicuous nature of some of these traits, more than one specimen should be observed for identification.

Only clear differences in the outline of the test can be used to differentiate closely related species, as it depends on the ratio between the test length and width and pseudostome width, which are quite variable. The number of oral and parietal scales does not either seem to be reliable traits to differentiate closely related species, as they are directly related to the dimensions of the scales compared to the size of test or width of the pseudostome and do vary. Furthermore, closely related species often have the same number of scales. The circularity of the aperture has been sometimes used to distinguish between similar morphospecies (Clarke 2003). However, as we could find both types of aperture in some of our strains, this does not seem to be a reliable trait for identification. As our results show that clear morphological differences in the shape and dimensions of the test or the idiosomes are sufficient to identify species, it is justified to describe new species based on morphology alone as long as the fine structure and disposition of the idiosomes are accurately represented.

In our strains, the CV of the length and width of the test were, except for a few cases, well below 10%. While hard thresholds are rarely useful, a CV of more than 10% for the length and width of the test could suggest that there may be more than one species in a population (at least it should give an incentive to study this question in more detail for the given sample). The fact that these measurements were taken mostly on cultured strains might be a bias that resulted in lower CV than could be expected in wild population. However, as we did find comparable CV in wild populations, it seems that strains that have been cultured for less than one year retain most of their range of morphological variation, at least in their dimension. Nonetheless, by comparing the current strain of *Euglypha rotunda* CCAP1520/1 to specimens pictured in 1973 (Hedley and Ogden 1973), it is clear that maintaining a culture for too long can alter its morphology as this strain presents nowadays a high proportion of abnormal specimens with disformed test or scales.

Taxonomic acts

We formally describe 23 new species and designate one strain as the neotype of *E. scutigera* Penard (Figure 3). We also designate *E. scutigera* as the type species of genus and subgenus *Euglypha,* due to the invalidation of *E. tuberculata* Dujardin, the previous type species. As our results show that slight discrepancies in the morphology of the scales or clear difference in the shape of the test are powerful diagnostic criteria (see Results - Species delineation), we also describe species that lack data genetic data but clearly differ in their morphology. Based on our review of the literature, we validate only eight species for which permanent material is available or which meet the following criteria: high resolution pictures or precise line drawing showing the outline as well as the shape and position of parietal and oral scales, and indication of sampling location. Conversely, we invalidate species whose original or subsequent descriptions do not meet these criteria. Strains that could not be formally described as new species are also presented in Supplementary figure 1.

We erect two new subgenera, *Euglypha* and *Calliglypha*, and 10 sections. We preferred erecting subgenera rather than splitting the genus into two genera because this allows classifying taxa into more morphologically and genetically consistent units for the usage of taxonomists while still maintaining the same well-known generic name. The names of poorly described morphospecies such as *Euglypha ciliata* were retained if the names are commonly reported in the scientific, especially ecological, literature; such names are now used to refer to sections. Each section was designed to correspond to a group with a consistent morphology, and when genetic data were available, they were designed to correspond as much as possible to monophyletic units. A section was erected only when it included at least two species that were accurately pictured using high resolution microscopy with one of them barcoded in this study or previous publications. Invalidated taxa, with the exclusion of junior synonyms and infrasubspecific names, for which the description was precise enough to allow a clear assignation to a section were also included, in this case it is indicated that the name refers to an invalid taxon. It must be noted that infrasubgeneric groups such as sections are not regulated by the ICZN and are thus informal. They are to be treated as a working concept for simplifying the reports of observations, but they are not real taxa.

Foissner and Schiller (2001) recognised the potential of using the morphology of parietal scales as a tool to split the genus *Euglypha* into smaller, more coherent division and created the genus *Scutiglypha* to accommodate species with scutiform parietal scales, which corresponds to our subgenus *Euglypha* minus section *Tuberculatae*. As the acceptance of this genus would render paraphyletic both the previous and current definition of genus *Euglypha*, due to the designation of *E. tuberculata* Dujardin as the type species by Loeblich and Tapan (1961), and would require splitting it in at least three genera, we invalidate the genus *Scutiglypha*. Furthermore, the scutiform and crenate scales might not be monophyletic characteristics as there seems to be transitions between the well scutiform to crenate and ellipsoid shapes, which was nonetheless acknowledged by Foissner and Schiller.

Discussion

The "*acanthophora* problem" as an illustration of the necessity of a taxonomic revision

One of the most striking results of our analyses is that morphologically similar species are consistently placed close to each other in our phylogenetic reconstruction, forming clades than can be characterised by synapomorphies. This allows subdividing the genus *Euglypha* into morphologically and genetically coherent clades that can be easily identified. However, these clades, which roughly correspond to the current interpretation of older species, are morphologically and genetically too diverse to be considered as single species. Furthermore, while some species can be identified thanks to the conspicuous morphology and disposition of their idiosomes, most can be differentiated only based on minutes details invisible at low magnification. Lastly, all our strains in section *Eurotundae* except one did not present any morphological characteristics that allow distinguishing them from each other despite being genetically distant enough to be considered as distinct species. This shows the existence of cryptic species in genus *Euglypha* too, which was foreseeable considered that most, if not all, groups of protists harbour species complex that cannot be distinguished based on morphology alone.

Most taxonomic work on *Euglypha* has been conducted during the 19th and early 20th century when modern tools were not available. As slight discrepancies in morphology can indicate distinct species, the true diversity of euglyphids has been largely underestimated because these differences could often not be observed, or when they were noticed they were often interpreted as being due to natural variability (as seen in Penard 1902, where he discusses the case of *E. alveolata*) or possibly response to local ecological conditions (as seen in Wallich 1885). As a result, species described without the use of modern tools were often not described accurately. These "older" species, together with their respective subspecific and infrasubspecific taxa, should therefore be regarded as aggregates of species that likely differ in their ecology and distribution pattern, a concept that has been consistently shown in other protist groups (see Kosakyan *et al.* 2013, Dumack *et al.* 2016 and Howe *et al*. 2009 where this is discussed for hyalospheniid testate amoebae, Tectofilosida and glissomonads, respectively).

As a result, the systematic of the genus *Euglypha* is a nomenclatural (i.e., what name should be used to refer to a taxon) and a taxonomical (i.e., what is the identity of the taxa behind those names) mess, the identity of most taxa remaining unclear often with several conflicting interpretations in the literature. This situation has led to an inflation of names, with most taxa never reported since their description. Furthermore, as the taxonomy of the genus *Euglypha* has not been re-evaluated since Decloitre's monograph (1962), the identification of a species relies mostly on the subjective interpretation of a mostly old, scattered literature that often contradict itself rather than well-established identification keys. As an example, in a study exploring the degree of confusion of testate amoebae identification, on 9 morphospecies of the genus *Euglypha*, 4 were considered as moderately confusing, and 4 were considered as very confusing (Mitchell et al. 2014). This confusion is even more obvious in Amesbury et al. (2016), one of the largest studies using testate amoebae as palaeoecological proxies. There morphospecies of *Euglypha* where lumped into two groups in a process that appears random, with at least one morphospecies appearing in the two groups under different names. It must be noted that this study used data from 31 other published studies, and in the process of harmonizing the taxonomy when merging different data sets, some pooling of species had to be done. The fact that the *Euglypha* species were not pooled in an appropriate way, despite the participation of several testate amoeba experts, illustrates the state of confusion in the community and thus the need for a clarification! As it stands, every report of a species without pictures allowing a clear appreciation of its morphology should be considered as unreliable and the state of knowledge of the taxonomy, ecology, and biogeography of the euglyphids should mostly be considered as a blank slate. Finally, because the boundaries between species were never established, new species are almost never recognized but rather considered as peculiar form of an older species, which leads to the stagnation of the knowledge of this group. As an example, during the past 40 years only one new species, *Euglypha (Scutiglypha) cabrolae* (De Smet 2009) has been described. It can thus be argued that the genus *Euglypha* is almost completely useless in applied ecological and palaeoecological studies and that any advances cannot be made without a taxonomic revision.

These nomenclatural and taxonomic issues are well illustrated by the case of *E. alveolata* Dujardin, one of the most iconic species of euglyphids whose name and identity were unclear since it was described together with *E. tuberculata*. Prior to this revision, *E. alveolata* Dujardin was usually considered as a junior synonym of *E. acanthophora* (Ehrenberg) but was also often considered as a synonym of *E. tuberculata*, and sometimes even considered as another species.

Although the current interpretation of *E. tuberculata* and *E. alveolata* is that the former does not have spines while the latter is spinous, the original description of *E. tuberculata* (Dujardin, 1841) indicated that both species counted specimens with and without spines: "*un têt vide avait [...] en arrière plusieurs pointes irrégulièrement placées comme dans l'espèce suivante [E. alveolata]* - an empty test [...] had on its fundus several irregularly placed spines like in the following species [*E. alveolata*]". Furthermore, it was indicated that the two species were very similar and that the only distinguishing character was the nature of the fine imprints of the test which were described as rounded tubercles in *E. tuberculata* and polygonal depressions (alveolae) in *E. alveolata*. Dujardin even admits "*peut-être même sera-t-on tenté de n'y voir qu'une variété [d'E. tuberculata]* - maybe could we be tempted to only consider it as a variety [of *E. tuberculata*]". Later the same year Ehrenberg (1841) argued that Dujardin's species included two species that he distinguished based on the presence of spines, the one without spines which he describes as *Difflugia areolata* (which corresponds to the current interpretation of *E. tuberculata*)*,* as he did not recognize the genus *Euglypha*, and the one with spines as *D. acanthophora*, which was later transferred to the genus *Euglypha* by Perty (1849).

This was the start of a long-lasting confusion regarding what name should be used to refer to the spinous specimens as during the same period *E. alveolata* Dujardin was 1) used interchangeably with *E. acanthophora* (Ehrenberg) to refer to the morphotype with spines, 2) used to refer to the spineless morphotype as a synonym of *E. tuberculata* Dujardin, 3) considered to be unrelated to *E. acanthophora* (Ehrenberg), even though Ehrenberg admitted *E. acanthophora* to be part of *E. alveolata*. Adding to the confusion, the name *E. acanthophora* (Ehrenberg) was briefly used by Penard (1902) to refer to *E. brachiata* Leidy while he was proposing *E. armata* (Penard and Wailes 1911) as a new name for *E. alveolata*.

This whole problem was acknowledged by Cash, Hopkinson & Wailes (1915) who proposed to keep the name *E. acanthophora* (Ehrenberg) to refer to the spinous species, as it was not clear what was the identity of Dujardin's *E. alveolata*. Another solution was proposed by Loeblich and Tapan (1961) who fixed the meaning of *E. alveolata* Dujardin by designating a spinous test as the lectotype amongst Dujardin's original figures - they also considered *E. tuberculata* as the type species for the genus, albeit cryptically. Afterwards, *E. tuberculata* Dujardin was used preferably to refer to the spineless specimens whereas *E. acanthophora* (Ehrenberg) was more often used to refer to the spinous form. However, maybe because those two proposed solutions were incompatible, no consensus was reached as the names *E. acanthophora* (Ehrenberg) and *E. alveolata* Dujardin both still appear in the scientific literature nowadays, sometimes as synonyms, sometimes as referring to distinct species. Nonetheless, as the name E. alveolata Dujardin was published before *E. acanthophora* (Ehrenberg) and both are to be treated as synonyms since the designation of a lectotype by Loeblich and Tapan (1961), *E. alveolata* Dujardin is the valid name.

While these nomenclature issues were ongoing, the understanding of the identity of *E. alveolata*, often referred to as *E. acanthophora* (Ehrenberg), expanded as specimens with distinct scale morphologies were discovered. When it was understood that euglyphids were covered with imbricated scales, the original interpretation of *E. alveolata* Dujardin was that of an elongated ovoid species with ensiform spines and circular to elliptic parietal scales. However, some early figures of these imbricated scales did show parietal scales that presented a nipple on their aboral margin or mucro on their oral margin, although this trait was often omitted in the textual description of the specimens (as seen in Leidy 1879). Furthermore Leidy (1879) already mentioned specimens with "cordate" parietal scales which he assigns to *E. alveolata*. However, Leidy's interpretation of *E. alveolata* was the same as Dujardin's (i.e., that *E. alveolata* could be spinous or not) and it is not possible to know whether these specimens with cordate scales should be attributed to *E. alveolata sensu* Loeblig and Tappan or a species akin to *E. scutigera*. Nonetheless, as specimen akin to *E. scutigera* with crenate scales have never been observed, it is likely that these specimens would today be assigned to *E. crenulata* Wailes (1912), a species described as similar to *E. alveolata* but bearing crenate instead of subcircular parietal scales. Afterwards, most representation of *E. alveolata*, including its subspecies and infrasubspecific taxa, before the usage of SEM imagery had circular or elliptic scales (as seen in Cash, Hopkinson & Wailes, 1915 and Decloitre 1962) and the scutiform to crenate scales were more assigned to *E. crenulata*. However, since the use of scanning electron microscopy, the interpretation of *E. alveolata* expanded again and specimens with either subcircular or crenate to scutiform scales were included in *E. alveolata* (as seen in Wylezich 2002, Ogden 1980, Ogden 1981).

Given our current knowledge of the taxonomy of this group all these specimens with different morphologies should be considered as belonging to distinct species. But as the distinction between these species relies on the shape of the parietal scales which could not be observed by Dujardin, it is not possible to determine which of these species, if any, could be the original *E. alveolata*. Finally, no precise locality of origin was given in the original description (Dujardin only mentioned that *E. alveolata* was found in vases where he stored water from swamps in Paris and aquatic plants whose origins are unknown) which renders searching the type locality for candidates impossible. This quest is further pointless as many habitats sampled in the 19th century are now destroyed or degraded, especially near large cities. Given the lack of identifiable morphological characteristics and type locality in the original description, we consider impossible to establish the identity of *E. alveolata* Dujardin and we therefore treat it as a *nomen dubium*. Furthermore, due to the confusion in its identification and the usage of its name, *E. alveolata* Dujardin and its synonyms are associated to unreliable ecological and biogeographical data that would persist even if an authoritative redescription was made. Any designation of a neotype to fix its identity would therefore only add another competing interpretation of this name in the taxonomic literature and prolong the confusion.

The case of *E. alveolata* Dujardin might be the most documented, as it was one of the first species of the genus to be described, but it is not isolated and it should be considered that these issues apply to the vast majority of euglyphids described before the appearance of modern tools. After much consideration we therefore concluded that every species of euglyphids described without the use of modern tools should either be considered *nomen dubium,* or *inquirenda* at best when there are reasonable expectations to establish its original identity based on a very conspicuous morphology and the mention of a precise type locality and habitat in the original description.

The taxonomical resolution limit of the 18S genetic marker

In this work, we used the nuclear gene SSU rRNA as a genetic marker. This marker is commonly used for assessing general protist diversity from isolated specimens (Pawlowski et al., 2012), environmental samples (Berney et al., 2017) and to a lesser extent for DNA barcoding (Pawlowski et al., 2012). In Euglyphida, it is conserved enough to allow the classification of evolutionarily divergent organisms (Dumack et al., 2021), but is also variable enough for taxonomic purposes including discriminating closely related and morphologically similar species (Chatelain et al., 2013). SSU rRNA evolves more slowly than mitochondrial cytochrome C oxydase (COI). For example, in genus *Cyphoderia*, the divergence of SSU rRNA was ca. three times lower than COI, but the difference still allowed to delineate species (Heger et al., 2011). Choosing the right genetic marker is a trade-off between obtaining fine taxonomic resolution and keeping a good phylogenetic signal. In our study, SSU rRNA generally performed as expected, separating most morphologically different isolates, even though some differed only by a few nucleotides. However, two species (*Euglypha AcUniNe* and *Euglypha regipurpurei*) had identical SSU rRNA sequences, including the V4 region – one of the most common markers used in metabarcoding to discriminate species (Burki *et al.* 2021) - but differed clearly in scale morphology. Thus, the SSU rRNA gene seems inappropriate as a marker to distinguish closely related species but is potentially sufficient to reconstruct the backbone of the Euglyphidae phylogenetic tree. Furthermore, it must be noted that the monophyly of genus *Euglypha* is not fully supported by phylogenetic reconstruction based on the SSU rRNA gene alone. Depending on the alignment and the taxa included, the phylogenetic reconstruction either recovers the monophyly of *Euglypha* or places other taxa between the subgenera *Euglypha* and *Calliglypha*. For example, in the studies of Chatelain et al. (2013), and Tsyganov et al. (2017), the Trinematidae, Sphenoderiidae and Assulinidae are placed within the Euglyphidae, while Lara et al. (2007), and Dumack et al. (2021) found Euglyphidae to be monophyletic, albeit with low support. These conflicting results might be explained by a long branch attraction caused by a high genetic divergence between the subgenera *Euglypha* and *Calliglypha*, and the problem might be solved by populating the phylogenetic tree. However, while the phylogenetic reconstruction does not fully support the monophyly of the Euglyphidae, the monophyly is well supported by the morphological consistency between its species (Lara et al., 2007).

Designation of sections for the end-user

Given the likely gigantic diversity of the genus *Euglypha* compared to the few species we described, and how difficult identification is in routine work conditions, an accurate reassessment of the ecological preferences and distribution of any of these species will take a lot of time. Furthermore, the diversity within the genus *Euglypha* is likely too high to expect a correct identification of most species even if the taxonomy is solved. However, most of these species can be assigned to easily recognizable broad morphotypes to which thecamoebologists are already accustomed. We thus erected sections to act as a simplified taxonomic framework to study and report observations of *Euglypha* taxa. We also provide an identification key to the sections that we defined here. These sections were based on both morphology and phylogeny to accommodate closely related species. We hope that these sections somewhat differ in their ecological preferences, and that identifying the section of a species could already provide some usable information. These sections are to be treated as working concepts and can be seen as analogue to a trait-based approach that also includes phylogeny, as species of the same sections will share most of their morphological characteristics. This level of identification corresponds to what we can realistically expect most (palaeo)ecologists to achieve, we therefore propose that this should be the minimum requirement for any applied use of *Euglypha*.

As infrasubgeneric groups, these sections are not regulated by the ICZN and their appellation does not have to follow its rules, we thus took the opportunity to name these sections after well-known morphospecies so as to be readily usable by the community (i.e., no new names to remember!), even if the corresponding morphospecies were invalidated. As an example, spinous species of the subgenus *Euglypha* are currently placed in the section *Acanthophorae*, as prior to this revision these species would mostly be identified as *E. acanthophora* (Ehrenberg) Perty. The difference being that, by referring to a section rather than to a species, it is acknowledged that the species could not be identified precisely but that key morphological traits were observed. Furthermore, as each section has a clear diagnosis, the identification of a section and the usage of its name will depend less on one's personal interpretation, whereas the names of older species most likely hide hundreds of distinct and potentially unrelated species.

These sections were only based on our collected specimens and the most easily recognizable morphotypes depicted in the literature, and as such many older morphospecies are currently not assigned to either one. A corollary of this is that, if and when new sections are erected, the diagnosis of pre-existing sections will likely have to be revised to avoid any overlap. We thus aim to regularly update these sections to reflect the advances in the taxonomy of the genus *Euglypha* to prevent the taxonomy of this beautiful genus to once more fall in an obscure mess.

Revised classification of genus *Euglypha*

Here we present our classification of the genus *Euglypha* with the diagnosis of each subgenus, sections, and newly described species. Validated species are also listed under their corresponding section.

Family – **Euglyphidae**

Euglyphida with one or more rows of flat or gibbous, serrated oral scales surrounding an axial pseudostome.

Genus – ***Euglypha***

Type species: *Euglypha scutigera* Penard

Subgenus - ***Euglypha***

Euglyphidae with one or more rows of flat, serrated oral scales whose denticulation originate on the margin. Parietal scales ranging from circular / elliptic to crenate / scutiform. Spines, when present, either acicular as in the morphotype *E. brachiata*, or ensiform (they take the form of modified, elongated parietal scales) as in *E. regipurpurei*. Ensiform spines in positions normally occupied by parietal scales on the body of the test. Test uncompressed.

Type species: *Euglypha scutigera* Penard

Section - ***Tuberculatae***

Test not compressed, ovoid to lanceolate. Circular to elliptic parietal scales. No spines. See Meisterfeld (2002) for an example.

Section - ***Scutigerae***

Test not compressed, ovoid to lanceolate. Parietal scales ranging from sub-circular to scutiform. No spines.

***Euglypha* (*Euglypha*) “*scutGC”* Duckert & Meisterfeld**

Diagnosis: Test not compressed, ovoid in broad view with maximal width at around one third from the fundus, pseudostome circular, nucleus granular. Length (minimum-maximum-average): 51-61-66 µm; width (minimum-maximum-average): 29-31.5-34 µm; pseudostome width (average): 11.5 µm. Parietal scales sub-circular to sub-scutiform and only slightly overlapping in the middle of the test, which is sometimes not completely covered by scales, slenderer and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales between 7-9, flat, oral end with three to five pairs of denticulations originating on the margin on each side, aboral end hemi-circular to slightly angled.

Differential diagnosis: *Euglypha “scutGC”* can be differentiated from *Euglypha scutigera* Penard by its smaller and more elongated test.

Type locality: Top soil in a fen in the Grande Cariçaie, near Yverdon-les-Bains, Switzerland. 46°78’52’’9N. 6°66’45’’3E

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

Section - ***Acanthophorae***

Test not compressed, ovoid to lanceolate. Parietal scales ranging from circular, usually with a mucro on their oral margin, to crenate. Ensiform spines present.

***Euglypha (Euglypha) cashii* Ogden**

Differential diagnosis: *Euglypha cashi* can be differentiated from other *Acanthophorae* by its wide pseudostome (more than 15 µm) and small spines compared to the size of the test.

***Euglypha* (*Euglypha*) *cabrolae* (De Smet)**

Differential diagnosis: *Euglypha cabrolae* can be differentiated from other *acanthophora*-like species by its rectangular parietal scales and its ovoid test.

***Euglypha* (*Euglypha*) *regipurpurei* Duckert**

Diagnosis: Test not compressed, lanceolate in broad view with maximal width at around one third from the fundus, pseudostome circular, nucleus granular. Length (minimum-maximum-average): 65-71-78 µm; width (minimum-maximum-average): 32-34-35 µm; pseudostome width (average): 12 µm. Parietal scales scutiform with a mucro on their oral pole and only slightly overlapping in the middle of the test, which is not completely covered by scales, slenderer and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales between 8-9, flat, oral end with three to five pairs of denticulations originating on the margin on each side, aboral end hemi-circular to slightly angled. Up to four flexuous, ensiform spines, around 20 µm long located near the fundus.

Differential diagnosis: *Euglypha regipurpurei* can be differentiated from similar species by its scutiform parietal scales barely overlapping each other.

Etymology: To be updated

Type locality: Top soil in a fen in the Grande Cariçaie, near Yverdon-les-Bains, Switzerland. 46°78’52’’9N. 6°66’45’’3E

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

***Euglypha* (*Euglypha*) *“AcUnine”* Duckert**

Diagnosis: Test not compressed, lanceolate in broad view with maximal width at around one third from the fundus, pseudostome circular, nucleus granular. Length (minimum-maximum-average): 62-66.5-70.5 µm; width (minimum-maximum-average): 31-33-35 µm; pseudostome width (average): 12.5 µm. Parietal scales elliptic with a mucro on their oral pole and a nipple on their aboral pole, slenderer closer to the pseudostome. Pseudostome scales between 7-9, flat, oral margin with four to five pairs of denticulations originating on the margin on each side, aboral end hemi-circular to slightly angled. Up to three straight, ensiform spines, around 20 µm long located near the fundus.

Type locality: Partially submerged vegetation in a pond near the university of Neuchâtel, Switzerland (47°00'00.4"N 6°57'01.5"E).

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

Species currently not attributed to a section

***Euglypha* (*Euglypha*) *aspera* Penard**

Subgenus - ***Calliglypha***

Euglyphidae with a single row of gibbous, serrated oral scales whose denticulation originate below the margin. Parietal scales ranging from ellipsoid to polygonal (i.e., rectangular, rhomboidal, or hexagonal). Spines, when present, acicular and inserted between the parietal scales as in *E. FilSeeon* or ribbon-like and arranged in a tuft as in *E. bryo*. Test often compressed.

Type species: *Euglypha “NeAL4”*

Section - ***Cristatae***

Tuft of ribbon-like spines on the fundus.

***Euglypha* (*Calliglypha*) “*Bryo*” Duckert & Meisterfeld sp. nov.**

Diagnosis: Test slightly compressed, pyriform in broad view with maximal width at around one third from the fundus, pseudostome circular, nucleus vesicular. Length (minimum-maximum-average): 45-50-55 µm; width (minimum-maximum-average): 24-25.5-28 µm; pseudostome width (average): 7.5 µm. Parietal scales elliptic in the middle of the test, more rhomboidal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales between 8-10, oral end with a large denticulation originating from behind the apex of the central knob, one pairs of well-developed denticulations plus a small denticulation on each side, aboral margin well digitate, oral pole presenting a bulge with two lobes. Ribbon-like spines, around 15 µm long, arranged in a tuft on the fundus of the test.

Etymology: To be updated.

Type locality: Terrestrial moist mosses in the Jura Mountains, Switzerland (47°04’53’’6N; 6°51’56’’6E)

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

Section - ***Compressae***

Test compressed, ovoid shorter acicular spines, often in pairs, present almost only on the margin. Parietal scales sub-hexagonal, sub-rectangular to elliptic.

Euglypha **(*Calligylpha*) “*P4D1*” sp. nov. Meisterfeld**

Diagnosis: Test compressed, ovoid in broad view with maximal width at around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average): 68-74.5-81 µm; width (minimum-maximum-average): 38-43-47 µm; pseudostome width (average): 15 µm. Parietal scales sub-elliptic with sometimes an indentation in the middle of the test, more hexagonal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales between 12-14, roughly triangular, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral end almost flat. Acicular spines around 5 µm long located on the margin from the fundus up to almost mid-length of the test.

Differential diagnosis: *Euglypha “P4D1”* can be differentiated from *Euglypha “StriNII”* by its more angular parietal scales and its more abundant and densely packed spines.

Etymology: To be updated.

Type locality: La Palma, Spain

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

Euglypha **(*Calliglypha*) “*CompSN*” sp. nov. Duckert**

Diagnosis: Test compressed, ovoid-lanceolate in broad view with maximal width at around one third from the fundus, pseudostome elliptic, nucleus ovular. Length (minimum-maximum-average): 61-66-70 µm; width (minimum-maximum-average): 36-39-44 µm; pseudostome width (average): 10.5 µm. Parietal scales between hexagonal and elliptic with sometimes an indentation in the middle of the test, more rhomboidal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales between 11-13, oral end with a large denticulation as a prolongation of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral end hemi-elliptic. Acicular spines around 10 µm long located on the margin from the fundus up to two thirds of the test.

Etymology: To be updated.

Type locality: *Sphagnum* mosses in the Sierra Nevada, Spain (37°5'15.09"N; 3°22'24.18"W)

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX.

urn:lsid:zoobank.org:act: To be updated.

***Euglypha* (*Calliglypha*) “*StriNII*” sp. nov. Duckert**

Diagnosis: Test compressed, ovoid in broad view with maximal width at around mid-length, pseudostome most often elliptic, nucleus ovular. Length (minimum-maximum-average): 66-74-80 µm; width (minimum-maximum-average): 37-41.5-44 µm; pseudostome width (average): 14.5 µm. Parietal scales sub-elliptic with sometimes an indentation in the middle of the test, more elliptic closer to the pseudostome. Pseudostome scales around 12-13, roughly triangular, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral end almost flat. Acicular spines around 5 µm long located on the margin from the fundus up to almost mid-length of the test.

Differential diagnosis: *Euglypha “StriNII”* can be differentiated from *Euglypha “P4D1”* by its more elliptic parietal scales and less abundant and sparse spines.

Etymology: To be updated.

Type locality: Dry mosses on rocks near the university of Neuchâtel, Switzerland (47°00'00.4"N 6°57'01.5"E).

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX.

urn:lsid:zoobank.org:act: To be updated.

Section - ***Ciliatae***

Test compressed, short and densely packed acicular spines present mostly on the margin but also on the rest of the test. See strains “StrigTemplin” and “StrigP2A6” in Supplementary Figure 1 for examples.

***Euglypha (Calliglypha) penardi* Meisterfeld**

Differential diagnosis: *Euglypha penardi* can be differentiated from other *Compressae* or *Ciliatae* by its yellowish-brownish color due to the thick organic cement supporting the scales.

Section - ***Filiferae***

Filifera-like, test compressed, pyriform-ovoid to lanceolate. Long and sparse acicular spines inserted mostly one by one. Parietal scales sub-rectangular to hexagonal.

Euglypha **(*Calliglypha*) "*FilCRK12*" sp. nov. Meisterfeld**

Diagnosis: Test slightly compressed, ovoid-lanceolate in broad view with maximal width at around one third from the fundus, pseudostome circular, nucleus granular. Length (minimum-maximum-average-(outlier)): 60.5-67.5-77.5-(80) µm; width (minimum-maximum-average): 31-37-42.5 µm; pseudostome width (average): 11.5 µm. Parietal scales sub-rectangular in the middle of the test, more elliptic closer to the pseudostome. Pseudostome scales around 11, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin hemi-elliptic. Acicular spines around 10 µm long, inserted one by one, located on the margin from the fundus up to mid-length of the test.

Differential diagnosis: *Euglypha "FilCRK12"* can be differentiated from *Euglypha "FilTully"* and *Euglypha "FilSeeon"* by its sub-rectangular parietal scales.

Etymology: To be updated.

Type locality: Mosses on tree in Costa Rica

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: AJ418787

Euglypha **(*Calliglypha*) "*FilSeeon*" sp. nov. Meisterfeld**

Diagnosis: Test compressed, lanceolate in broad view with maximal width at around one third from the fundus, pseudostome circular, nucleus granular. Length (minimum-maximum-average-(outlier)): 73.5-78-83 µm; width (minimum-maximum-average): 33-36-40 µm; pseudostome width (average): 12.5 µm. Parietal scales sub-hexagonal in the middle of the test, slenderer closer to the pseudostome. Pseudostome scales around 10, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin hemi-circular to slightly angular. Acicular spines around 15 µm long, inserted one by one, located on the margin from the fundus up to mid-length of the test.

Differential diagnosis: *Euglypha "FilSeeon"* can be differentiated from *Euglypha "FilCRK12"* by its sub-rectangular parietal scales, and from *Euglypha "FilTully"* by its oral scales with an hemi-circular aboral margin and its spines stopping at around mid-length of test.

Etymology: To be updated.

Type locality: Griessee, Seeon, Germany

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

Euglypha **(*****Calligypha*****) “*****FilTully*****” sp. nov. Meisterfeld**

Diagnosis: Test compressed, lanceolate in broad view with maximal width at around one third from the fundus, pseudostome circular, nucleus ovular. Length (minimum-maximum-average-(outlier)): (49.5)-54.5-58.5-64.5 µm; width (minimum-maximum-average): 21.5-25-27.5 µm; pseudostome width (average): 7 µm. Parietal scales hexagonal in the middle of the test and only slightly overlapping, slenderer and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales between seven and nine, oral end with a large denticulation originating from behind the apex of the central knob, three pairs of well-developed denticulations plus a small denticulation on each side, aboral margin slightly digitate to slightly angular. Thick acicular spines up to 20 µm long, inserted one by one, located on the margin from the fundus almost up to the pseudostome.

Differential diagnosis: *Euglypha “FilTully”* can be differentiated from *Euglypha “FilCRK12”* by its hexagonal parietal scales, and from *Euglypha “FilTully”* by its oral scales with an slightly digitate to angular aboral margin.

Etymology: To be updated.

Type locality: Mats of algae in Tully, Australia

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: AJ418786

Section - ***Eurotundae***

Rotunda-like, test compressed, often very small, ovoid. Parietal scales elliptic, aboral margin of the oral scales hemi-circular to slightly digitate. Nucleus vesicular.

Euglypha **(*****Calligypha*****) “*****CRK4*****” sp. nov. Meisterfeld**

Diagnosis: Test compressed, ovoid in broad view with maximal with around mid-length, pseudostome circular, nucleus vesicular. Length (average): 31.2 µm with CV of 4.8; Width (average): 16.4 µm with a CV of 9.8; pseudostome width (average): 6.5 µm. Parietal scales sub-elliptic in the middle of the test, more rhomboidal closer to the pseudostome. Pseudostome scales around ten, oral end with a large denticulation originating from behind the apex of the central knob, one pair of well-developed denticulations plus a small denticulation on each side, aboral margin slightly digitate.

Etymology: To be updated

Type locality: Mosses on trees in Selvatica Rara Avis, Costa Rica

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: AJ418782

Section - ***Rhomboidalae***

Rotunda-like, test compressed, ovoid. Parietal scales rhomboidal, aboral margin of the oral scales angular to slightly digitate. Nucleus ovular.

***Euglypha (Calliglypha) hyalina* Coûteaux**

Differential diagnosis: *Euglypha capsiosa* can be differentiated from other *Rhomboidalae* by its very small test and its oral scales with a central knob that is not as prominent.

***Euglypha* (*Calliglypha*) *pantokratori* Duckert & Lara**

Diagnosis: Test compressed, ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average): 40-43-45 µm; width (minimum-maximum-average): 22-23.5-24 µm; pseudostome width (average): 7.5 µm. Parietal scales sub-rhomboidal in the middle of the test, more elliptic closer to the pseudostome. Pseudostome scales around eight-nine, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin angular to very slightly digitate.

Differential diagnosis: *Euglypha pantokratori* can be differentiated from *Euglypha "NeEch4"* and *Euglypha "P4ASQ"* by its wider parietal scales that overlap more closer to the pseudostome, and its oral scales with a less angled aboral margin. It can be differentiated from *Euglypha "P6B4"* by its slightly larger test, more rhomboidal parietal scales and oral scales with a less angled aboral margin.

Etymology: This species was named in reference to the mount Pantokrator, close to which it was found.

Type locality: Dry mosses on rocks on Corfu Island, Greece (39°75'20''4N. 19°84'72''5E).

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX. To be updated.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

***Euglypha* (*Calliglypha*) "*NeEch4*" sp. nov. Duckert**

Diagnosis: Test compressed, ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average): 40-43.5-48 µm; width (minimum-maximum-average): 22-23.5-24 µm; pseudostome width (average): 7 µm. Parietal scales sub-rhomboidal in the middle of the test, more rhomboidal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales around eight-nine, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin angular to slightly digitate.

Differential diagnosis: *Euglypha "NeEch4"* can be differentiated from *Euglypha pantokratori* and *Euglypha "P6B4"* by its slenderer parietal scales and which overlap only slightly on their poles closer to the pseudostome. It can be differentiated from *Euglypha "P4A6SQ"* by its wider and slightly shorter test.

Etymology: To be updated

Type locality: Dry mosses on rocks near the university of Neuchâtel, Switzerland (47°00'00.4"N 6°57'01.5"E).

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX. To be updated.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

***Euglypha* (*Calliglypha*) *"P6B4"* sp. nov. Meisterfeld**

Diagnosis: Test compressed, ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average): 35-40.5-44 µm; width (minimum-maximum-average): 17-20-22 µm; pseudostome width (average): 9 µm. Parietal scales sub-rhomboidal in the middle of the test, more rhomboidal closer to the pseudostome. Pseudostome scales around 10, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin slightly digitate.

Differential diagnosis: *Euglypha "P6B4"* can be differentiated from *Euglypha "NeEch4"* and *Euglypha "P4A6SQ"* by its wider parietal scales and which overlap more closer to the pseudostome. It can can be differentiated from *Euglypha pantokratori* by its slightly smaller test, more elliptic parietal scales and oral scales with a more digitate aboral margin.

Etymology: To be updated

Type locality: Terrestrial mosses near Brunssum, Germany (50°55'45.3"N 6°0'09.5"E).

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

***Euglypha* (*Calliglypha*) *"P4A6SQ"* sp. nov. Meisterfeld**

Diagnosis: Test compressed, elongated ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average): 41-46-51 µm; width (minimum-maximum-average): 19-21-23 µm; pseudostome width (average): 8.5 µm. Parietal scales sub-rhomboidal in the middle of the test, more rhomboidal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales around nine-ten, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin slightly angular to slightly digitate.

Differential diagnosis: *Euglypha "P4A6SQ"* can be differentiated from *Euglypha pantokratori* and *Euglypha "P6B4"* by its slenderer test and its parietal scales that are slenderer and overlap only slightly on their poles closer to the pseudostome. It can be differentiated from *Euglypha "NeEch4"* by its slenderer and slightly longer test.

Etymology: To be updated

Type locality: Mosses on trees in Aachen, Sieben Quellen, Germany.

Type material: We designate the specimen on Figure 3 as the type. To be updated

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

***Euglypha* (*Calliglypha*) *"P205B4"* sp. nov. Meisterfeld**

Diagnosis: Test small, compressed, ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average-(outlier)): 24-27-31-(34) µm; width (minimum-maximum-average): 10-12-15 µm; pseudostome width (average): 5 µm. Parietal scales sub-rhomboidal in the middle of the test, more rhomboidal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales between eight-ten, oral end with a large denticulation originating from behind the apex of the central knob, one pair of well-developed denticulations plus a small denticulation on each side, aboral margin slightly digitate.

Differential diagnosis: *Euglypha "P205B4"* can be differentiated from *Euglypha "EP106A2"* by its smaller test and its oral scales which have a less pronounced digitation.

Etymology: To be updated

Type locality: Hirschauer-Bucht, Germany

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

***Euglypha* (*Calliglypha*) *"EP106A2"* sp. nov. Meisterfeld**

Diagnosis: Test compressed, ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average-(outlier)): 30-33-36-(39) µm; width (minimum-maximum-average-(outlier)): 14-15-17-(21) µm; pseudostome width (average): 5.5 µm. Parietal scales broad, sub-rhomboidal in the middle of the test, more rhomboidal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales around eight, oral end with a large denticulation originating far from the apex of the central knob, one pair of well-developed denticulations plus a small denticulation on each side, aboral margin digitate.

Differential diagnosis: *Euglypha "EP106A2"* can be differentiated from *Euglypha "P205B4"* by its larger test and its oral scales which have a more pronounced digitation.

Etymology: To be updated

Type locality: Samana, Dominican Republic

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

Section - **Quadratae**

Rotunda-like, test compressed, ovoid. Parietal scales rectangular, aboral margin of the oral scales hemi-circular to slightly angled. Nucleus ovular.

***Euglypha* (*Calliglypha*) *"TenK2"* sp. nov. Meisterfeld**

Diagnosis: Test compressed, elongated ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (average): 36 µm with CV of 3.9; Width (average): 16.6 µm with a CV of 8.4; pseudostome width (average): 7 µm. Parietal scales thin, elongated rectangular and only slightly overlapping in the middle of the test, more rhomboidal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales between 10-12, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations on each side, aboral margin hemi-elliptic to slightly angular.

Differential diagnosis: *Euglypha "TenK2"* can be differentiated from *Euglypha "NeAL4"* and *Euglypha "P205A2"* by its slenderer test and its very slender and rectangular parietal scale that only slightly overlap.

Etymology: To be updated

Type locality: Mosses in Tenerife, Spain

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: AJ418784

Euglypha (_Calliglypha_) _"NeAL4"_ sp. nov. Duckert

Diagnosis: Test small, compressed, ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average): 45-48.5-51 µm; Width (minimum-maximum-average): 23-24-26 µm; pseudostome width (average): 9 µm. Parietal scales rectangular in the middle of the test, more elliptic and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales around 9-11, roughly elliptic, oral end with a large denticulation originating far from the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin hemi-circular to hemi-elliptic.

Differential diagnosis: *Euglypha "NeAL4"* can be differentiated from *Euglypha "TenK2"* by its larger test and its parietal scales that are less rectangular and overlap more. It can be differentiated from *Euglypha "P205A2"* by its larger test and its more rectangular parietal scales. It can be differentiated from *Euglypha "CAB"* by its more rectangular, smaller parietal scales and its oral scales that are more rounded on their aboral margin.

Etymology: To be updated

Type locality: Dry mosses on rocks near the university of Neuchâtel, Switzerland (47°00'00.4"N 6°57'01.5"E).

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX. To be updated

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

Species currently not attributed to a section

Euglypha (_Calliglypha_) _capsiosa_ Coûteaux

Differential diagnosis: *Euglypha capsiosa* can be differentiated from other *rotunda*-like species by its drop-shaped oral scales.

Euglypha (_Calliglypha_) _dickensii_ Ogden

Differential diagnosis: *Euglypha dickensii* can be differentiated from other *rotunda*-like species by its parietal scales that are sometimes elongated, similarly to ensiform spines.

***Euglypha* (*Calliglypha*) “*Eungella*” sp. nov. Meisterfeld**

Diagnosis: Test slightly compressed, ovoid-lanceolate in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average): 26-29-32 µm; width (minimum-maximum-average): 10-12-15 µm; pseudostome width (average): 5.5 µm. Parietal scales between rectangular and elliptic in the middle of the test, more rhomboidal closer to the pseudostome. Pseudostome scales around eight, oral end with a large denticulation as a prolongation of the central knob, one pair of well-developed denticulations plus a small denticulation on each side, aboral margin slightly digitate.

Etymology: To be updated

Type locality: Eungella, Australia

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

***Euglypha* (*Calliglypha*) *miquettae* Duckert sp. nov.**

Diagnosis: Test slightly compressed, elongated pyriform in broad view with maximal width at around one third from the fundus, sometimes curved, pseudostome circular. Length (minimum-maximum-average): 45-46-49 µm; width (minimum-maximum-average): 21-22.5-23 µm; pseudostome width (average): 6.5 µm. Parietal scales sub-rectangular in the middle of the test, more rhomboidal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales around seven, roughly circular, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin hemi-circular to slightly angular.

Etymology: As this species was found in the previous botanical garden of the University of Neuchâtel, we dedicate this species to the local botanist Marie-Marguerite “Miquette” Duckert-Henriod.

Type locality: Partially submerged vegetation in a pond near the university of Neuchâtel, Switzerland (47°00'00.4"N 6°57'01.5"E). This seems to be an aquatic species, as it was found only in a pond and not in the terrestrial mosses next to it.

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

***Euglypha* (*Calliglypha*) “*P205A2*” sp. nov. Meisterfeld**

Diagnosis: Test compressed, ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus vesicular. Length (minimum-maximum-average): 28-30-32 µm; width (minimum-maximum-average): 12-13-15 µm; pseudostome width (average): 5.5 µm. Parietal scales sub-rectangular in the middle of the test, more rhomboidal and only slightly overlapping on their poles closer to the pseudostome. Pseudostome scales around eight, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin very slightly digitate.

Differential diagnosis: *Euglypha "P205A2"* can be differentiated from *Euglypha "TenK2"* and *Euglypha "NeAL4"* by its smaller test, more elliptic parietal scales and oral scale that are slightly digitate on their aboral margin.

Etymology: To be updated

Type locality: Hirschau, Germany

Type material: We designate the specimen on Figure 3 as the type.

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated.

***Euglypha* (*Calliglypha*) *"CAB"* sp. nov. Duckert**

Diagnosis: Test compressed, ovoid in broad view with maximal width around mid-length, pseudostome circular, nucleus ovular. Length (minimum-maximum-average): 43-46.5-50 µm; width (minimum-maximum-average): 24-26-28.5 µm; pseudostome width (average): 8 µm. Parietal scales sub-rectangular in the middle of the test, more elliptic closer to the pseudostome. Pseudostome scales around nine, roughly almond shaped, oral end with a large denticulation originating from behind the apex of the central knob, two pairs of well-developed denticulations plus a small denticulation on each side, aboral margin hemi-elliptic to slightly angled.

Differential diagnosis: *Euglypha "CAB"* can be differentiated from *Euglypha "NeAL4"* by its more elliptic and larger parietal scales and its almond-shaped oral scales.

Etymology: To be updated

Type locality: Dry mosses on rocks near the university of Neuchâtel, Switzerland (47°00'00.4"N 6°57'01.5"E).

Type material: We designate the specimen on Figure 3 as the type. The SEM stub has been deposited in XXX with ID XXX. To be updated

urn:lsid:zoobank.org:act: To be updated. Genbank ID: To be updated

Conclusion

Rather than describing a plethora of new species accompanied by extensive ecological and distribution data, the goal of this revision was to provide a sound basis for future taxonomic works on the genus *Euglypha*. We hope that, by establishing clearer boundaries between species, identifying diagnostic traits, and clarifying the nomenclature, euglyphids appear less hermetic and that this work will motivate the description of new species, thus clarifying the diversity, biogeography and ecology of these organisms and their use in applied ecological research.

We acknowledge that accurate species identification is currently impractical and that the newly described species will not be reported before long. However, we erected informal sections based on morphology and phylogeny to be used instead, and we encourage researchers to re-evaluate the ecological value of euglyphids using this system.

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Tables and figures

Table 1. Sampling location and dimensions of the strains characterized during this study.

Figure 1. Scanning electron microscopy pictures of the idiosomes of *Euglypha*: Flat oral scales (A), gibbous oral scales (B), elliptic parietal scales (C), rectangular parietal scales (D), rhomboidal parietal (E), scutiform parietal scales (F), ensiform spines (G), acicular spines (H) and ribbon-like spines (I). Scale bar=5 µm.

Figure 2. Schematic phylogenetic tree of the genus *Euglypha* showing the relation between the sections defined in this work (upper left corner). Maximum likelihood phylogenetic tree of the genus *Euglypha* based on the SSU rRNA gene. RaxML bootstrap values (bs) and MrBayes posterior probabilities (pp) are indicated next to their node. Black dots indicate nodes with high support (bs/pp at least 95/0.99). The bs and pp values of low with low support (bs/pp below 50/0.5) were omitted.

Figure 3. Scanning electron microscopy pictures and schematic drawings of the test and the outline and disposition of parietal scales of the species formally described in this work. Scale bar on the left = 5 µm, scale bar on the right = 20 µm. The scales on the right are not to scale.

Supplementary Figure 1. Scanning electron microscopy pictures of the strains that were not formally described in this work. Scale bar = 20 µm.

Supplementary Table 1. Compilation of the names pertaining to *Euglypha* appearing in the literature with the evaluation of their status and validity.

Table 1

Strains ID	Sampling location	Genbank ID	Length min-average-max(-outlier)	Average Length	Width min-average-max(-outlier)	Average Width	Average pseudostome width
Euglypha pantokratori sp. nov.	Greece, Perama, Corfu Island	Will be updated	40-43-45	43	22-23.5-24	23.5	7.3
Euglypha NEECH4 sp. nov	Switzerland, Neuchâtel	Will be updated	40-43.5-48	43.5	22-23.5-24	23.5	6.8
Euglypha P6B4 sp. nov.	Netherlands, Brunsumer Heide	Will be updated	35-40.5-44	40.5	17-20-22	20	9.1
Euglypha P4A6S.Q sp. nov.	Germany, Aachen, Sieben Quellen	Will be updated	41-46-51	46	19-21-23	21	8.5
Euglypha P205B4 sp. nov.	Germany, Hirschauer Bucht	Will be updated	24-27-31-(34)	27	10-12-15	12	5.1
Euglypha EP106A2 sp. nov.	Dominican Republic, Samana	Will be updated	30-33-36-(39)	33	14-15-17-(21)	15	5.5
Euglypha TenK2 sp. nov.	Spain, Teneriffe	AJ418784	36	36	16.6	16.6	7.2
Euglypha NEAL4 sp. nov.	Switzerland, Neuchâtel	Will be updated	45-48.5-51	48.5	23-24-26	24	9
Euglypha Eungella sp. nov.	Australia, Eungella	Will be updated	26-29-32	29	10-12-15	12	5.6
Euglypha P205A2 sp. nov	Germany, Hirschau	Will be updated	28-30-32	30	12-13-15	13	5.5
Euglypha CRK4 sp. nov.	Costa Rica, Selvatica Rara Avis	AJ418782	31.2	31.2	16.4	16.4	6.3
Euglypha CAB sp. nov.	Switzerland, Neuchâtel	Will be updated	43-46.5-50	46.5	24-26-28.5	26	8.1
Euglypha scutGC	Switzerland, Yverdon, Grande Caricaie	Will be updated	56-61-66	61	29-31.5-34	31.5	11.5
Euglypha AcUnine sp. nov.	Switzerland, Neuchâtel	Will be updated	62-66.5-70.5	66.5	31-33-35	33	12.7
Euglypha regipurpurei sp. nov.	Switzerland, Yverdon, Grande Caricaie	Will be updated	65-71-78	71	32-34-35	34	12.1
Euglypha P4D1 sp. nov.	Spain, La Palma	Will be updated	68-74.5-81	74.5	38-43-47	43	14.8
Euglypha SNcomp sp. nov.	Spain, Sierra Nevada	N.a	61-66-70	66	36-39-44	39	10.5
Euglypha STriNII sp. nov.	Switzerland, Neuchâtel	N.a	66-74-80	74	37-41.5-44	41.5	14.5
Euglypha miquettae sp. nov	Switzerland, Neuchâtel	Will be updated	45-46-49	46	21-22.5-23	22.5	6.7
Euglypha FilCRK12 sp. nov.	Costa Rica	AJ418787	60.5-67.5-77.5-(80)	67.5	31-37-42.5	37	11.5
Euglypha FilSeeon sp. nov.	Germany, Seon, Griessee	Will be updated	73.5-78-83	78	33-36-40	36	12.7
Euglypha FilTully sp. nov.	Australia, Tully	AJ418786	(49.5)-54.5-58.5-64.5	58.5	21.5-25-27.5	25	7
Euglypha Bryo sp. nov.	Switzerland, Jura Mountains, Les Convers	Will be updated	45-50-55	50	24-25.5-28	25.5	7.4
rot GomeraSPK3	Spain, La Gomera, track to El Cedro	Will be updated	38-41.5-45	41.5	21-23-26	23	9.7
rot P2a5	Spain, La Palma	Will be updated	39-43-49-(55)	43	21-25-27-(32)	25	8.9
rot P3C4	Spain, La Palma	Will be updated	33-36-40	36	17-20-22	20	7.5
rot SPK1	Spain, La Gomera	AJ418783	25-28-30	28	12-15-17	15	6.1
rot P306C6	Ecuador, San Francisco	Will be updated	28-31-33	31	13-15.5-17	15.5	5.7
rot P2D4	Spain, La Palma, Los Tilos	Will be updated	29-31-33	31	11-15-18	15	6.7
rot P2C2	Spain, La Palma, Los Tilos	Will be updated	29-30.5-33	30.5	13-16-17	16	6.4
rot P5D3	Germany, Aachen, Wurmtal 5	Will be updated	30-32-35	32	15-17-19	17	6.9
rot P6C6	Germany, Klaber	Will be updated	30-32.5-37	32.5	11-15-18	15	7.6
fil P106B5Samana	Dominican Republic, Samana	Will be updated	42-47.5-51	47.5	18.5-22.5-25	22.5	8.3
Comp TENK3	Spain	Will be updated	57-63-68-(74)	63	35-40-44-(48)	40	13.6
comp Spha	Switzerland	N.a	62-67.5-72	67.5	39.5-42-43.5	42	15.2
strig P2A6	Spain, La Palma, El Pilar	Will be updated	(62)-65-70-74	70	37-40.5-43	40.5	14
strig Templin	Germany, Templin	Will be updated	N.a	N.a	N.a	N.a	N.a
bryo Gi	Germany, Giessen, Bergwerkswald	Will be updated	45-49.5-54	49.5	23-26-29	26	7.9
scut Seeon	Germany, Giessen, Bergwerkswald	Will be updated	60-66.5-71-(74)	66.5	28-31-33	31	11.3
ac BWGi	Germany, Giessen, Bergwerkswald	Will be updated	59-64-69	64	29-31-33	31	14.5
Comp Seeon	German, Seoon, Griessee	Will be updated	69-76-81	76	40-45-50	45	13

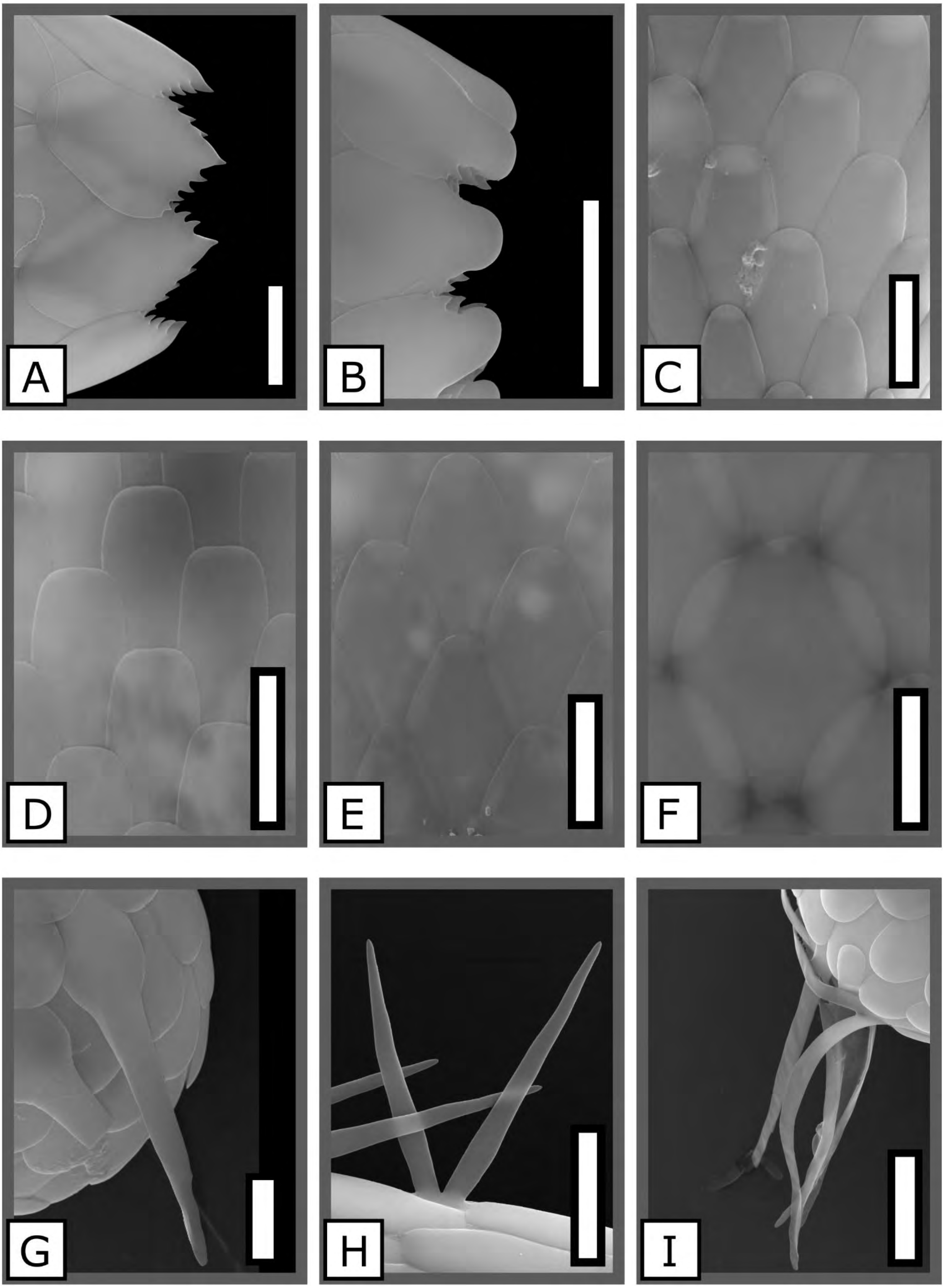
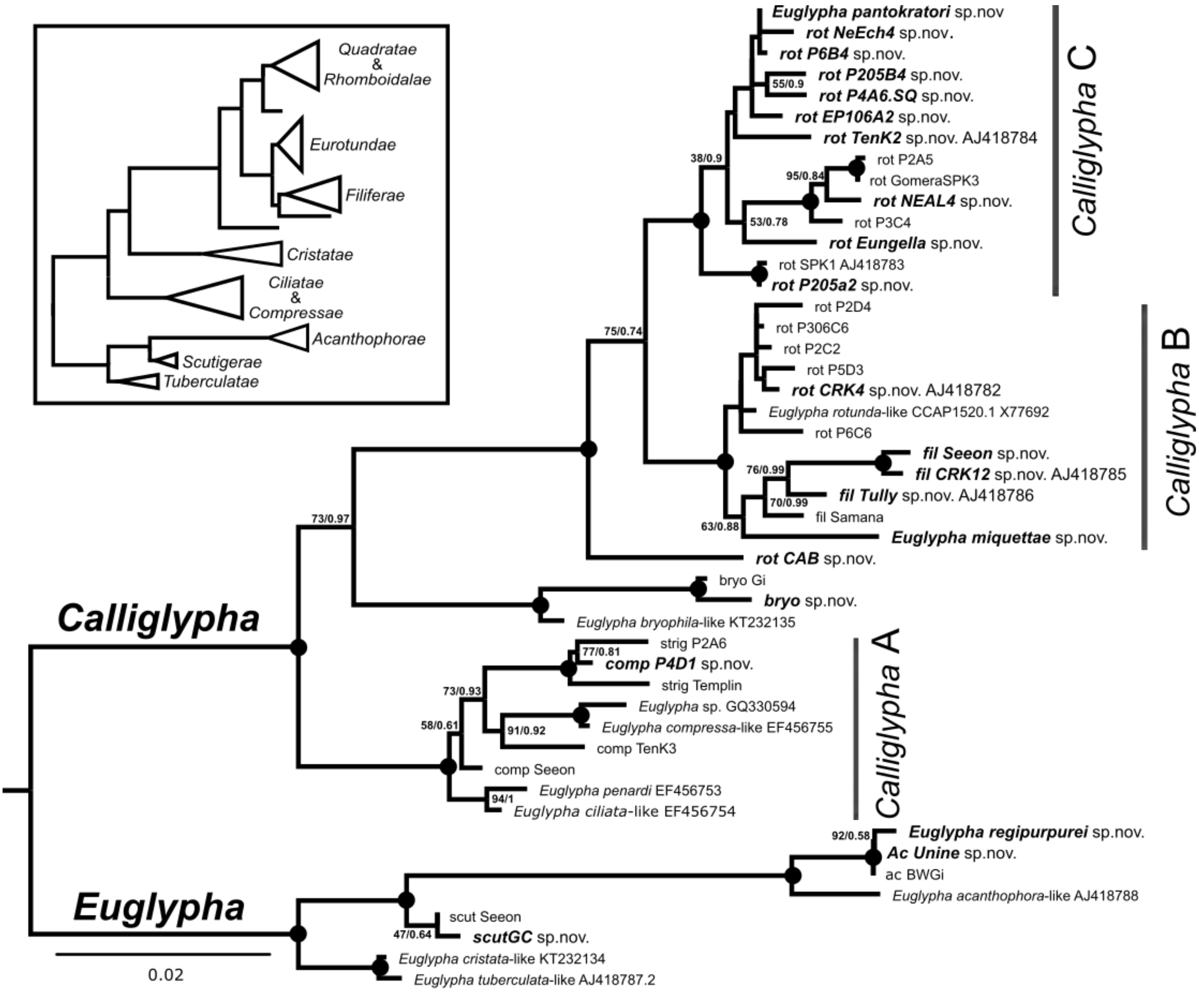


Figure 1


Quadratae
&
Rhomboidalae
Eurotundae
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Cristatae
Ciliatae
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Compressae
Acanthophorae
Scutigerae
Tuberculatae
Euglypha pantokratori sp.nov
rot NeEch4 sp.nov.
rot P6B4 sp.nov.
55/0.9
rot P205B4 sp.nov.
rot P4A6.SQ sp.nov.
rot EP106A2 sp.nov.
rot TenK2 sp.nov. AJ418784
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rot P2A5
95/0.84
rot GomeraSPK3
rot NEAL4 sp.nov.
53/0.78
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rot Eungella sp.nov.
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rot CRK4 sp.nov. AJ418782
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rot P6C6
fil Seeon sp.nov.
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70/0.99
fil Tully sp.nov. AJ418786
fil Samana
63/0.88
Euglypha miquettae sp.nov.
Calliglypha B
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bryo Gi
bryo sp.nov.
Calliglypha
Euglypha bryophila-like KT232135
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comp P4D1 sp.nov.
strig Templin
73/0.93
Euglypha sp. GQ330594
58/0.61
91/0.92
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comp TenK3
comp Seeon
Euglypha penardi EF456753
94/1
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ac BWGi
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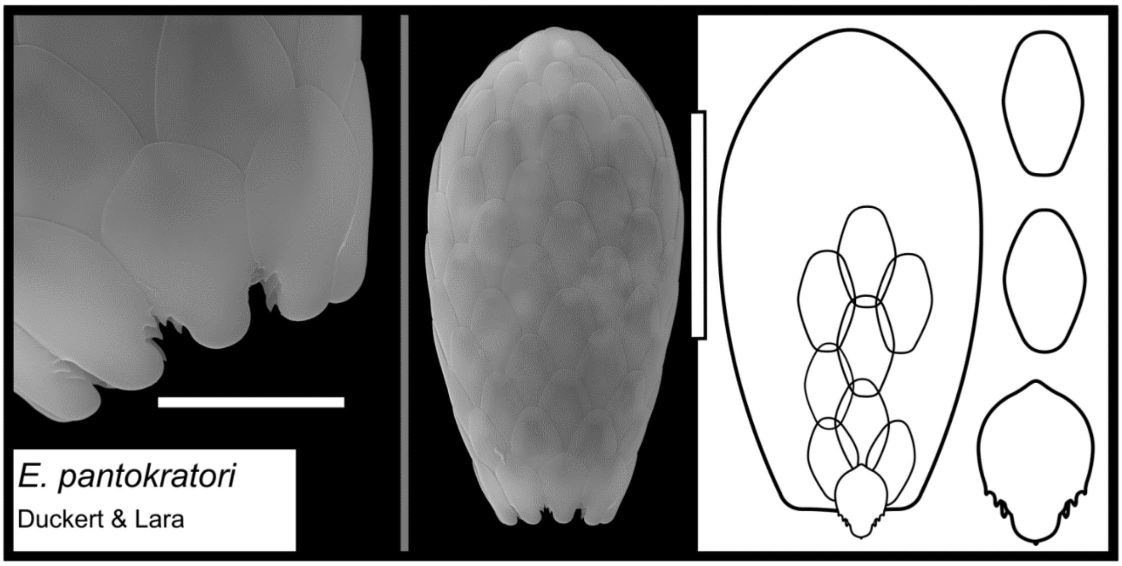
Figure 2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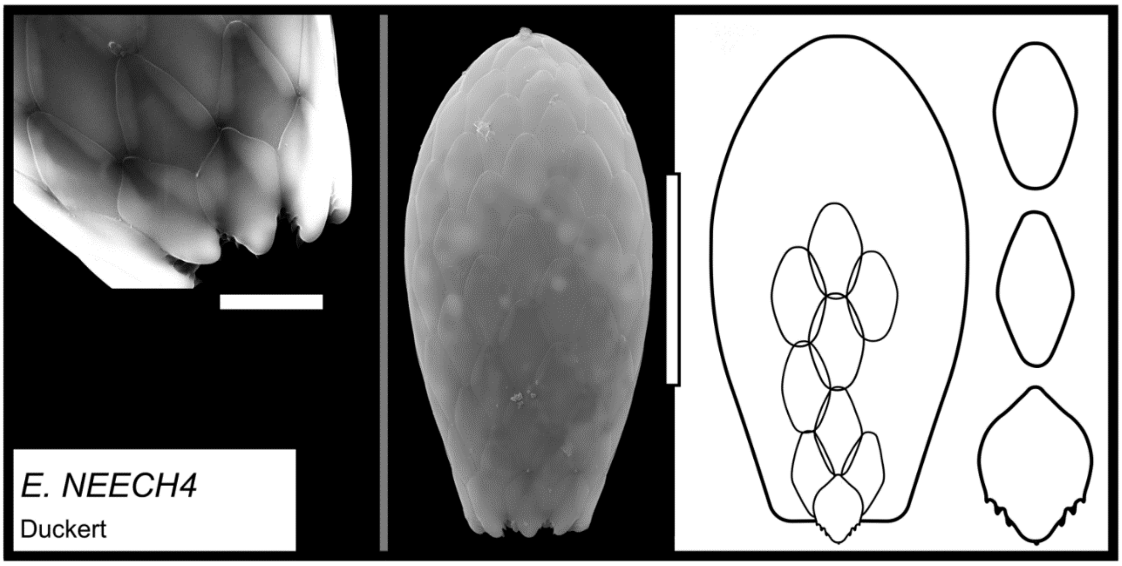
Duckert & Lara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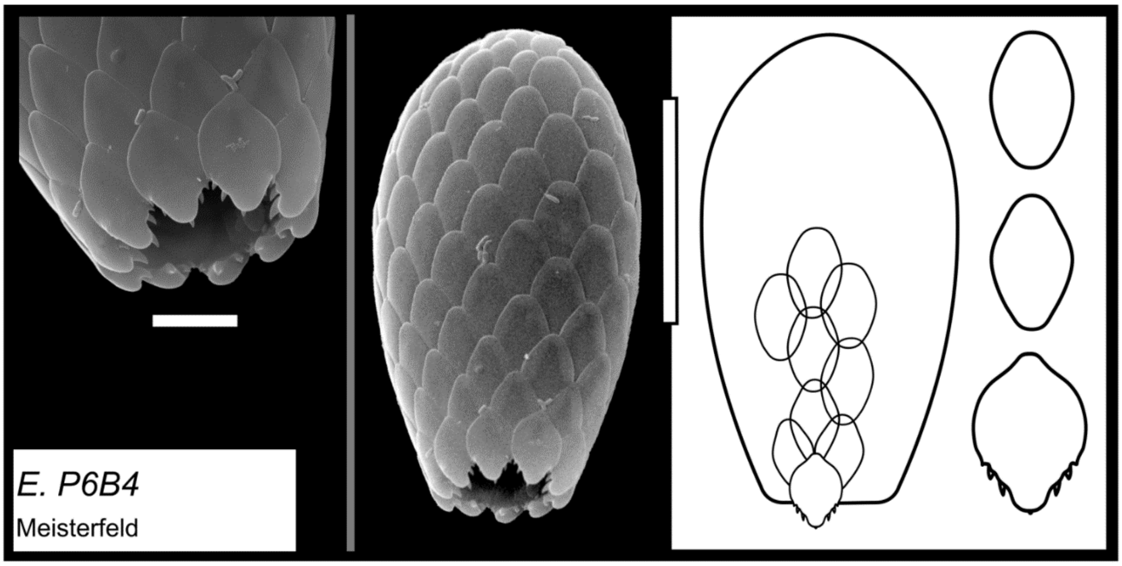
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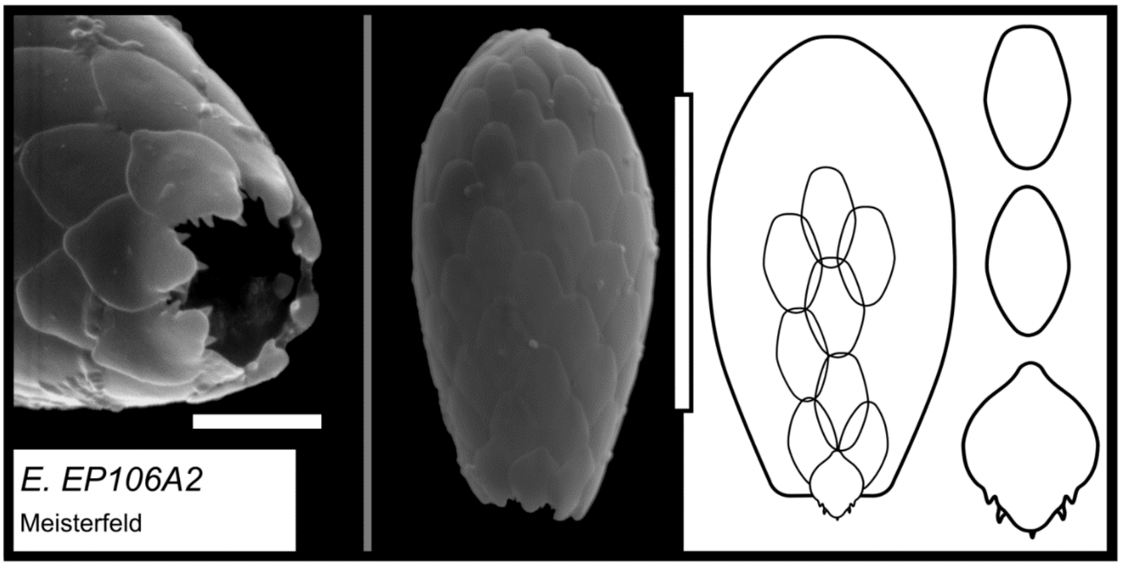

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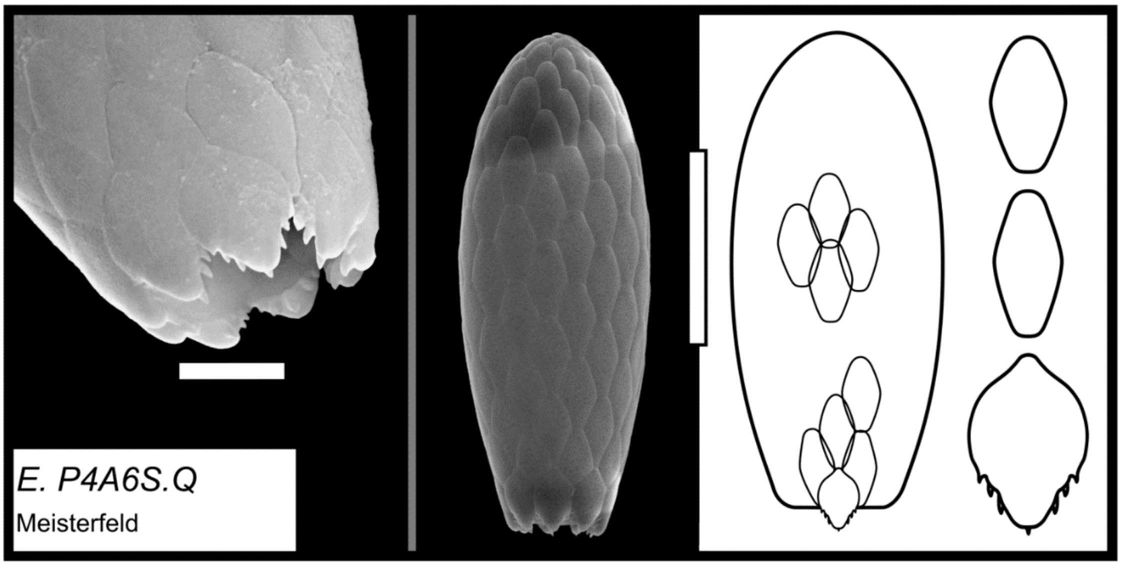

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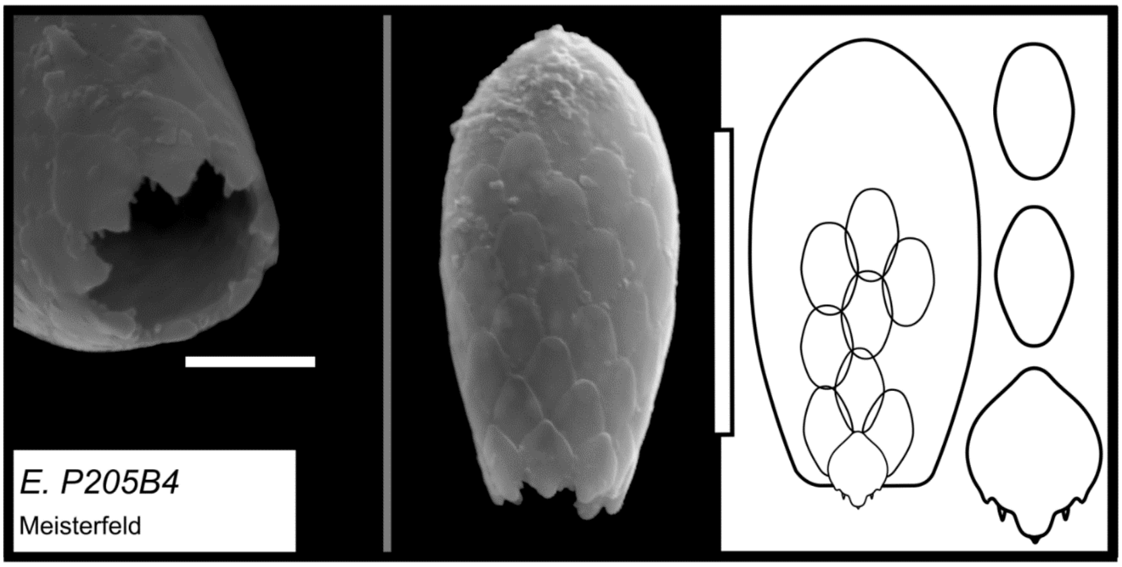

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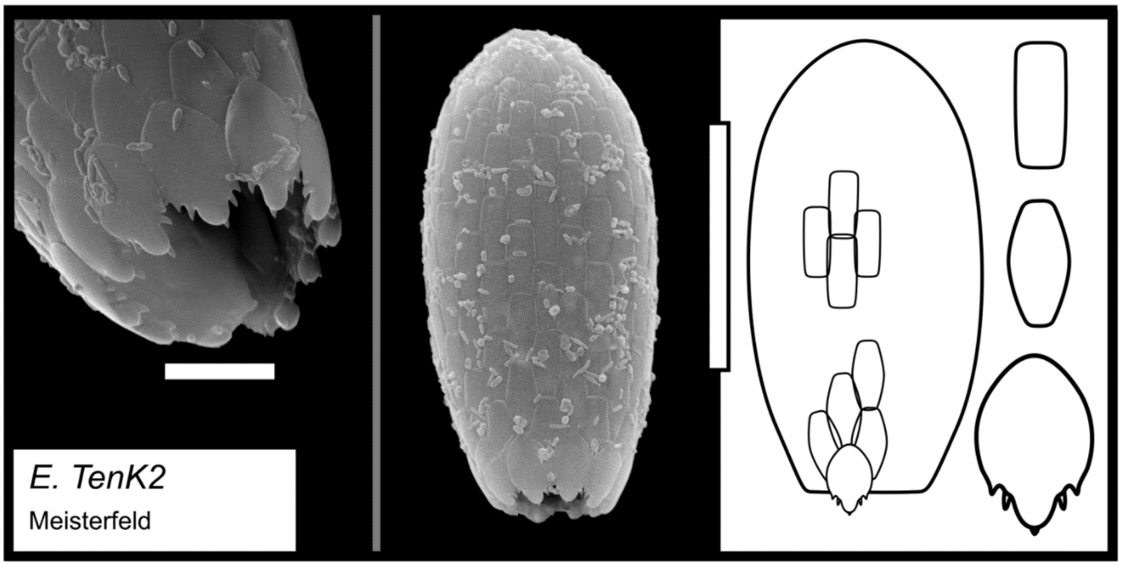
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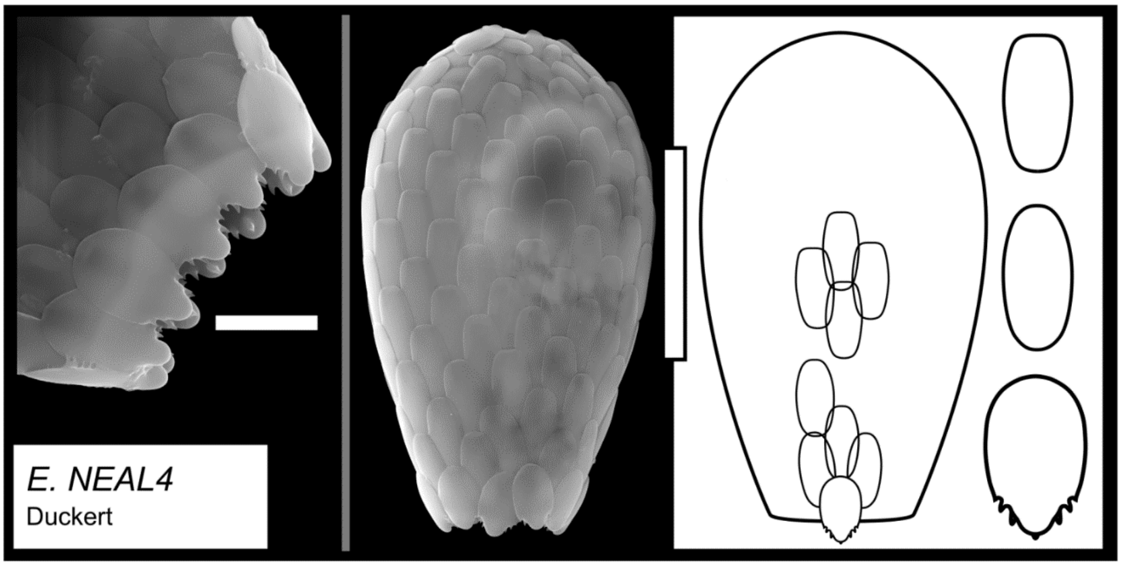
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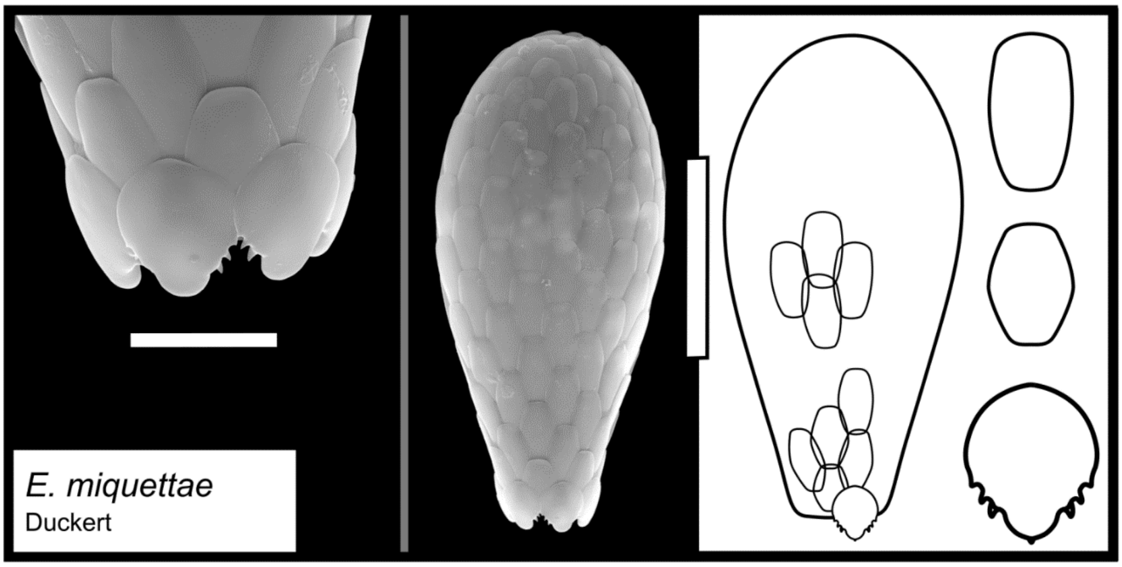
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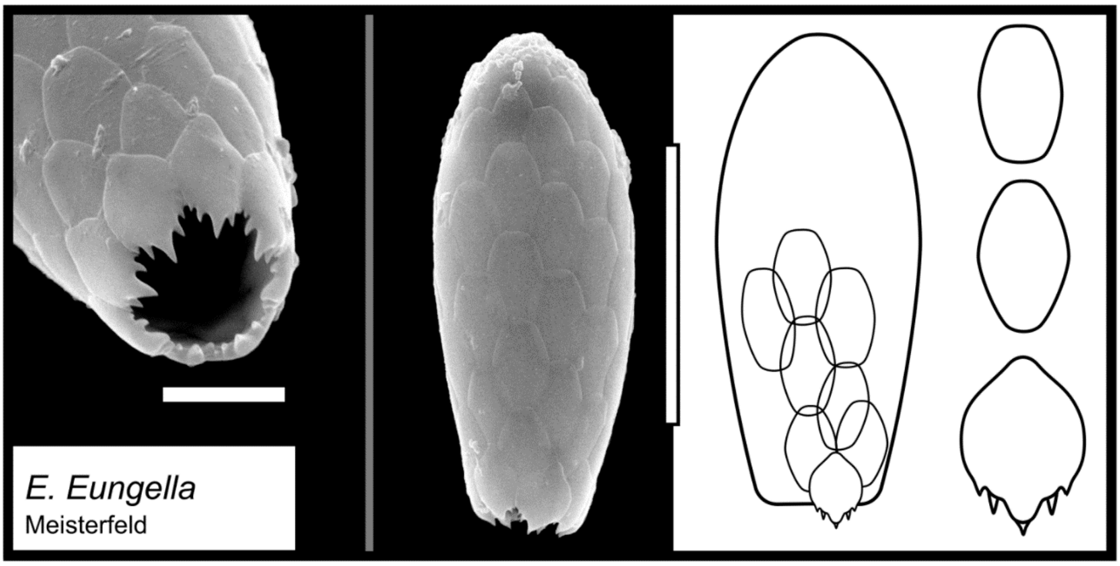
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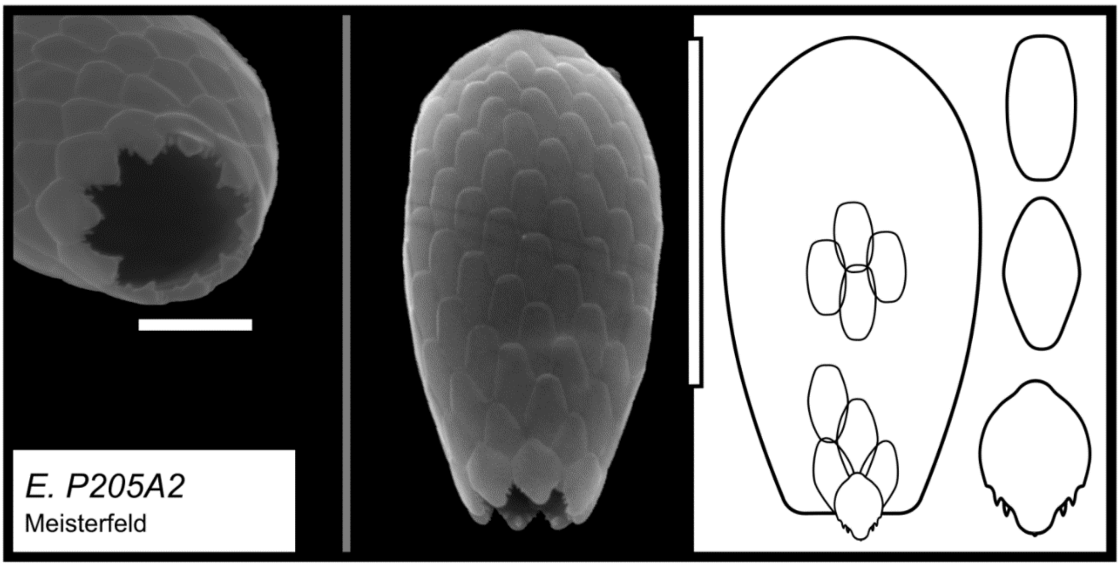
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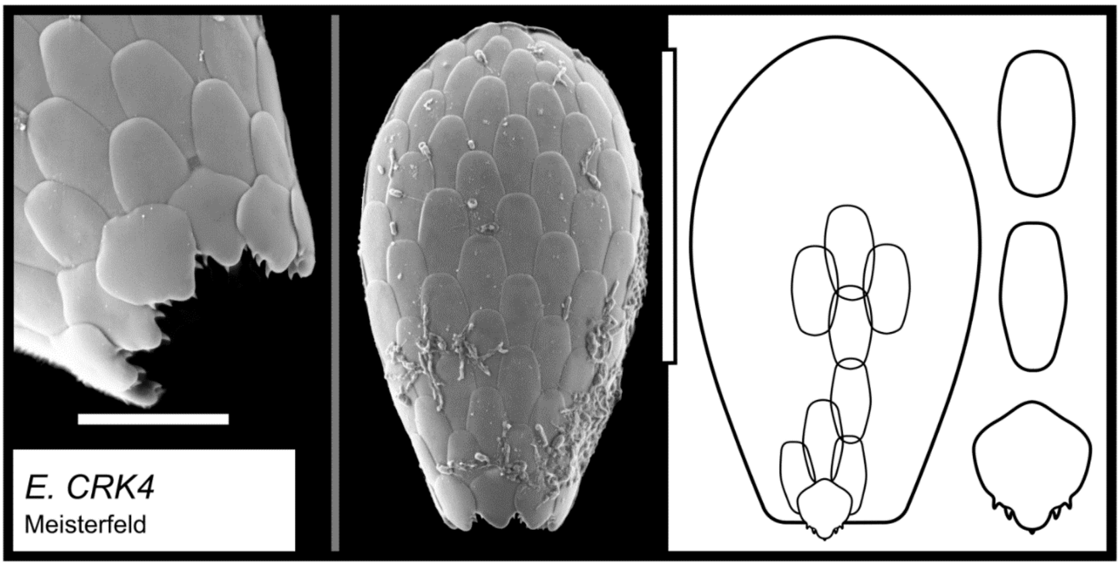
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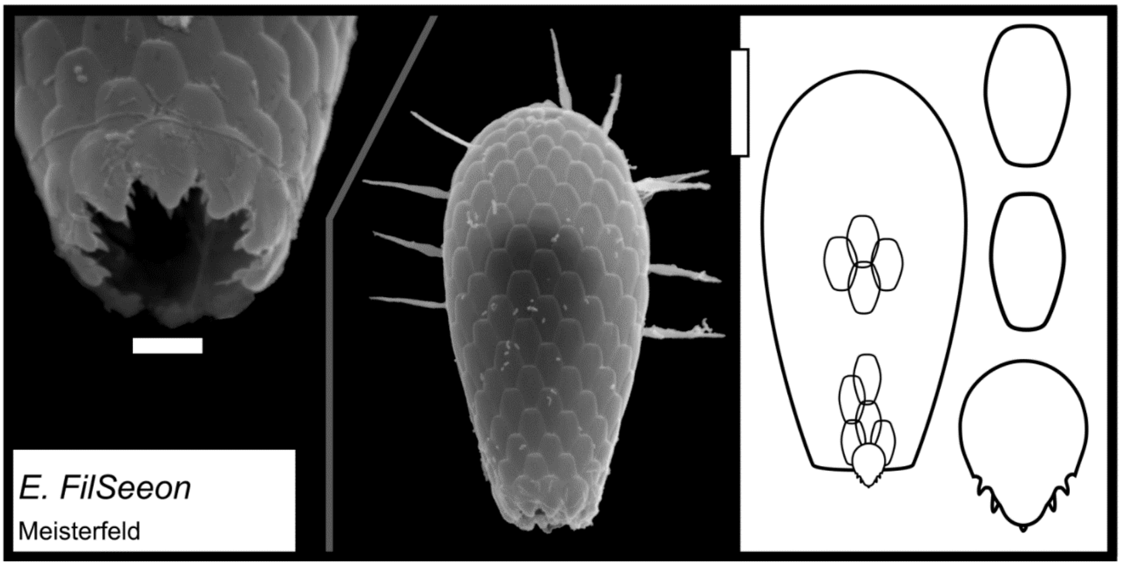
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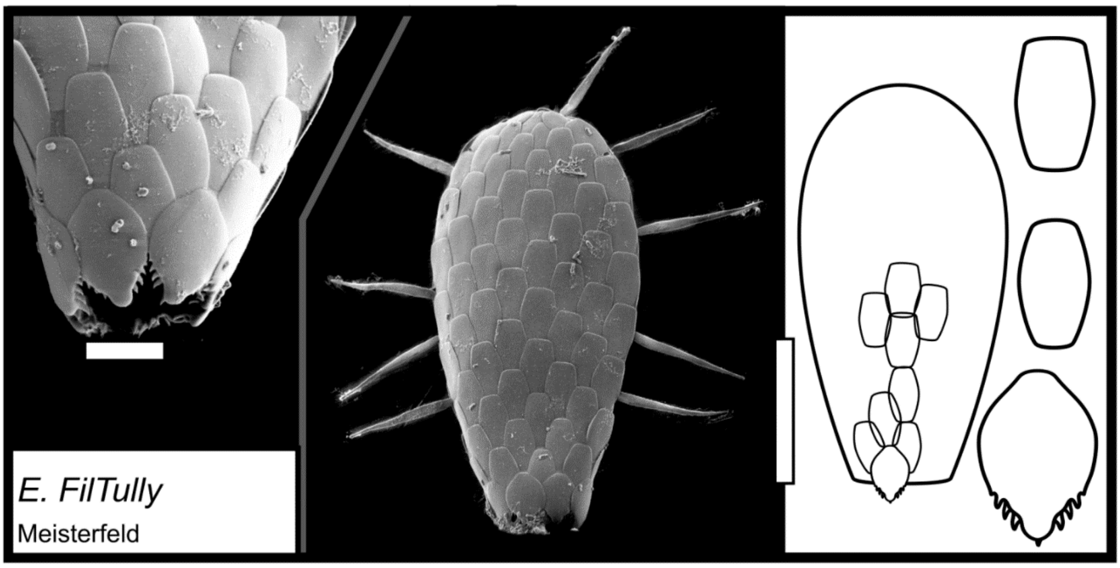
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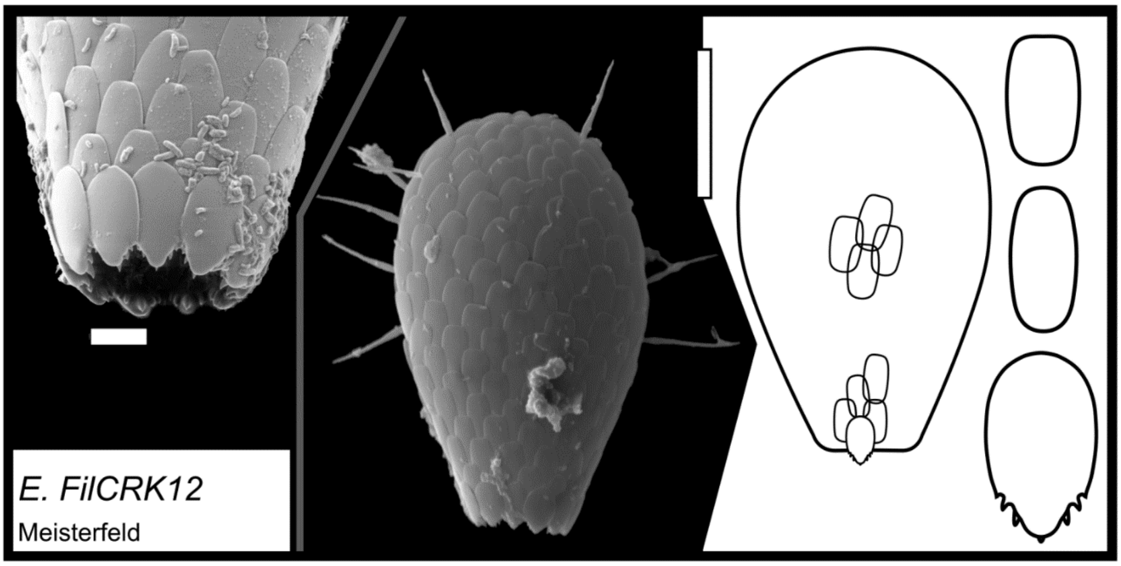
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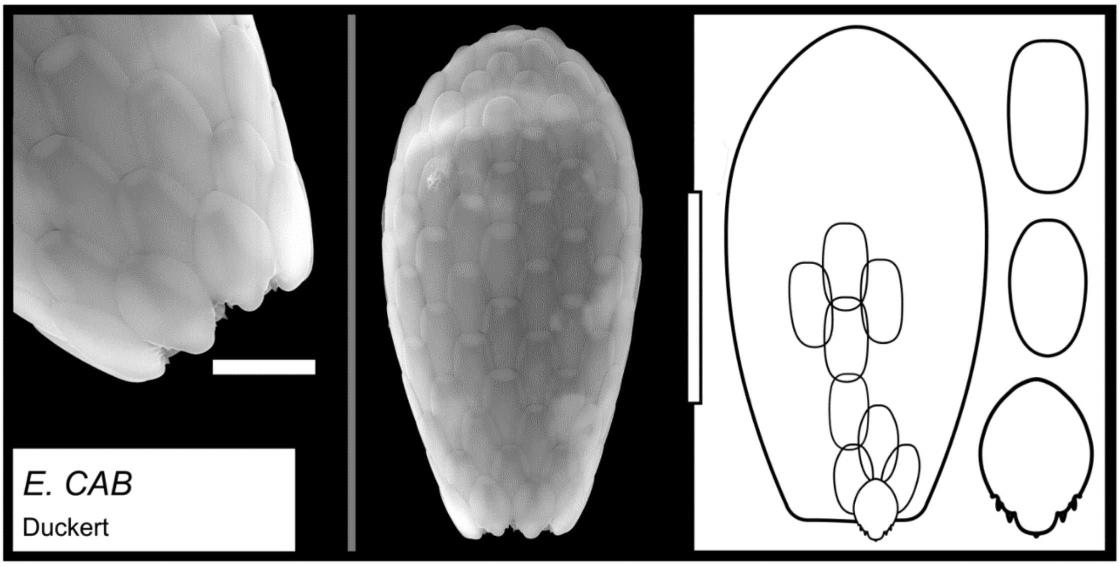
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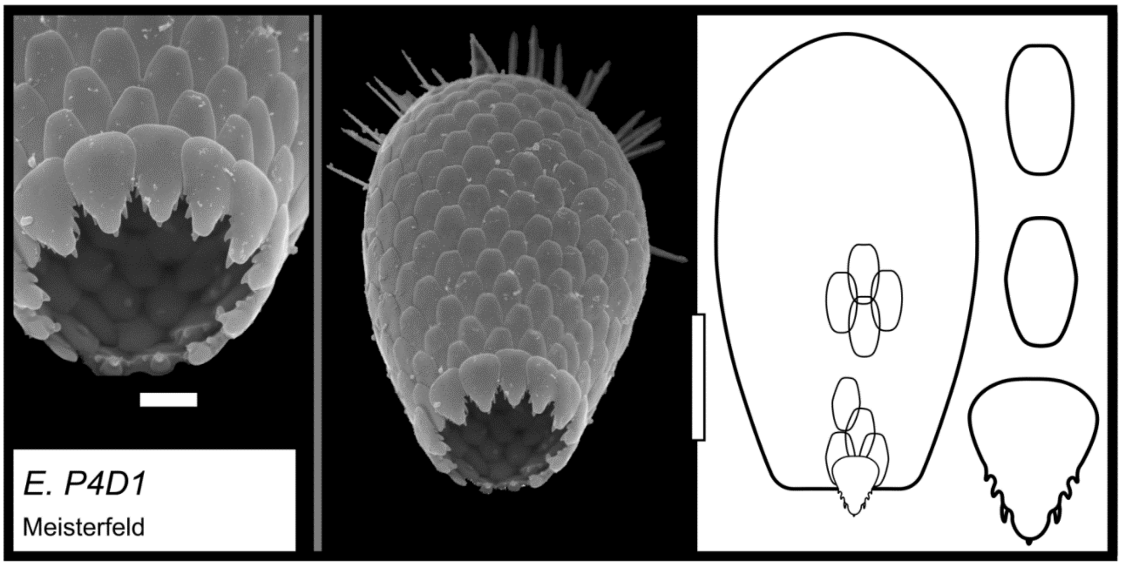
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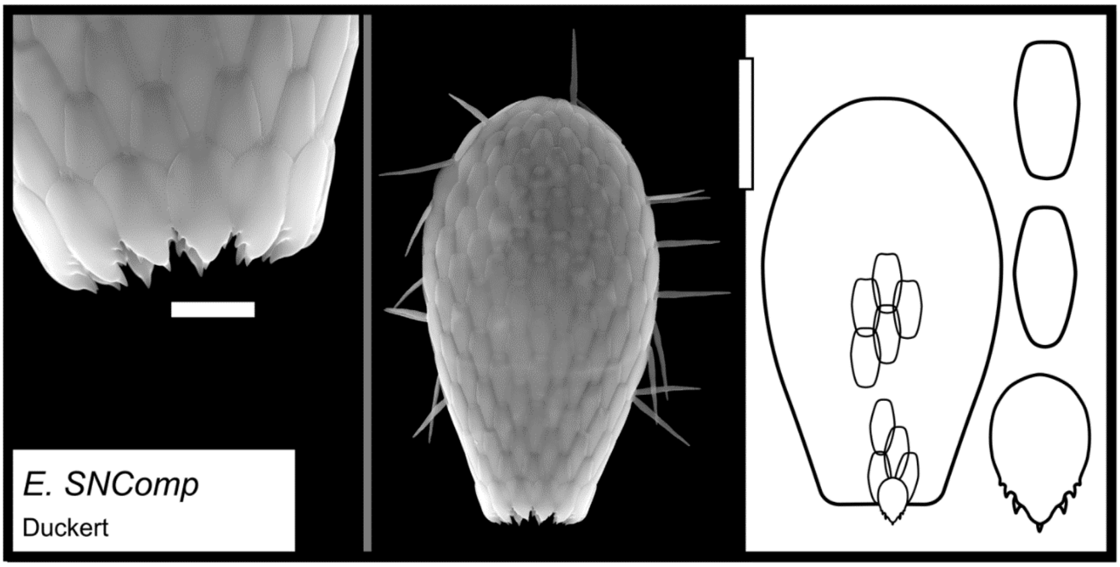
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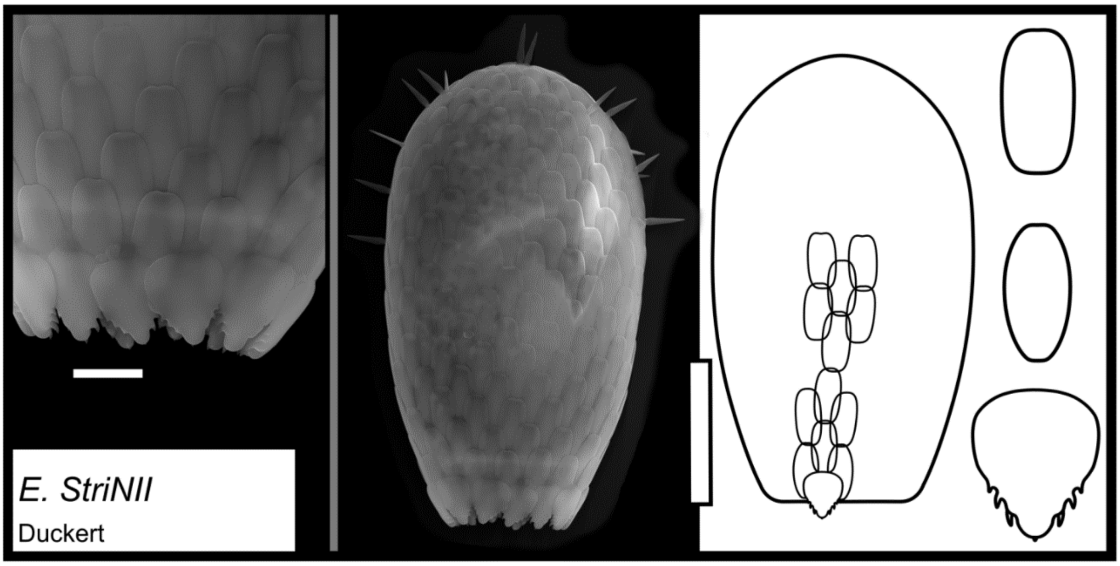
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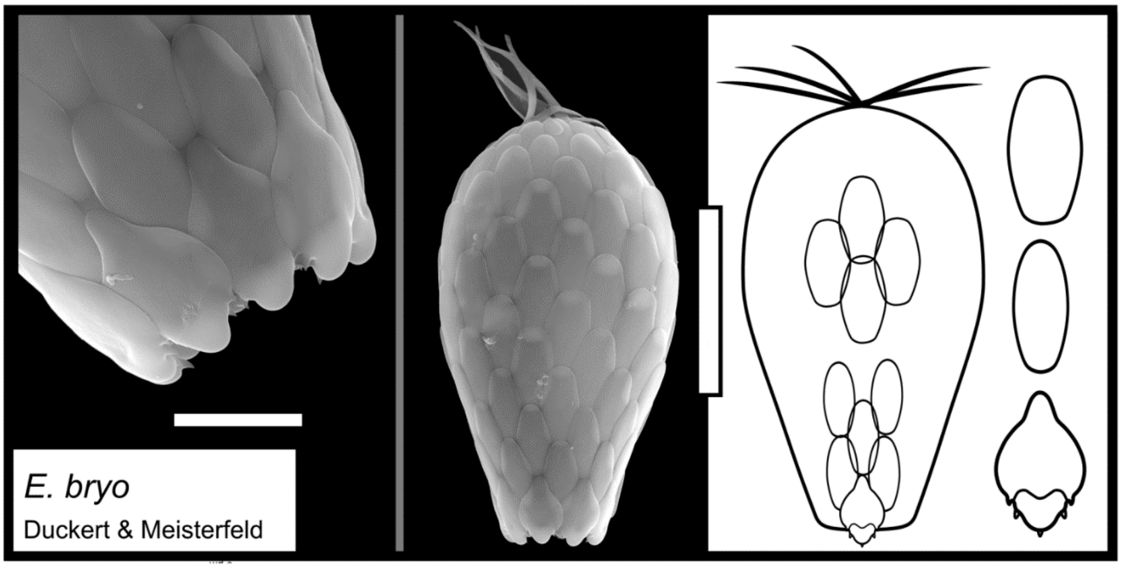
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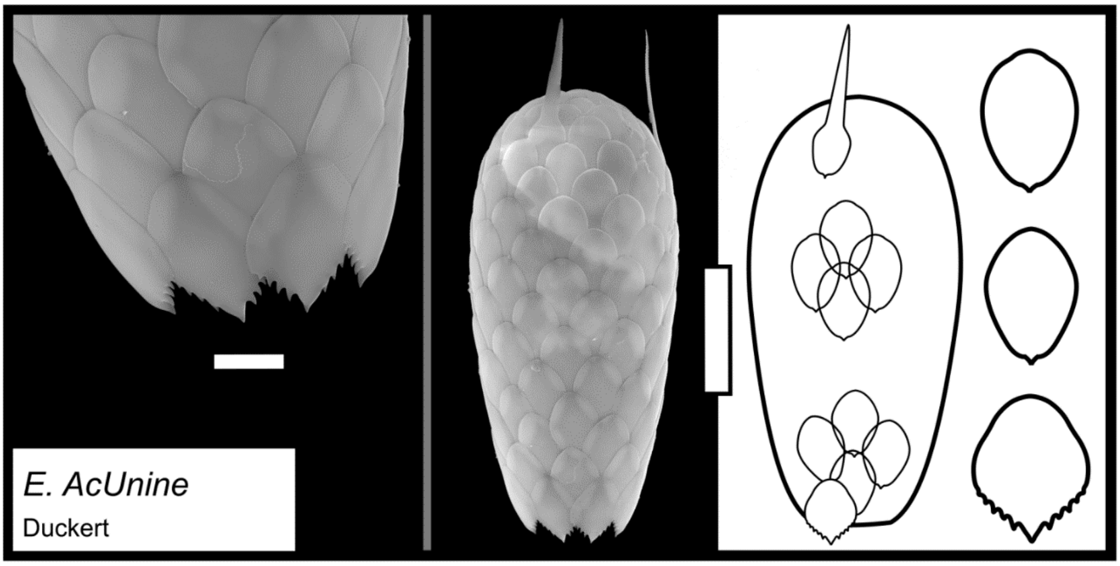
Duckert & Meisterfeld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Duckert

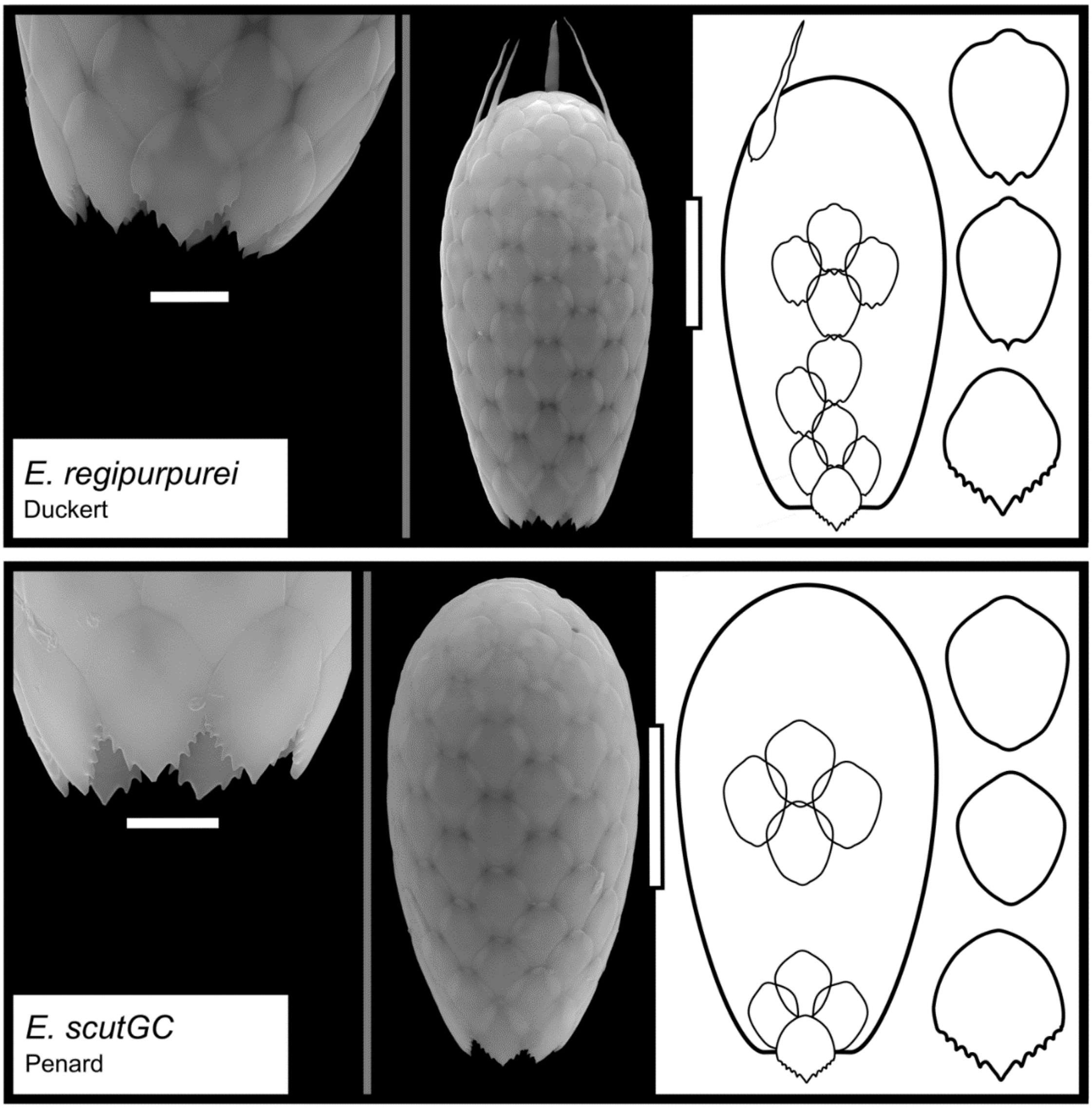


Figure.3

Supplementary Table 1.

Taxon	**Status**	**Original publication**
E. acanthophora (Ehrenberg) Perty	Junior synonym of *E. alveolata* Dujardin sensu Loeblich and Tapan. Originally described as *Difflugia acanthophora*.	Ehrenberg, C. G. (1841). Verbreitung und Einflufs des mikroskopischen Lebens in Süd- und Nord-Amerika. *Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin*, pp. 413-444.
E. alveolata Dujardin	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Dujardin, F. (1841). *Histoire naturelle des zoophytes: Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope: Ouvrage accompagné de planches,* p. 252. Roret.
E. areolata Ehrenberg	Junior synonym of *E. tuberculata* Dujardin sensu Loeblich and Tapan. Originally described as *Difflugia areolata*.	Ehrenberg, C. G. (1841). Verbreitung und Einflufs des mikroskopischen Lebens in Süd- und Nord-Amerika. *Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin*, pp. 413-444.
E. tuberculata Dujardin	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Misspelled as *E. tuberculosa* in the figures.	Dujardin, F. (1841). *Histoire naturelle des zoophytes: Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope: Ouvrage accompagné de planches,* p. 252. Roret.
E. ciliata (Ehrenberg) Leidy	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Ehrenberg, C. G. (1848). *Bericht über die zur Bekanntmachung geeigneten Verhandlungen der Konigl.Preuss.Akademie der Wissenschaften zu Berlin*, p. 379.
E. setigera Perty	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. No figures in the original description.	Perty, M. (1849). Mikroskopische Organismen der Alpen und der italienischen Schweiz. *Mittheilungen der Naturforschenden Gesellschaft in Bern*, *164*(165), 153-176.

E. laevis Perty	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. No figures in the original description. Sometimes referred to as *E. laevis* (Ehrenberg) Perty or misspelled as *E. loevis* Perty.	Perty, M. (1849). Uber verticale Verbreitung mikroskopischer Lebensformen. *Mittheilungen der naturforschenden Gesellschaft in Bern*, 146, 17-45.
E. curvata Perty	Not Euglypha, but *Cyphoderia* according to Decloître (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. minima Perty	Not *Euglypha*, but *Trachelocorythion* according to Decloitre (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. pleurostoma Carter	Not *Euglypha*, but *Trinema*.	Carter, H.J. (1857). On the freshwater Infusoria of Bombay. *The Annals and magazine of natural history; zoology, botany, and geology*, p. 41.
E. compressa Carter	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Carter, H.J. (1864). On freshwater Rhizopoda of England and India. *The Annals and magazine of natural history; zoology, botany, and geology*, p. 32.
E. globosa Carter	Not *Euglypha,* but *Sphenoderia.*	Carter, H.J. (1865). On the fresh- and salt-water Rhizopoda of England and India. *The Annals and magazine of natural history; zoology, botany, and geology*, p. 290.
E. spinosa Carter	Not *Euglypha*, but *Placocista.*	Carter, H.J. (1865). On the fresh- and salt-water Rhizopoda of England and India. *The Annals and magazine of natural history; zoology, botany, and geology*, p. 290.
E. strigosa (Ehrenberg) Leidy	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Originally described as *Difflugia strigosa* Ehrenberg.	Ehrenberg, C. G. (1871). Nachtrag zur Ubersicht der organischen Atmosphärilien, *Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin*, p. 257.

E. *ampullacea* Hertwig and Lesser	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Hertwig, R., Lesser, E. (1874). *Ueber Rhizopoden und denselben nahestehende Organismen: Morphologische Studien von Richard Hertwig und E. Lesser* (Vol. 10). Max Cohen & Sohn.
E. cristata Leidy	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. No figure in the original publication, first depiction shows several distinct species, of which likely *E. bryophila.* No illustration in the original publication. Only shown in Leidy, J. (1879) Freshwater Rhizopods of North America for his oldest plates of E. brachiata	Leidy, J. (1874). *Proceedings of the Academy of Natural Sciences of Philadelphia*, p.226.
E. brunnea Leidy	Not *Euglypha*, but *Assulina*.	Leidy, J. (1874). *Proceedings of the Academy of Natural Sciences of Philadelphia*, p.226.
E. brachiata Leidy	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. No figure in the original publication, first depiction shows several distinct species. No illustration in the original publication. Only shown in Leidy, J. (1879) Freshwater Rhizopods of North America for his oldest plates of E. brachiata	Leidy, J. (1878). *Proceedings of the Academy of Natural Sciences of Philadelphia*, p.172.
E. mucronata Leidy	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. No illustration in the original publication. Only shown in Leidy, J. (1879) Freshwater Rhizopods of North America for his oldest plates of E. brachiata	Leidy, J. (1878). *Proceedings of the Academy of Natural Sciences of Philadelphia*, p.172.
E. alveolata var. *minor* Taranek	Transferred to *E. tuberculata* as *E. tuberculata var. minor* Taranek by Decloitre.	Taranek, K. J. (1881). Beiträge zur Kenntniss der Süsswasser-Rhizopoden Böhmens. *Sitzungsberichte der Königlichen Böhmischen Gesellschaft der Wissenschaften in Prag*, pp. 220-235.
E. alveolata var. *gracilis* Taranek	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Taranek, K. J. (1881). Beiträge zur Kenntniss der Süsswasser-Rhizopoden Böhmens. *Sitzungsberichte der Königlichen Böhmischen Gesellschaft der Wissenschaften in Prag*, pp. 220-235.

E. longispina Taranek	Senior synonym of *E. filifera* Penard. Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Taranek, K. J. (1881). Beiträge zur Kenntniss der Süsswasser-Rhizopoden Böhmens. *Sitzungsberichte der Königlichen Böhmischen Gesellschaft der Wissenschaften in Prag*, pp. 220-235.
E. zonata Maggi	Synonymized with E. compressa by Cash, Wailes and Hopkinson (1915)	Reference not known. Appears in Cash, J., Wailes, H., & Hopkinson, J. (1915). The British Freshwater Rhizopoda and Heliozoa Ray Society. London.
E. filifera Penard	Junior synonym of *E. longispina* Taranek. Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Penard, E. (1890). *Études sur les Rhizopodes d'eau douce* (Vol. 31, No. 2). Imprimerie Aubert-Schuchardt.
E. heterospina Penard	Transferred into *E. strigosa* as *E. strigosa* f. *heterospina* by Wailes and Penard.	Penard, E. (1890). *Études sur les Rhizopodes d'eau douce* (Vol. 31, No. 2). Imprimerie Aubert-Schuchardt.
E. laevis var. *minor* Penard	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Penard, E. (1890). *Études sur les Rhizopodes d'eau douce* (Vol. 31, No. 2). Imprimerie Aubert-Schuchardt.
E. alveolata var. *aspera* Penard	Elevated to species level as *E. aspera* Penard	Penard, E. (1891). Etudes des rhizopodes du Léman.
E. aspera Penard	Valid species. The original description present peculiar scales that are accurately depicted and congruent with permanent slides left by Penard.	Penard, E. (1899). Les Rhizopodes de faune profonde dans le lac Léman. W. Kündig.
E. lens Penard	Not *Euglypha*, but *Placocista.*	Penard, E. (1900). Rhizopodes des eaux profondes dans le lac Léman.
E. brachiata Leidy var. *flexuosa* Penard	Admitted error. Was corrected into *E. armata* var. *flexuosa* Penard and later *E. acanthophora* (Ehrenberg) Perty var. *flexuosa* Penard.	Penard, E. (1902). Faune rhizopodique du bassin du Léman.
E. brachiata Leidy var. *brevispina* Penard	Admitted error. Was corrected into *E. armata* var. *brevispina* Penard and later *E. acanthophora* (Ehrenberg) Perty var. *brevispina* Penard.	Penard, E. (1902). Faune rhizopodique du bassin du Léman.

E. strigosa (Ehrenberg) Leidy f. *heterospina* Penard	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H., Penard, E. (1911). A biological survey of Clare Island in the county of Mayo, Ireland and the Adjoining district, Rhizopoda. *Proceedings of the Royal Irish academy.*
E. armata Wailes and Penard	Junior synonym of *E. alveolata* sensu Loeblich and Tapan.	Wailes, G. H., Penard, E. (1911). A biological survey of Clare Island in the county of Mayo, Ireland and the Adjoining district, Rhizopoda. *Proceedings of the Royal Irish academy.*
E. bryophila Brown	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Brown, J. M. (1911). Observations on some new and little-known British rhizopods. Zoological Journal of the Linnean Society, 32(212), 77-85.
E. cristata var. *major* Wailes	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H., Penard, E. (1911). A biological survey of Clare Island in the county of Mayo, Ireland and the Adjoining district, Rhizopoda. *Proceedings of the Royal Irish academy.*
E. rotunda Wailes and Penard	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H., Penard, E. (1911). A biological survey of Clare Island in the county of Mayo, Ireland and the Adjoining district, Rhizopoda. *Proceedings of the Royal Irish academy.*
E. scutigera Penard	Valid species. The original description present peculiar scales that are accurately depicted and congruent with permanent slides left by Penard.	Wailes, G. H., Penard, E. (1911). A biological survey of Clare Island in the county of Mayo, Ireland and the Adjoining district, Rhizopoda. *Proceedings of the Royal Irish academy.*
E. strigosa f. *glabra* Wailes	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H., Penard, E. (1911). A biological survey of Clare Island in the county of Mayo, Ireland and the Adjoining district, Rhizopoda. *Proceedings of the Royal Irish academy.*
E. strigosa var. *muscorum* Wailes and Penard	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H., Penard, E. (1911). A biological survey of Clare Island in the county of Mayo, Ireland and the Adjoining district, Rhizopoda. *Proceedings of the Royal Irish academy.*

E. armata Wailes and Penard var. *flexuosa* Penard	Admitted error. Was corrected into *E. acanthophora* (Ehrenberg) Perty var. *flexuosa* Penard.	Wailes, G. H., Penard, E. (1911). A biological survey of Clare Island in the county of Mayo, Ireland and the Adjoining district, Rhizopoda. *Proceedings of the Royal Irish academy.*
E. armata Wailes and Penard var. *brevispina* Penard	Admitted error. Was corrected into *E. acanthophora* (Ehrenberg) Perty var. *brevispina* Penard.	Wailes, G. H., Penard, E. (1911). A biological survey of Clare Island in the county of Mayo, Ireland and the Adjoining district, Rhizopoda. *Proceedings of the Royal Irish academy.*
E. alveolata Dujardin var. *cirrata* Wailes	Transferred into *E. acanthophora* as *E. acanthophora* var. *cirrata* Wailes.	Wailes, G. H., (1912). Freshwater Rhizopoda and Heliozoa from the States of New York, New Jersey, and Georgia, USA with Supplemental Note on Seychelles Species. *Journal of the Linnean Society of London*, *Zoology*, 32(214), pp. 121-161.
E. *brachiata* Leidy var. *librata* Wailes	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H., (1912). Freshwater Rhizopoda and Heliozoa from the States of New York, New Jersey, and Georgia, USA with Supplemental Note on Seychelles Species. *Journal of the Linnean Society of London*, *Zoology*, 32(214), pp. 121-161.
E. crenulata Wailes	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Scales are represented, however oral scales are irrealistic and the textual description is too broad, including also specimens akin to *E. alveolata*. Furthermore, he includes under this name specimens that are depicted in Leidy (1879) and does not fit the description of the species.	Wailes, G. H., (1912). Freshwater Rhizopoda and Heliozoa from the States of New York, New Jersey, and Georgia, USA with Supplemental Note on Seychelles Species. *Journal of the Linnean Society of London*, *Zoology*, 32(214), pp. 121-161.
E. crenulata var. *minor* Wailes	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H., (1912). Freshwater Rhizopoda and Heliozoa from the States of New York, New Jersey, and Georgia, USA with Supplemental Note on Seychelles Species. *Journal of the Linnean Society of London*, *Zoology*, 32(214), pp. 121-161.
E. cristata var. *acicularis* Wailes	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H., (1912). Freshwater Rhizopoda and Heliozoa from the States of New York, New Jersey, and Georgia, USA with Supplemental Note on Seychelles Species. *Journal of the Linnean Society of London*, *Zoology*, 32(214), pp. 121-161.
E. denticulata Brown	Likely not *Euglypha,* but Assulinidae.	Brown, J. M. (1912). Further contribution to our knowledge of the Rhizopoda and Heliozoa of Scotland. *Scottish Naturalist*.

E. filifera Penard var. *spinosa* Wailes	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H. (1912). Freshwater Rhizopoda and Heliozoa from the States of New York, New Jersey, and Georgia, USA with Supplemental Note on Seychelles Species. *Journal of the Linnean Society of London, Zoology*, 32(214), pp. 121-161.
E. filifera Penard var. *pyriformis* Wailes	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Wailes, G. H. (1913). Freshwater Rhizopoda from North and South America.
E. alveolata var. *hamulifera* Playfair	Transferred to *E. dentata* as *E. dentata* var. *hamulifera* Playfair and later synonymized with *E. tuberculata* Dujardin var. *ovoidea* Decloitre.	Playfair, G. I. (1914). Contributions to a knowledge of the biology of the Richmond River. *Proceedings of the Linnean Society of New South Wales.*
E. alveolata Dujardin var. *laevis* (Perty) Playfair	Admitted error, synonymized with *E. laevis* Perty.	Playfair, G. I. (1914). Contributions to a knowledge of the biology of the Richmond River. *Proceedings of the Linnean Society of New South Wales.*
E. acanthophora (Ehrenberg) Perty var. *brevispina* Penard	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Originally described as *E. brachiata* var. *brevispina* Penard.	Cash, J., Hopkinson, J., & Wailes, G. H. (1915). *The British freshwater rhizopoda and heliozoa, volume* 3. Ray society.
E. acanthophora (Ehrenberg) Perty var. *flexuosa* Penard	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Originally described as *E. brachiata* var. *flexuosa* Penard.	Cash, J., Hopkinson, J., & Wailes, G. H. (1915). *The British freshwater rhizopoda and heliozoa, volume* 3. Ray society.
E. ciliata (Ehrenberg) Leidy f. *glabra* Cash, Wailes, Hopkinson	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Cash, J., Hopkinson, J., & Wailes, G. H. (1915). *The British freshwater rhizopoda and heliozoa, volume* 3. Ray society.
E. ciliata (Ehrenberg) Leidy f. *heterospina* Cash, Wailes, Hopkinson	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Cash, J., Hopkinson, J., & Wailes, G. H. (1915). *The British freshwater rhizopoda and heliozoa, volume* 3. Ray society.

E. compressa Carter f. *glabra* Cash, Wailes, Hopkinson	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Cash, J., Hopkinson, J., & Wailes, G. H. (1915). *The British freshwater rhizopoda and heliozoa, volume* 3. Ray society.
E. acanthophora var. *gracillima* Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. acanthophora var. *elliptica* Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. australica Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. australica var. *elegans* Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Only a single scale is represented.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. In Proc. Linn. Soc. NSW (Vol. 42, pp. 633-675).
E. australica var. *cylindracea* Playfair	Transferred to *E. acanthophora* as *E. acanthophora* var. *cylindracea* (Playfair) Decloitre	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. compressa var. *obscura* Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. cristata var. *lanceolata* Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).

E. filifera var. *cuneata* Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. filifera var. *cylindracea* Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. filifera var. *elegans* Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. laevis var. *lanceolata* Playfair	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. dentata var. *elongata* Playfair	Not *Euglypha*, but *Tracheleuglypha.*	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. dentata var. *hamulifera* Playfair	Synonymized with *E. tuberculata* Dujardin var. *ovoidea* Decloitre under the name *E. alveolata var. hamulifera* Playfair.	Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. *Proceedings of the Linnean Society of New South Wales* (Vol. 42, pp. 633-675).
E. dentata (Penard) Moniez(?)	Not *Euglypha*, but *Tracheleuglypha.*	Reference not known. Appears in Playfair, G. I. (1917). Rhizopods of Sydney and Lismore. Proceedings of the Linnean Society of New South Wales (Vol. 42, pp. 633-675).
E. acanthophora var. *subcylindracea* Deflandre	Synonymized with E. acanthophora var. cylindracea (Playfair) Decloitre	Deflandre, G. (1926). Notes sur quelques Rhizopodes et Heliozoaires du Venezuela. *Bulletin de la société zoologique de France*, 51, 515-553.
E. acanthophora var. *cirrata* Wailes	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Originally described as *E. alveolata* Dujardin var. *cirrata* Wailes	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.

E. robusticornis Bradley	Not *Euglypha*.	Bradley, W. H. (1931). *Origin and microfossils of the oil shale of the Green River Formation of Colorado and Utah* (Vol. 168). US Government Printing Office.
E. hutchinsoni van Oye	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	van Oye, P. (1932). Neue Rhizopoden aus Afrika. *Zoologischer Anzeiger*, p. 324.
E. rotunda f. *toxodera* Jung	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Jung, W. (1941). Südchilenische Thekamöben. *Archive für Protistenkunde.*
E. rotunda f. *curvata* Jung	Synonym of *E. rotunda* f. *toxodera* Jung.	Jung, W. (1941). Südchilenische Thekamöben. *Archive für Protistenkunde.*
E. cristata var. *decora* (Ehrenberg) Jung	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Originally described as *Difflugia decora* Ehrenberg.	Jung, W. (1941). Südchilenische Thekamöben. *Archive für Protistenkunde.*
E. acanthophora var. *heterospina* Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1949). Matériaux pour une faune rhizopodique d'AOF. *Bulletin I.F.A.N.*, p. 295, fig. 8
E. tuberculata var. *minor* (Taranek) Decloitre f. *subcylindrica* Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1955). Rhizopodes thecamoebiens du Venezuela. *Hydrobiologia*, *7*(4), 325-372.
E. *tuberculata* var. *ovoidea* Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1955). Speleologia Africana. Thécamoebiens de la grotte des Singes r Segea (Guinee). *Bulletin I.F.A.N*, *17*(4), 989-1019.

E. acanthophora var. *equeis* Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1956). Les thécamoebiens de L'Eqe (Groenland) (Vol. 1242). Hermann et Cie.
E. rotunda var. *obliqua* Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloître, L. (1956). Le Parc National de Niokolo-Koba, Rhizopodes Thécamoebiens. *Mémoires de l'Institut Français d'Afrique Noire*, *48*, 233-258.
E. crenulata var. *elongata* Thomas	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Thomas, R. (1958). Sur quelques Euglypha nouvelles ou peu connues observées en Afrique. *Bulletin de la société d'histoire naturelle de l'Afrique du Nord*, 49, 83-92.
E. curvata van Oye	Invalid name, renamed *E. van oyei* (van Oye) Decloitre.	van Oye, P. (1958). Etude sur les rhizopodes des marais du sud-ouest d'Uvira. *Hydrobiologia*, 10.
E. filifera Penard var. *magna* van Oye	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	van Oye, P. (1958). Etude sur les rhizopodes des marais du sud-ouest d'Uvira. *Hydrobiologia*, 10.
E. gauthieri Thomas	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Thomas, R. (1958). Sur quelques Euglypha nouvelles ou peu connues observées en Afrique. *Bulletin de la société d'histoire naturelle de l'Afrique du Nord*, 49, 83-92.
E. marginata Van Oye	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	van Oye, P. (1958). Etude sur les rhizopodes des marais du sud-ouest d'Uvira. *Hydrobiologia*, 10.
E. symoensi Van Oye	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	van Oye, P. (1958). Etude sur les rhizopodes des marais du sud-ouest d'Uvira. *Hydrobiologia*, 10.
E. cuspidata Bonnet	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Bonnet, L. (1959). Nouveaux thécamoebiens du sol. *Bulletin société histoire naturelle de Toulouse*, 94.

E. dolioliformis Bonnet	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Bonnet, L. (1959). Nouveaux thécamoebiens du sol. *Bulletin société histoire naturelle de Toulouse*, 94.
E. umbilicata Bonnet	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Bonnet, L. (1959). Nouveaux thécamoebiens du sol. *Bulletin société histoire naturelle de Toulouse*, 94.
E. rotunda Wailes var. *polylepis* Bonnet	Elevated to species level as *E. polylepis* (Bonnet) Decloitre	Bonnet, L. (1959). Nouveaux thécamoebiens du sol. *Bulletin société histoire naturelle de Toulouse*, 94.
E. anodonta Bonnet	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Bonnet, L. (1960). Thécamoebiens des sols d'Angola. *Publicações culturais da Companhia de Diamantes de Angola, Lisboa*, *51*, 79-86.
E. alveolata var. *oblonga* Brodsky	Synonymized with *E. tuberculata* var. *subcylindracea* (Playfair) Decloitre.	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. binucleolus Moncilo	Considered as dubious by Decloitre (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. compressa Carter f. *inermis* Lepsi	Synonymized with *E. compressa* var. *glabra* by Decloitre (1962)	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. *cristata* var. *curvata* Jung	Nomen nudum according to Decloitre (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. pusilla Entz	Synonymized with *E. tuberculata* by Decloitre (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.

E. tincta Archer	Not *Euglypha*, but *Assulina* according to Decloitre (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. triacantus Escomel	Synonymized with E. bryophila by Decloitre (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. sacciformis Archer	Considered as dubious by Decloitre (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. mitrata Fantham and Porter (?)	Considered as dubious by Decloitre (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. lineare Bartos	Considered as dubious by Decloitre (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. margaritacea Wallich	Not *Euglypha*, but *Cyphoderia*	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. rotunda var. *palma* Decloitre	Invalid. Variety published after 1960.	Decloitre, L. (1961). Thecamoebiens des sols aériens des palmiers du Maroc.
E. rotunda var. *tunna* Decloitre	Elevated to species level as *E. tunna* Godeanu.	Decloitre, L. (1961). Matériaux pour une faune thécamoebienne du maroc.
E. tuberculata var. *minor* (Taranek) Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Originally described as *E. alveolata* Dujardin var. *minor* Taranek.	Decloitre, L. (1962). Le genre Euglypha Dujardin. *Archiv für Protistenkunde*, *106*, 51-100.
E. acanthophora var. *cylindracea* (Playfair) Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Only a single scale is represented. Originally described as *E. australica* var. *cylindracea* Playfair.	Decloitre, L. (1962). Le genre Euglypha Dujardin. *Archiv für Protistenkunde*, *106*, 51-100.

E. tuberculata var. *subcylindrica* Decloitre	Invalid. Variety published after 1960.	Decloitre, L. (1962). Le genre Euglypha Dujardin. *Archiv für Protistenkunde*, *106*, 51-100.
E. acanthophora var. *deflandrei* Decloitre	Invalid. Variety published after 1960.	Decloitre, L. (1962). Le genre Euglypha Dujardin. *Archiv für Protistenkunde*, *106*, 51-100.
E. van oyei (van Oye) Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Originally described as *E. curvata* van Oye.	Decloitre, L. (1962). Le genre Euglypha Dujardin. *Archiv für Protistenkunde*, *106*, 51-100.
E. polylepis (Bonnet) Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Originally described as *E. rotunda* var. *polylepis* Bonnet	Decloitre, L. (1962). Le genre Euglypha Dujardin. *Archiv für Protistenkunde*, *106*, 51-100.
E. acanthophora var. *dorsalis* Schonborn	Invalid. Variety published after 1960.	Schonborn, W. (1962). Neue testacean aus dem Grossen Stechlinsee und dessen Umgebung. *Limnologica* 1, pp.83-91
E. pseudociliata Chardez	Species inquirenda due to potentially existing type material.	Chardez, D. (1962). Sur les thécamoebiens des sols forestiers du Sart-Tilman (Liège)
E. pseudociliata var. *glabra* Chardez	Species inquirenda due to potentially existing type material.	Chardez, D. (1962). Sur les thécamoebiens des sols forestiers du Sart-Tilman (Liège)
E. anodonta var. *magna* Schonborn	Invalid. Variety published after 1960.	Schonborn, W. (1962). Die Okologie der Testacean im oligotrophen See, dargestellt Beispiel des Grossen Stechlinsees. *Limnologica*, 1, pp.111-182

E. ciliata (Ehrenberg) Leidy var. *strigosa* Leidy (?)	Synonymized with *E. strigosa* by Decloitre 1962.	We did not find it in Leidy's work. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. maggii Longhi	Not *Euglypha*, but tintinnid according to Decloître (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. baltica Wallich	Not *Euglypha*, but *Cyphoderia* according to Decloître (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. enchelys Wallich	Not *Euglypha*, but *Trinema*.	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. tegulifera Barnard	Not Euglypha, according to Decloitre (1962) citing Leidy.	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. striata Lepsi	Not *Euglypha*, but *Tracheleuglypha* according to Decloître (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. reticulata (Ehrenberg)	Not *Euglypha*, but *Nebela* according to Decloitre (1962). However, this is dubious as Ehrenberg did not recognize the genus *Euglypha*.	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.
E. sphaerica Lepsi	Not *Euglypha*, but *Tracheleuglypha* according to Decloître (1962).	Reference not known. Appears in Decloitre, L. (1962). Le genre Euglypha Dujardin. Archiv für Protistenkunde, 106, 51-100.

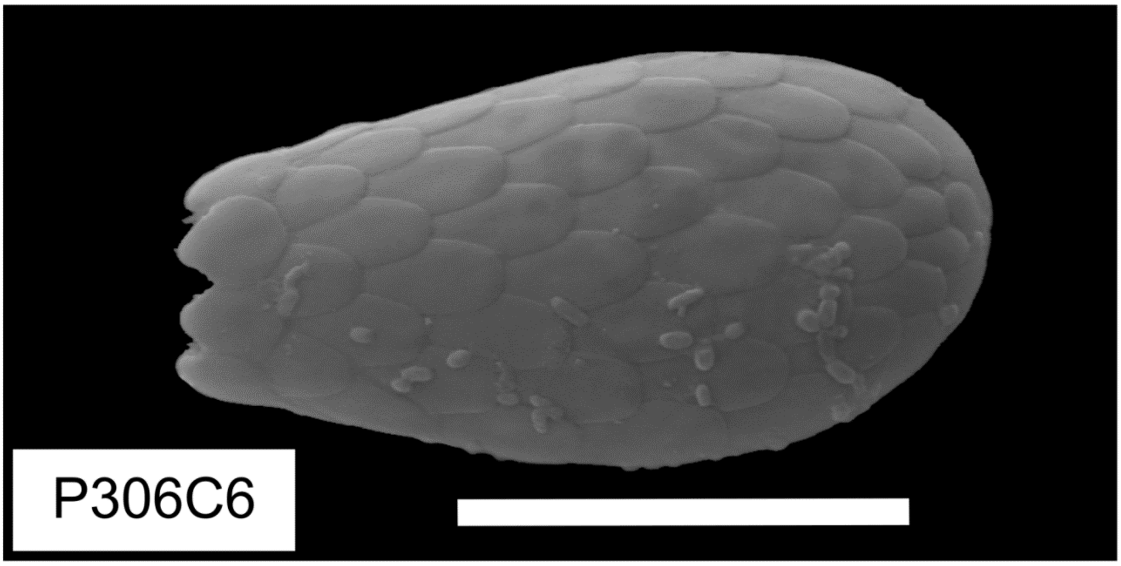
E. acanthophora var. *elegans* Stepanek	Invalid. Variety published after 1960.	Štěpánek, M. (1963). *Die Rhizopoden aus Katanga (Kongo-Afrika). Koninklijk Museum voor Midden-Afrika.*
E. macrodonta Bartos	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Reference not known. Appears in Decloitre, L. (1976). Le genre Euglypha. Compléments à jour au 31. Décembre 1974 de la Monographie du genre parue en 1962. Archiv Für Protistenkunde, 118, 18–33.
E. dentisecara Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloître, L. (1964). *Théccamoebiens de la XIIe Expédition antarctique française* (Vol. 259). Expéditions polaires françaises. Missions Paul-Emile Victor.
E. dentisecara var. *glabra* Decloitre	Invalid. Variety published after 1960.	Decloître, L. (1964). *Théccamoebiens de la XIIe Expédition antarctique française* (Vol. 259). Expéditions polaires françaises. Missions Paul-Emile Victor.
E. rotunda var. *minor* (Unknown authority)	Nomen dubium. Several authors have been cited for this taxon, mostly Wailes and Bonnet.	This name potentially appeared around 1964 in the work of Bonnet. Appears in Decloitre, L. (1976). Le genre Euglypha. Compléments à jour au 31. Décembre 1974 de la Monographie du genre parue en 1962. Archiv Für Protistenkunde, 118, 18–33.
E. nana Lepsi	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions, based on Decloitre (1976) citing Godeanu.	Reference not known. Appears in Decloitre, L. (1976). Le genre Euglypha. Compléments à jour au 31. Décembre 1974 de la Monographie du genre parue en 1962. Archiv Für Protistenkunde, 118, 18–33.
E. rotunda var. *spinosa* Decloitre	Invalid. Variety published after 1960.	Reference not known. Appears in Decloitre, L. (1976). Le genre Euglypha. Compléments à jour au 31. Décembre 1974 de la Monographie du genre parue en 1962. Archiv Für Protistenkunde, 118, 18–33.
E. simplex Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1965). Contribution à la faune du Congo-Brazza. Mission Descarpentries-Villiers. *Bulletin I.F.A.N., 17*(1).

E. cristata var. *turbo* Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1965). Contribution a la faune du Congo (Brazzaville). Mission A. Dexarpentries et A. Villiers. III Rhizopodes Thecamoebiens. *Bull. Inst. Fr. Afr. Noire. A*, *27*, 165-184.
E. nana Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1965). Contribution a la faune du Congo (Brazzaville). Mission A. Dexarpentries et A. Villiers. III Rhizopodes Thecamoebiens. *Bull. Inst. Fr. Afr. Noire. A*, *27*, 165-184.
E. pseudotuberculata Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1965). Contribution a la faune du Congo (Brazzaville). Mission A. Dexarpentries et A. Villiers. III Rhizopodes Thecamoebiens. *Bull. Inst. Fr. Afr. Noire. A*, *27*, 165-184.
E. rectilineata Lepsi	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions, based on Decloitre (1976) citing Godeanu.	Reference not known. Appears in Decloitre, L. (1976). Le genre Euglypha. Compléments à jour au 31. Décembre 1974 de la Monographie du genre parue en 1962. Archiv Für Protistenkunde, 118, 18–33.
E. acanthophora (Ehrenberg) Perty var. *fantastica* Decloitre	Invalid. Variety published after 1960.	Decloitre, L. (1966). Compléments à The Zoology of Iceland. Vol. II. Part I. Amoebida Testacea (Rhizopoda). *Videnskabelige Meddelelser fra dansk naturhistorisk forening*, *129*, 67-71.
E. castrii Bonnet	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Bonnet, L. (1966). Le peuplement Thécamoebien de quelques sols du Chili. *Protistologica*, *2*(2), 113-140.
E. quadrata Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1966). Compléments à The Zoology of Iceland. Vol. II. Part I. Amoebida Testacea (Rhizopoda). *Videnskabelige Meddelelser fra dansk naturhistorisk forening*, *129*, 67-71.
E. recurvispina Bonnet	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Bonnet, L. (1966). Le peuplement Thécamoebien de quelques sols du Chili. *Protistologica*, *2*(2), 113-140.

E. tiscia Gal	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Gal, D. (1969). Zooplankton-Untersuchungen im Östlichen-Hauptkanal. *Acta Biologica Szeged*, 15, 93-100.
E. insolita Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1969). Thecamoebien bryomadicoles dans le Var.
E. leclerqi Chardez	Species inquirenda due to potentially existing type material.	Chardez, D. (1969). Contribution à la faune Thécamoebienne de l'Islande. *Bulletin de l'Institut Royal des Sciences naturelles de Belgique*, *45*, 1-16.
E. megastoma Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1969). Thecamoebien bryomadicoles dans le Var.
E. rotunda var. *dorsalis* Decloitre	Invalid. Variety published after 1960.	Decloitre, L. (1969). Thecamoebiens du sol dans le Var. Pinède de Six-Fours-la-Plage.
E. rotunda var. *madera* Decloitre	Invalid. Variety published after 1960.	Decloitre, L. (1969). Thecamoebien bryomadicoles dans le Var.
E. nipponica Hada	The original description could not be accessed.	Hada, Y. (1969). The Protozoa in the Moss-colonies distributed in the Sandankyo Gorge and the Yawata Highland. *Limnological Researches of the Sandankyo District* :225-259
E. tuberculata var. *lanceolata* Decloitre	Invalid. Variety published after 1960.	Decloitre, L. (1970). Thecamoebiens des Lichens et des Mousses, Régions méditéranéennes. *An. SSNATV*, *22*, 145-152.
E. ciliata var. *alpestris* Laminger	Invalid. Variety published after 1960.	Laminger, H. (1971). Über das Vorkommen von Schalenamöben (Protozoa, Rhizopoda testacea) in Hochgebirgsböden. *Zoologische Anzeiger, 186*, 329-332.
E. multidentata Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Decloitre, L. (1971). Thécamoebiens de Sollies-Pont (Var). *Extraits des annales de la S.S.N.A.T.V.*
E. tunna Godeanu	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Described first as *E. rotunda* var. *tunna* Decloitre	Godeanu, S. (1972). Citeva date asupra unor Testacee (Rhizopodea, Arcellinida, si Gromiida) din Romania. *St. si Cerc. Biol. seria Zoologie*, *24*(5), 401-408.
E. rotunda var. *tumuliformis* Decloitre	Invalid. Variety published after 1960.	Decloitre, L. (1972). Thécamoebiens du Var. *Ann. Soc. Sci. Nat. Arch. Toulon et Var*, 165-171.
E. acus Vucetich	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions.	Vucetich, M. C. (1973). Tecamebianos de los lagos General Vintter y Puelo (Chubut, Argentina). *Neotropica*, *19*(58), 1-10.
E. ovuliformis Decloitre	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. Misspelled *E. ovuliformia* in Decloitre 1979.	Decloitre, L. (1973). Thécamoebiens des iles Galapagos. *Ciencia y Naturaleza*, *19*(1), 11-20.

E. tuberculata var. *curvata* Vucetich	Invalid. Variety published after 1960.	Vucetich, M. C. (1973). Estudio de tecamebianos argentinos en especial los del dominio pampasico. *Revista del Museo de la Plata*, *11*(108), 287-332.
E. constricta Godeanu	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. No figure.	Godeanu, S. (1974). Contribuţii la cunoaşterea sinecologică a zoocenozelor tinovului Gemenele din Parcul Naţional Retezat. *Sargetia, Acta Mus. Dev., Ser. Sci. Nat*, 155–175.
E. cristata Leidy *elongata* Godeanu	Invalid. Variety published after 1960.	Godeanu, S. (1974). Contribuţii la cunoaşterea sinecologică a zoocenozelor tinovului Gemenele din Parcul Naţional Retezat. *Sargetia, Acta Mus. Dev., Ser. Sci. Nat*, 155–175.
E. acanthophora var. *longispina* Decloitre	Invalid. Variety published after 1960.	Reference not known. Appears in Decloitre, L. (1976). Le genre Euglypha. Compléments à jour au 31. Décembre 1974 de la Monographie du genre parue en 1962. Archiv Für Protistenkunde, 118, 18–33.
E. compressa Carter var. *heterospina* Decloitre	Invalid. Variety published after 1960.	Decloitre, L. (1976). Le genre Euglypha. Compléments à jour au 31. Décembre 1974 de la Monographie du genre parue en 1962. *Archiv Für Protistenkunde*, *118*, 18–33.
E. cristata Leidy f. *glabra* Bonnet	Invalid. Variety published after 1960.	Bonnet, L. (1977). Faunistique et biogéographie des thécamoebiens. II. Thécamoebiens des sols de la Guadeloupe et de la Martinique. *Bulletin de La Société d'histoire Naturelle de Toulouse*, *113*(1, 2).
E. capsiosa Couteaux	Valid species.	Couteaux, M. M. (1978) Quelques Thécamoebiens Du sol du Japon. *Revue d'écologie et de biologie du sol, 15.* pp. 119−128.
E. hyalina Couteaux	Valid species.	Couteaux, M. M. (1978) Quelques Thécamoebiens Du sol du Japon. *Revue d'écologie et de biologie du sol, 15.* pp. 119−128.
E. laevis f. *conica* Suxena	Invalid. Variety published after 1960. Misspelled as *E. loevis*.Type material?	Suxena, M. R. (1979). Algae and Testacea from high altitudes of Himalayas-I (collected by NCC Punch Chulli Expedition, W. Himalayas 1970). *Hydrobiologia*, *65*(2), 107-128.
E. rotunda var. *elongata* Decloitre	Invalid. Variety published after 1960.	Reference not known. Appears in Decloitre, L. (1982). Compléments aux publications précédentes Mise à jour au 31. XII. 1981 des genres *Arcella, Centropyxis, Cyclopyxis, Euglypha, Nebela* et *Trinema*. The Genera *Arcella, Centropyxis, Cyclopyxis, Euglypha, Nebela* and *Trinema*. Supplements closed 31. X11. 1981. *Archiv für Protistenkunde, 126(4)*, 393-407.
E. penardi Meisterfeld	Valid species.	Meisterfeld, R. (1979). Zur Systematik der Testaceen (Rhizopoda, Testacea) in Sphagnum. Eine REM-Untersuchung. *Archiv für Protistenkunde*, *121*(3), 246-269.
E. cashii Ogden	Valid species.	Ogden, C. G., & CG, O. (1981). Observations of clonal cultures of Euglyphidae (Rhizopoda, Protozoa).
E. dickensii Ogden	Valid species.	Ogden, C. G., & CG, O. (1981). Observations of clonal cultures of Euglyphidae (Rhizopoda, Protozoa).
E. filifera Penard var. *glabra* Bunescu et Matic	Invalid. Variety published after 1960.	Bunescu, V., & Matic, Z. (1982). Nouvelles especes et varietes de thecamoebiens (Protozoa: Rhizopoda Testacea) dans les sols des Monts Bucegi (Roumanie). Buletinul Institutului Agronomic Cluj-Napoca. Seria agricultura.

E. tubulosa (Unknown authority)	Nomen dubium due to the inaccuracy of the original description and the absence of authoritative redescriptions. No figure.	Reference not known. Appears in Decloitre, L. (1986). Compléments aux publications précédentes au 31.XII.1984 des genres *Arcella, Centropyxis, Cyclopyxis*, *Euglypha* et *Nebela*. Archiv Für Protistenkunde, 132, 131–136.
Scutiglypha cabrolae De Smet and Gibson	Valid species.	De Smet, W. H. (2009). On a new species of euglyphid testate amoeba, *Scutiglypha cabrolae*, from the Licancabur Caldera lake, Central Andes. *Acta Protozoologica*, *48*(2).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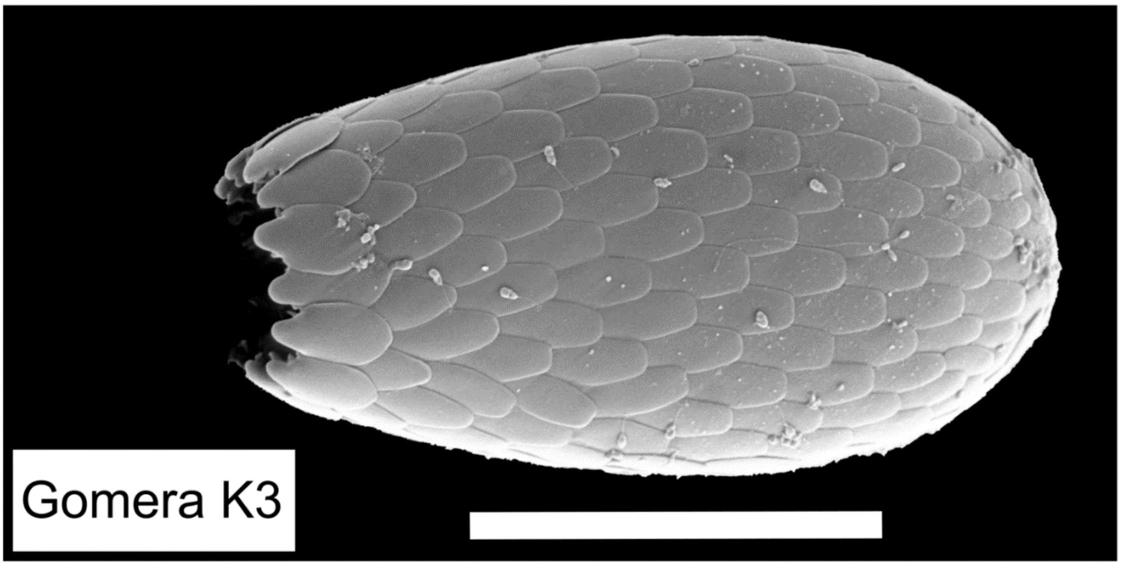
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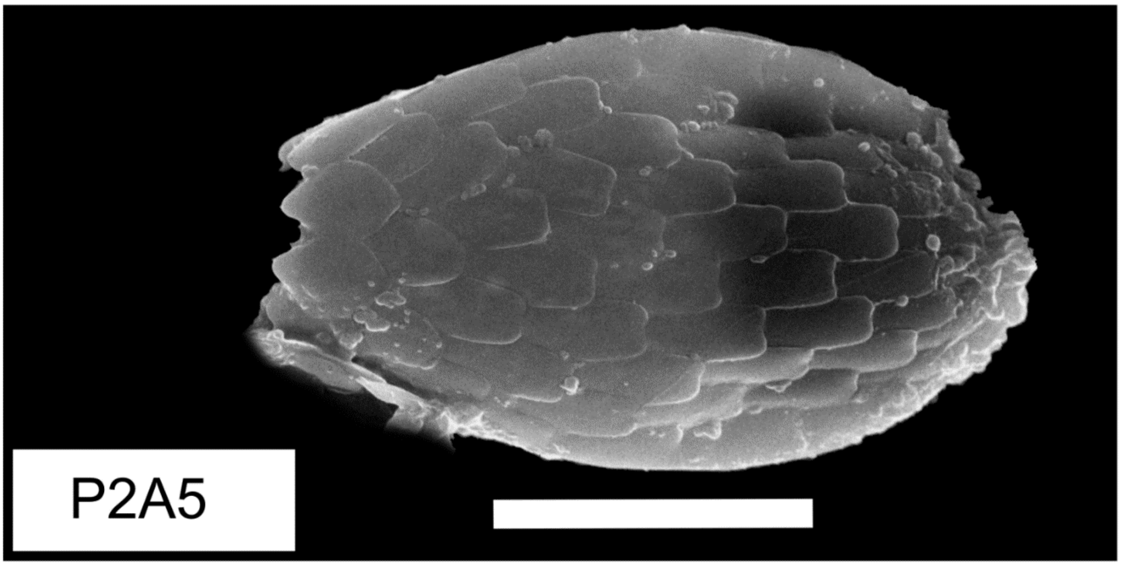
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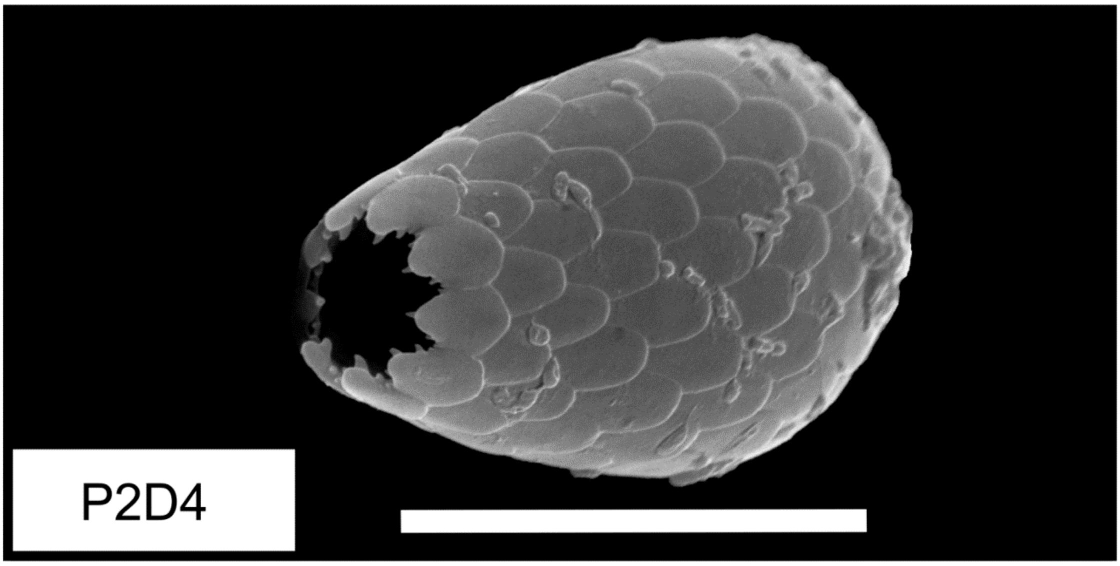

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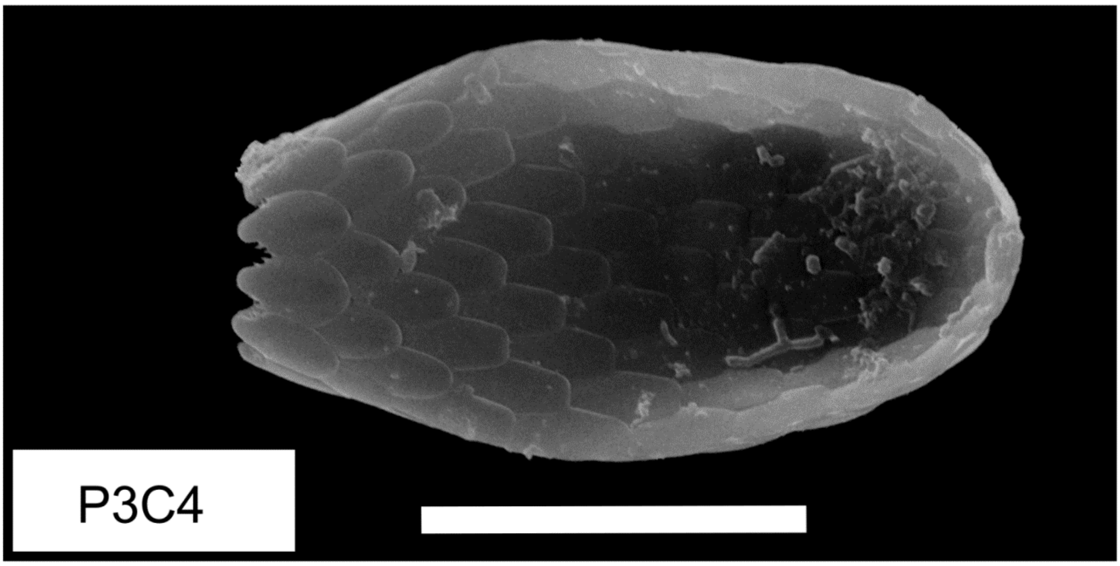

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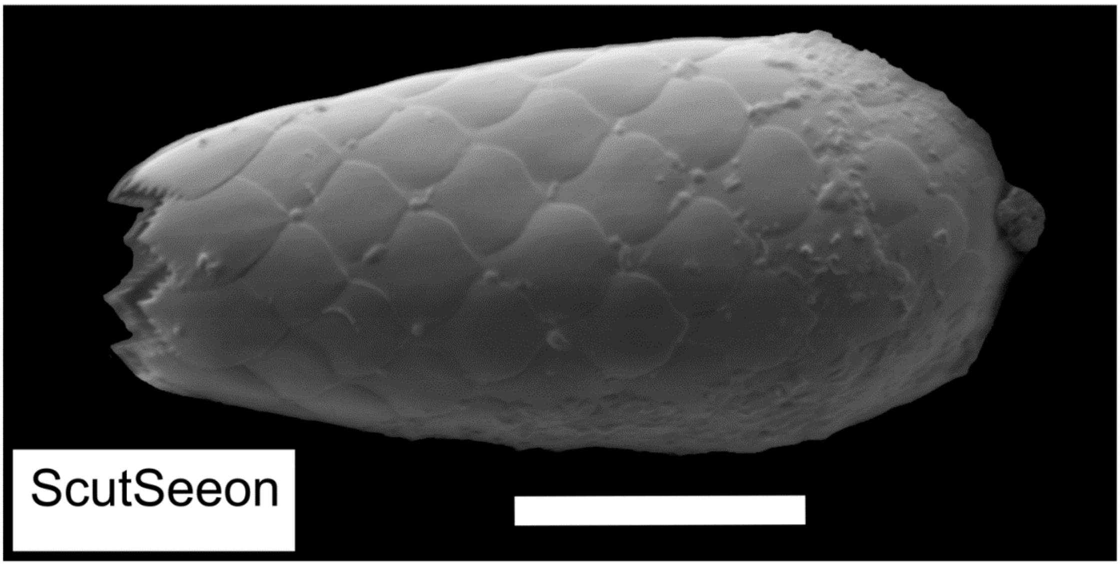

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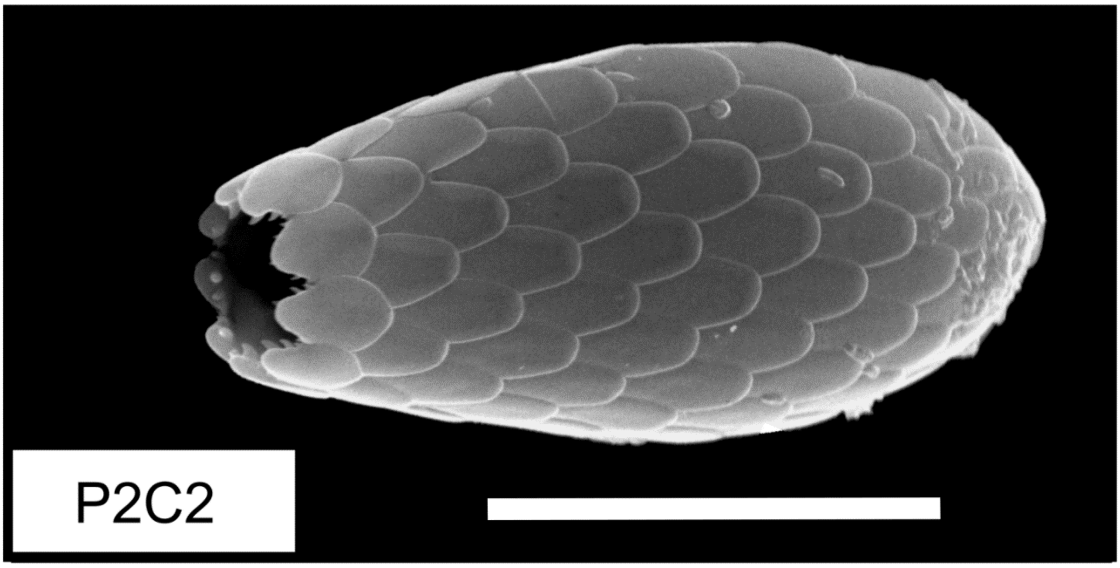

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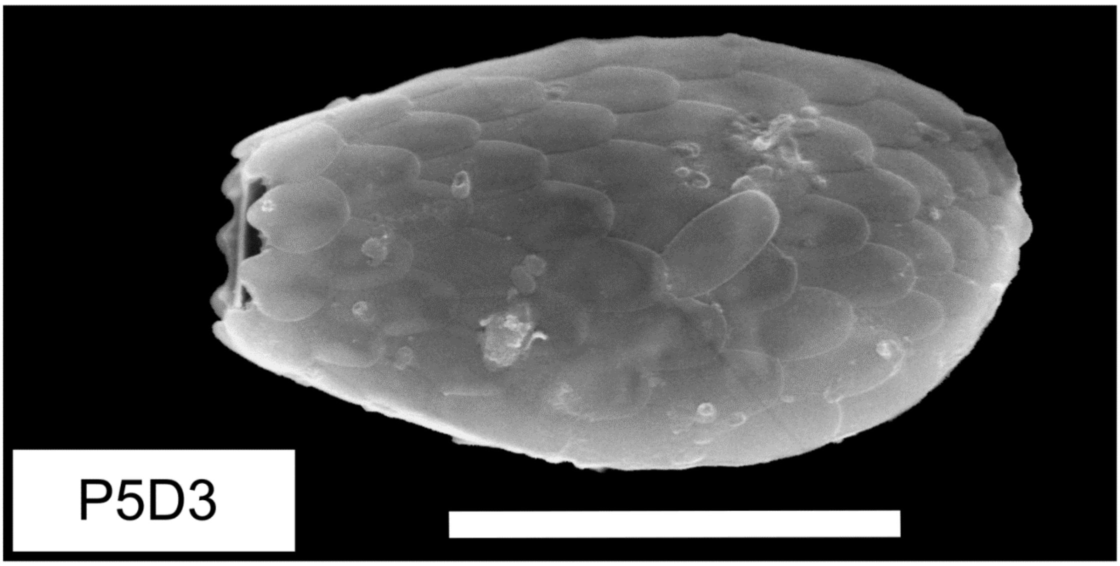

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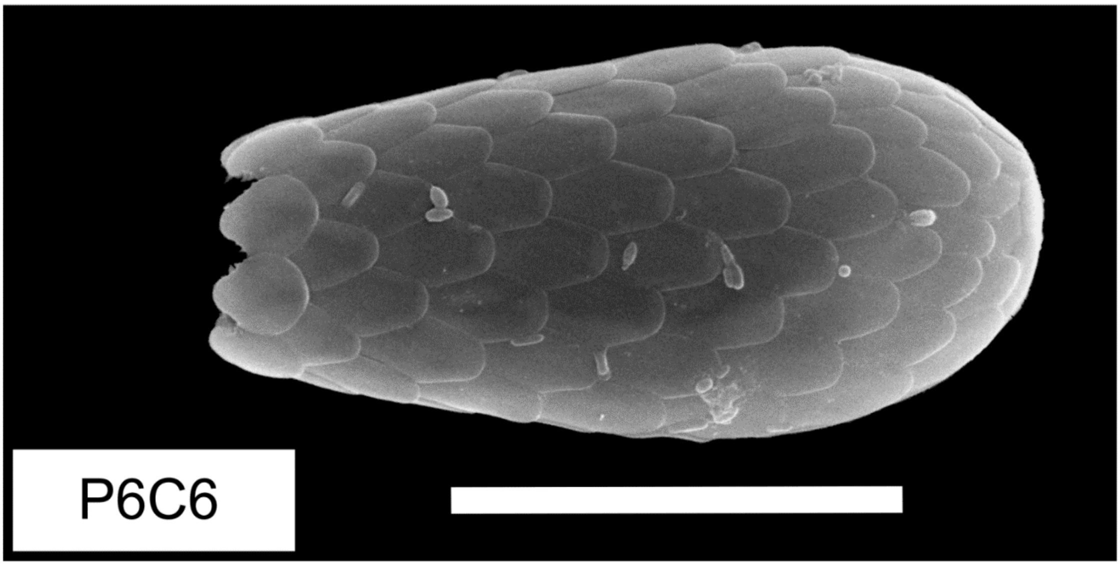

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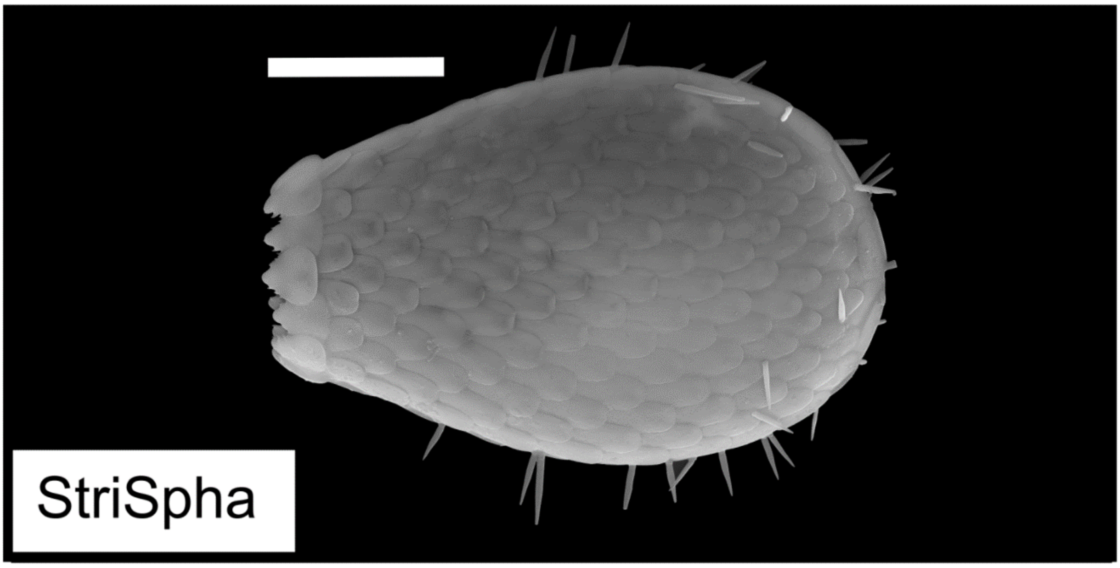
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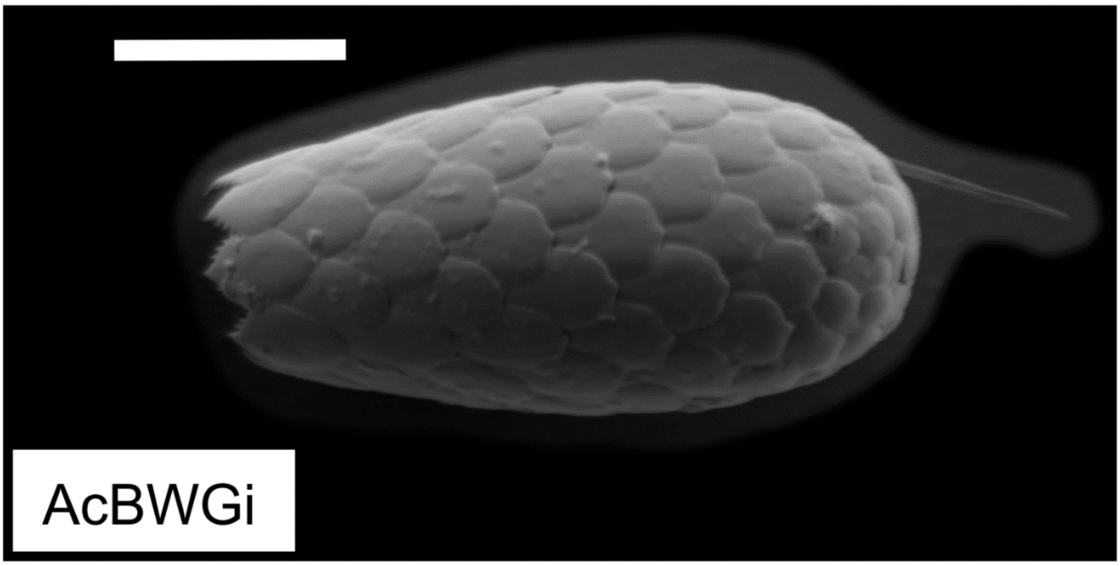
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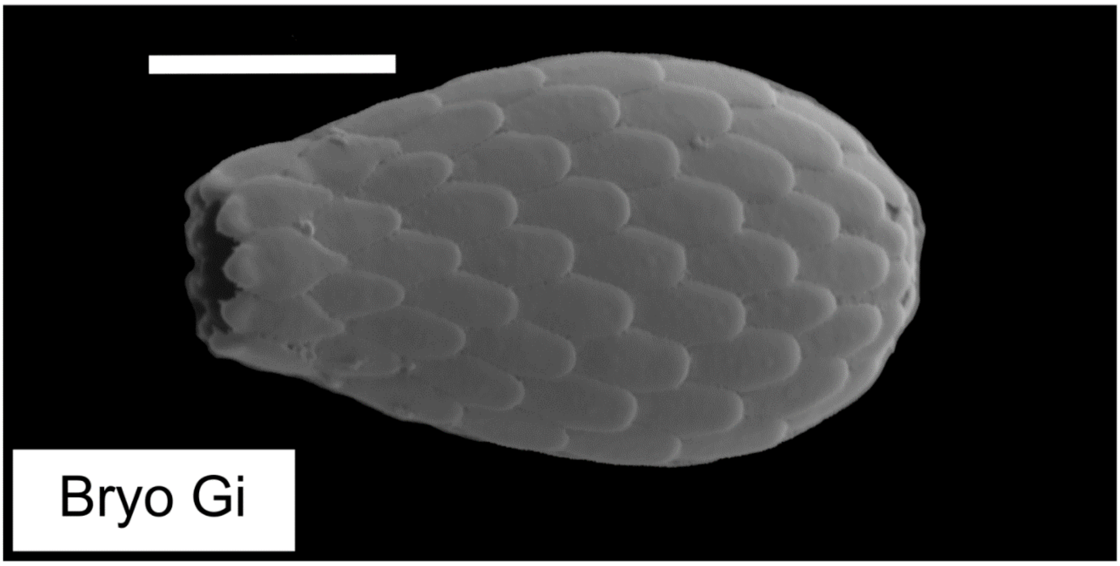
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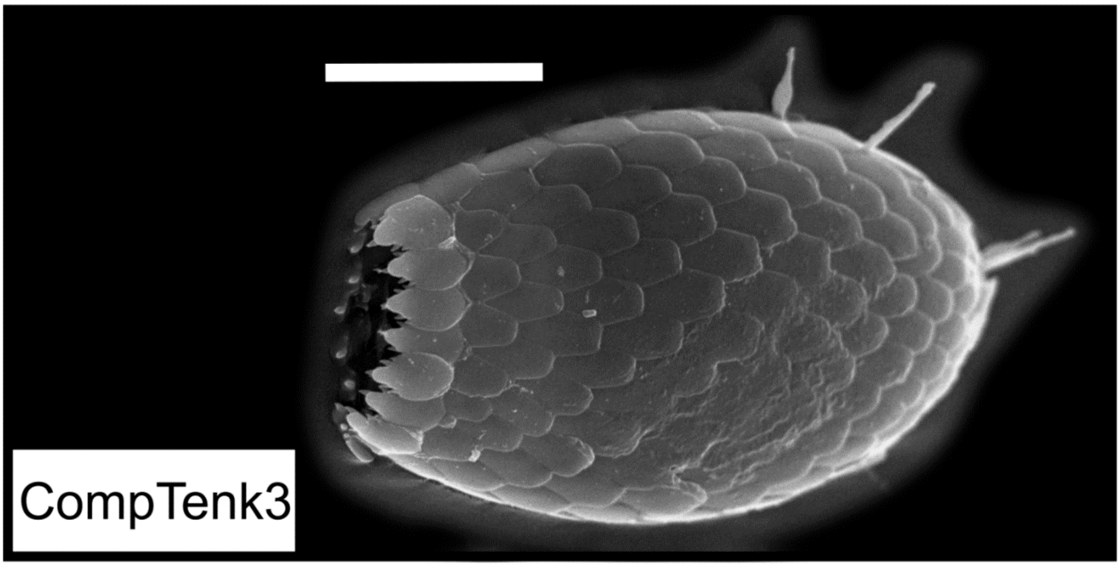
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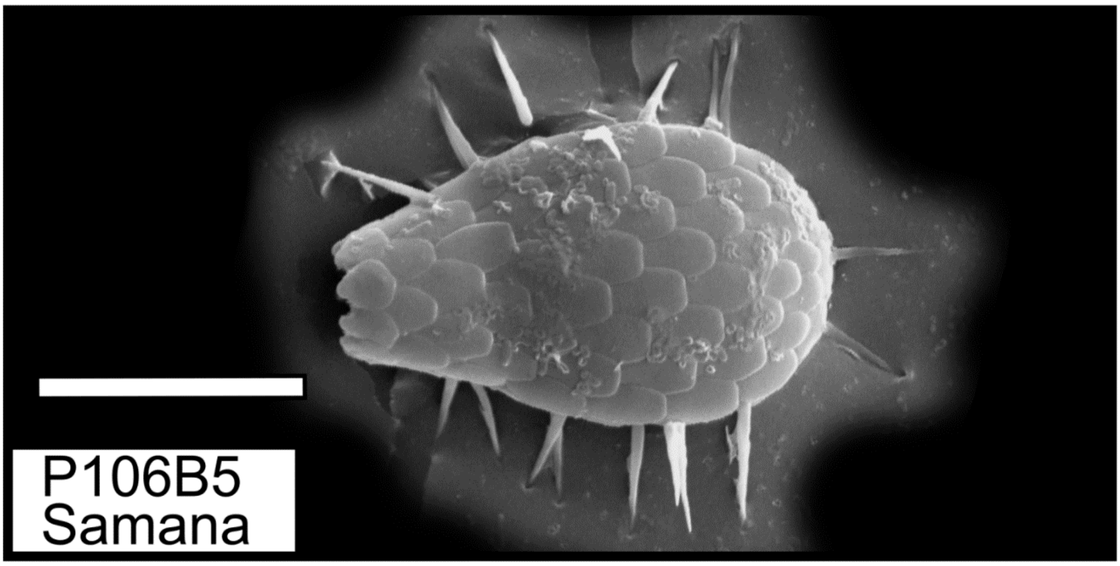
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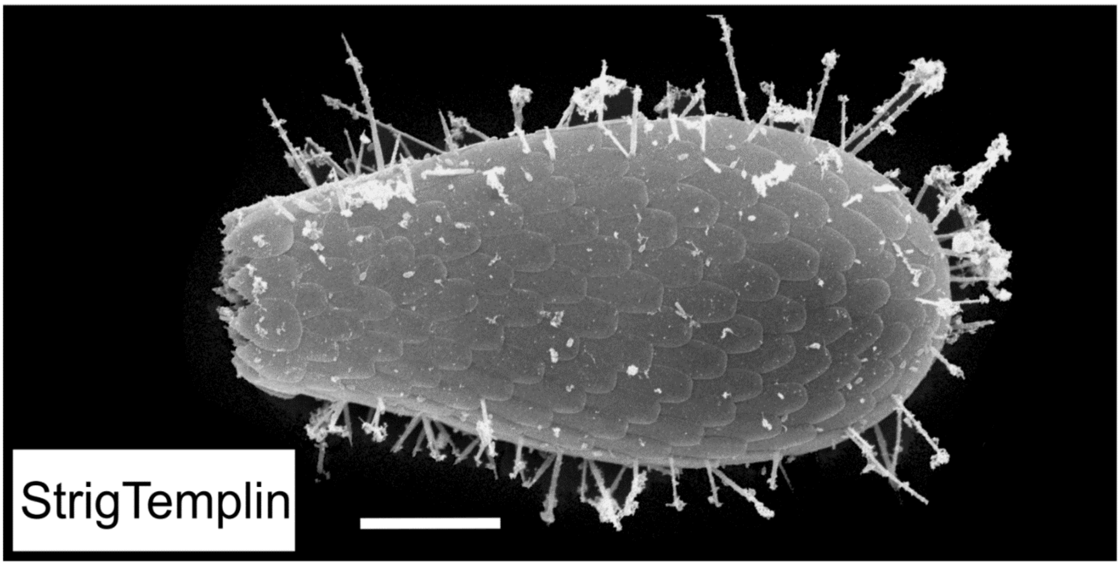
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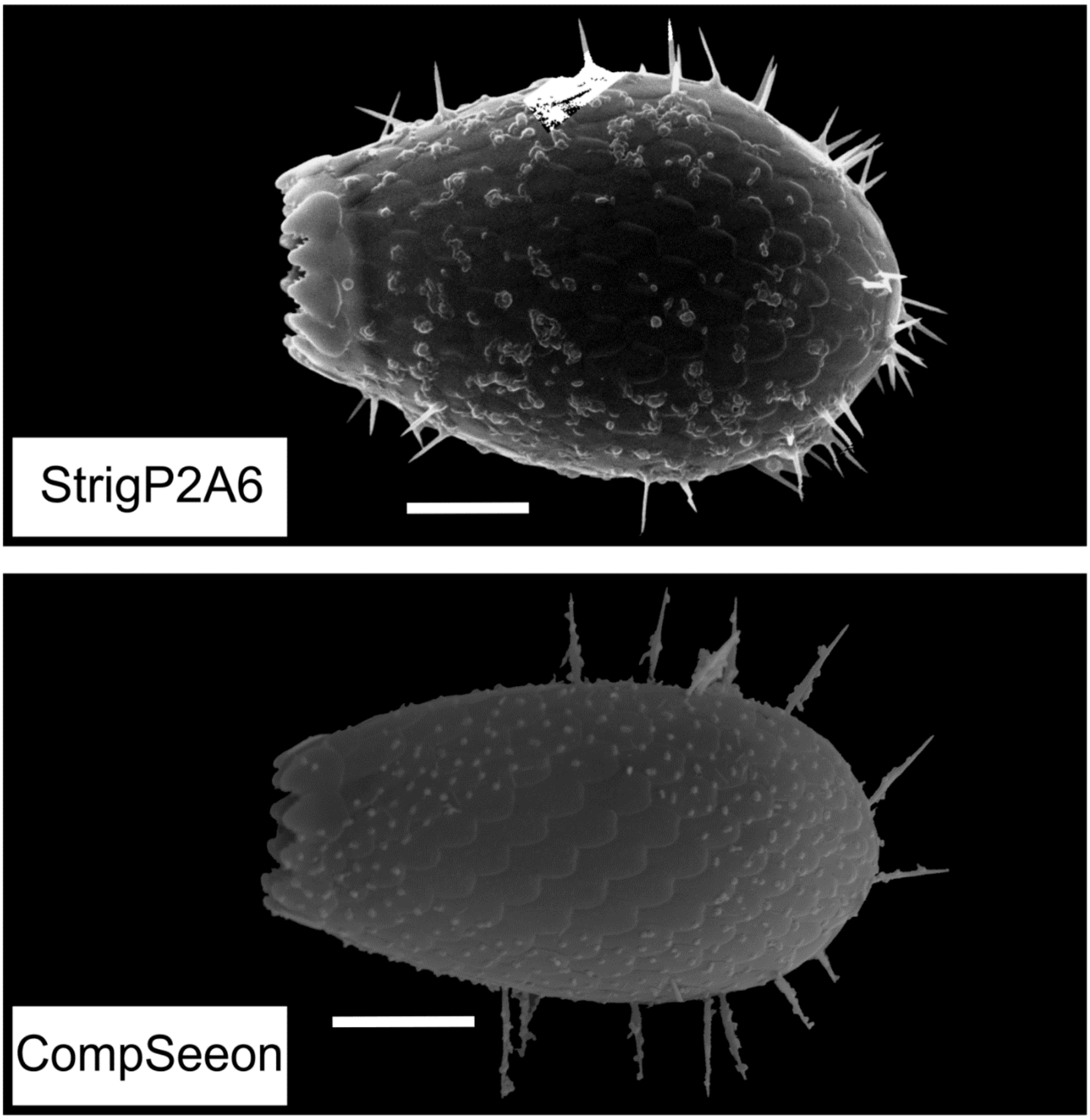

Supplementary figure 1

Chapter 9 -High-throughput sequencing of litter and moss eDNA reveals a positive correlation between the diversity of Apicomplexa and their invertebrate hosts across alpine habitats (published)

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Abstract

A high diversity of Apicomplexa was recently found in tropical soils presumably reflecting the diversity of their invertebrate hosts, but such patterns have not been explored in colder regions. We analysed the diversity of Apicomplexa and their potential metazoan hosts in litter and mosses collected in 11 different alpine habitats using an eDNA metabarcoding approach. The abundance and diversity of Apicomplexa phylotypes and of their potential invertebrate hosts were positively correlated. This confirms that eDNA metabarcoding is a useful tool to explore the unknown biodiversity of free-living eukaryotes, as well as potential host-parasite interactions. Future studies should aim at describing this diversity using a combination of morphological and molecular approaches.

Keywords

Soil biodiversity; Soil microbes; Invertebrates; Swiss Alps; Protists; Parasites

Introduction

Metabarcoding of environmental DNA (eDNA) is a powerful tool to explore the diversity of soil organisms, as shown by recent studies revealing that soils host an immense diversity of protists (Bates, 2013, de Araujo, 2018, Oliverio, 2020) and Metazoa (Fierer, 2017, Müller, 2019). This approach is especially useful to evaluate the diversity of poorly known groups such as Apicomplexa (Mahé et al., 2017, Geisen, 2015). Apicomplexa are obligate host-specific parasites of invertebrates, as well as vertebrates, including livestock and humans (del Campo et al., 2019; Simdyanov et al., 2017). It has been suggested that the richness of Apicomplexa-related sequences should be proportional to the diversity of their hosts, as illustrated by the immense diversity found in Neotropical forest soils (Mahé et al., 2017).

If this hypothesis were correct, such correlations should also be observed in other biomes. To test this hypothesis, we conducted a study at ca. 2500 m a.s.l. In the Furka pass region of the Swiss Alps. This region is characterised by a contrasted topography, bedrock and soil types resulting in high diversity of alpine habitats, plants and invertebrate communities across short distances (Hiltbrunner and Körner, 2018). We collected three to four samples of litter or mosses from 11 different habitats surrounding the Alpine Research Station Furka (ALPFOR) (Table 1, Supp. Table 1) in July 2012. We extracted eDNA using a MoBio PowerSoil extraction kit (Carlsbad, CA, USA) according to the manufacturer instructions. We assessed the phylotype richness of eukaryotes using a metabarcoding approach targeting the V9 region of the 18S rRNA gene using the eukaryotic primers 1380F/1510R (Amaral-Zettler et al., 2009). The PCR amplicons were sequenced with Illumina HiSeq 2000 (Fasteris, Geneva, Switzerland).

Table 1. Habitat type and phylotype richness of Apicomplexa parasites and their hosts in the central Swiss Alps close to the Furka pass.

Habitat No.	Habitat description/Dominant plant species	Elevation [m.a.s.l]	Latitude N#	Longitude E#	Metazoa± SD	Apicomplexa ± SD
1	Acidic grassland (*Nardus stricta*)	2455	46°34’41”	8°25’14”	54 ± 35	48 ± 13
2	Acidic species-rich grassland *(Nardus stricta/Calluna vulgaris)*	2493	46°34’43”	8°25’13”	09 ± 49	74 ± 21
3	Acidic ridge (*Loiseleuria procumbens*)	2468	46°34’42”	8°25’11”	20 ± 42	63 ± 18
4	Species-rich grassland on calcareous soil (*Festuca violacea*)	2392	46°34’33”	8°25’19”	91 ± 71	60 ± 20
5	Acidic grassland steep N-facing solifluction (*Carex curvula*)	2427	46°34’18”	8°25’12”	73 ± 22	59 ± 13
6	Acidic grassland (*Carex curvula*)	2491	46°34’01”	8°24’48”	77 ± 36	55 ± 11
7	Nutrient-rich grassland (*Agrostis schraderiana*)	2494	46°33’45”	8°24’48”	10 ± 75	57 ± 16

Habitat No.	Habitat description/Dominant plant species	Elevation [m.a.s.l]	Latitude N#	Longitude E#	Metazoa± SD	Apicomplexa ± SD
8	Snow-bed on acidic soil (*Salix herbacea*)	2432	46°34’41”	8°25’19”	18 ± 43	63 ± 16
9	Glacier forefield	2508	46°33’27	8°24’49”	73 ± 24	58 ± 13
10	Calcareous ridge (*Elyna myosuroides*)	2468	46°34’22”	8°24’49”	04 ± 59	70 ± 20
11	Fen	2433	46°34’32”	8°25’07”	58 ± 35	65 ± 17

#Coordinates are given for the centre of the selected habitat areas.

The eDNA reads were filtered, quality-checked, clustered into phylotypes using SWARM (Mahé et al., 2015) and taxonomically assigned with the PR^2 database (Guillou et al., 2012) using VSEARCH (Rognes et al., 2016). We extracted all phylotypes assigned to Metazoa and Apicomplexa. As 18S rDNA sequences of parasites such as Apicomplexa are highly divergent, classical assignation based on pairwise similarity can often be unreliable. Therefore, we constructed a reference tree that comprises all complete Apicomplexa 18S rRNA gene sequences available on GenBank as well as some *bona fide* Alveolata and other phyla as outgroups. Then, we used the Evolutionary Placement Algorithm as implemented in RAxML v.8.2.10 to determine the phylogenetic position of the potential apicomplexan phylotypes (Stamatakis, 2014). The data are available in the NCBI Sequence Read Archive under the BioProject number PRJNA623507 and Supplementary Table 1.

We used linear regression models to test whether the abundance of phylotypes of Apicomplexa and of their putative metazoan hosts were correlated. In order to circumvent potential methodological biases due to the variation of read numbers per sample, we calculated a corrected value of the phylotype richness as the normalized residuals of the number of phylotypes minus the predicted number of phylotypes based on the total number of reads in that same sample, following a similar logic as Tedersoo and co-authors (2014) (see Supplementary methods 1). All statistical analyses were performed in R version 3.5.1.

We obtained a total of 181 phylotypes of Apicomplexa (879,886 reads) and 856 phylotypes of Metazoa (1,180,871 reads). Phylogenetic reconstruction clustered Apicomplexa phylotypes into two main lineages (Fig. 1), the Actinocephaloidea and the Gregarinoidea (superfamilies proposed by Simdyanov et al., 2017), which include species that are mainly reported as parasites of terrestrial invertebrates, especially insects. While most phylotypes were associated to insect (e.g. *Gregarina*) or Annelida (*Monocystis*) parasites, two were associated to vertebrate parasites (*Nematopsis* and *Cryptosporidium*) and one phylotype was associated to a clade that recently transitioned from terrestrial to marine invertebrate hosts (*Caliculium*) (Wakeman et al., 2014). The majority of the apicomplexan reads (78.8%) were clustered into a single phylotype associated to genus *Gregarina* (GenBank L31799 and L31841), while 4.5% of the reads (39,407 sequences), could not be assigned with confidence to any known Apicomplexa lineage.

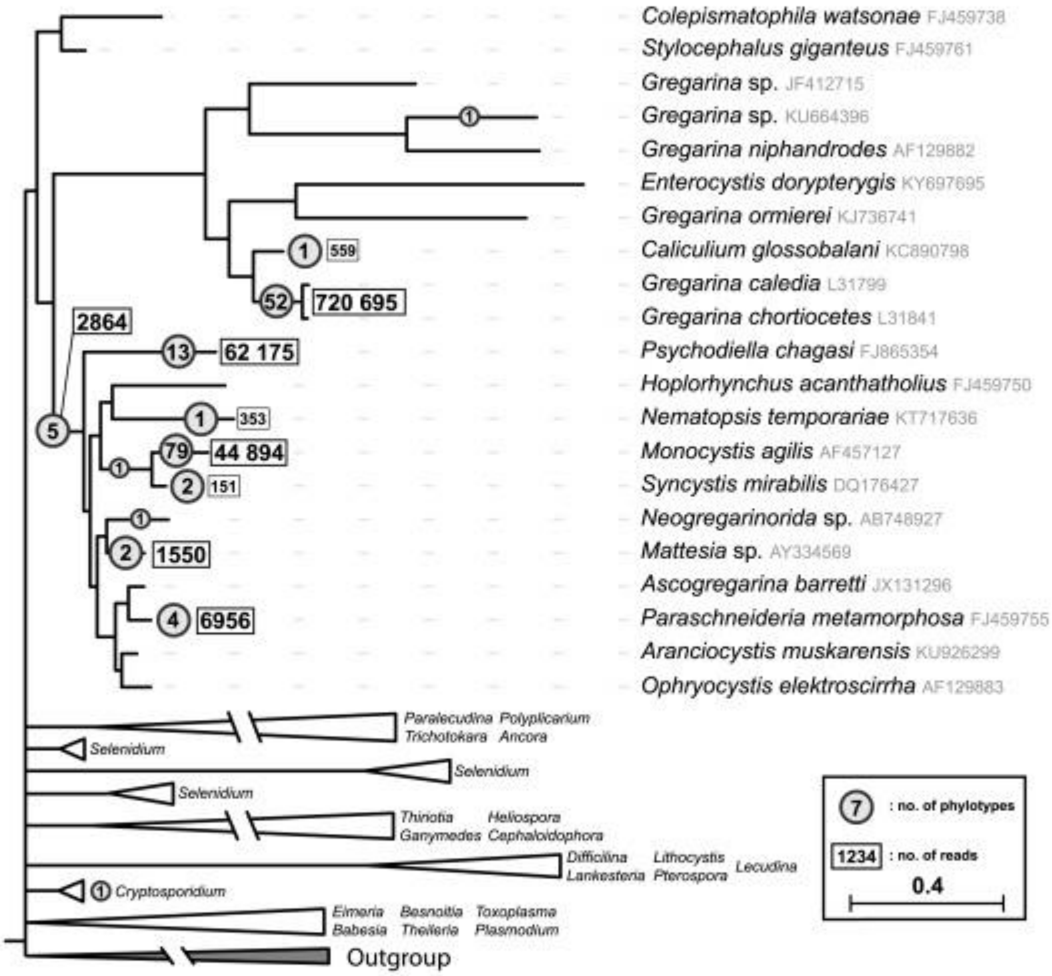


Fig. 1. Phylogenetic tree of DNA reference sequences of Apicomplexa and phylotypes from an eDNA Illumina sequencing of the V9 region of the 18S rRNA gene from 41 soil litter and moss samples collected in 11 different alpine habitats in the Furka Pass region (Switzerland). Only phylotypes with over 100 reads are shown. Numbers in circles represent numbers of phylotypes affiliated to an identified organism and numbers in squares represent the total number of reads.

As a whole, the number of Apicomplexa reads was significantly correlated to the number of Metazoa reads, as shown in a simple linear regression model (adjusted $R^2 = 0.64$, $p < 0.001$; Fig. 2). The correlation between the corrected diversity of Apicomplexa and Metazoa remained significant, which strengthens the validity of the analysis by removing potential methodological biases (Supp. Fig. 1). The correlations were also significant for litter samples and for both data sets combined but not for moss samples ($p = 0.11$; Supp. Fig. 1). Although we observed a positive correlation between the number of, respectively, Apicomplexa and Metazoa phylotypes *versus* all eukaryotes phylotypes, which can be interpreted as sequencing biases (Supp. Methods 1), this correlation disappeared when the correction factor was applied to the alpha diversity (Supp. fig 2; Supp. Methods 1), demonstrating the robustness of our results.

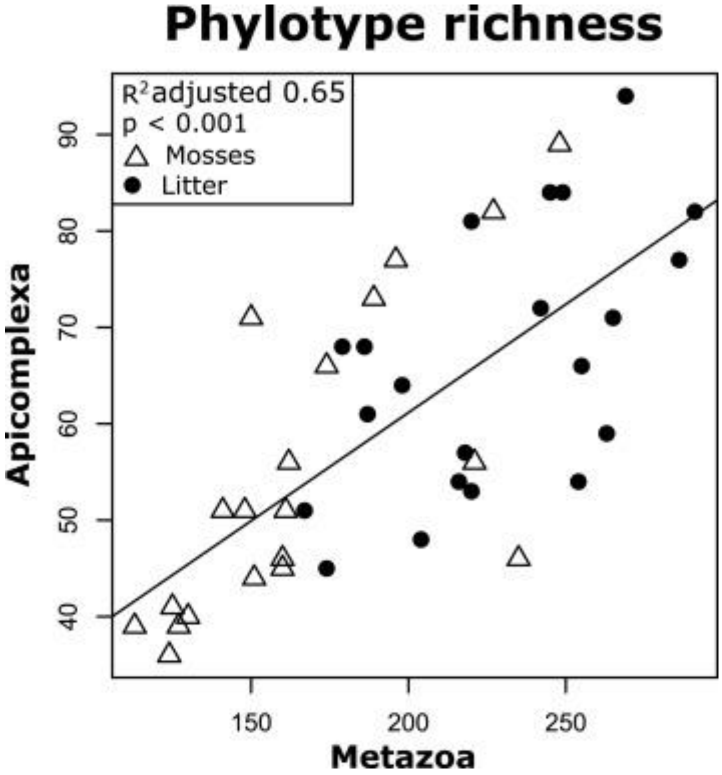


Fig. 2. Relationship between the phylotype (V9 region of the 18S rRNA) richness of Apicomplexa and Metazoa from 41 soil litter and mosses samples collected in 11 alpine habitats in the Furka Pass region (Switzerland). The line shows the slope of the linear regression.

The highest number of Apicomplexa phylotypes was found in the acidic grassland with *Calluna* spots (habitat no.2) and in the calcareous ridge (habitat no. 10): two contrasting habitats that do not share similar vascular plant or moss species (Table 1). The highest number of Metazoa phylotypes was detected in the fen (habitat no. 11) which had the highest number of individual flies and midges, and the highest number of families of Diptera (Hiltbrunner and Körner, 2018).

Our results support the hypothesis that the diversity of soil Apicomplexa may reflect the diversity of Metazoa (and especially invertebrates) in an ecosystem (Mahé et al., 2017). Although Apicomplexa diversity cannot be directly compared between the two studies, notably because of the genetic markers used (V9 in our study *versus* V4 in the study of Mahé et al. (2017), see (Dunthorn et al., 2012)) the genetic diversity obtained in our samples (Fig. 1) was also high and included many different clades of Apicomplexa. Even though the number of samples and sequencing depth in the study of Mahé et al. (2017) are larger than in this study, we sampled 11 contrasted alpine habitats differing in their characteristics and two contrasted types of samples litter versus mosses. Still, while most Neotropical Apicomplexa reads and phylotypes were branching at internal nodes in the reference tree, the majority of alpine Apicomplexa were placed on or very close to the tips (Fig. 1). This suggests the existence of a higher genetic novelty in Neotropical Apicomplexa, probably reflecting also a lack of knowledge of their hosts.

This study shows that inferred host-parasite relationships based on eDNA metabarcoding is a powerful approach to explore the diversity of poorly known taxa and for inferring potential host-parasite or other biotic interactions. These findings call for further research on host-parasite interactions by combining traditional methods (species morphology and barcoding) of soil fauna analysis with meta-barcoding of eDNA to characterise the level of interactions between the hosts and their parasites.

Supplementary methods – Calculation of a corrected phylotype richness

As sequencing depth (i.e., the number of reads per sample) is often uneven across samples, samples with low sequencing depth will have fewer reads of any group and vice versa. Furthermore, the number of reads often impacts the number of retrieved phylotypes. As a result, a correlation analysis based on either raw number of reads or phylotypes could result in an artefactual positive correlation between any two groups (Fig. supp. methods 1A).

One solution to overcome this bias is to model and remove the influence of the number of reads on the number of phylotypes. We applied a linear regression that predicts the number of phylotypes in a sample in function of the log-transformed number of reads of the same sample. We then analysed the correlation with the normalized residuals (i.e., the actual number of phylotypes minus the predicted number of phylotypes) which can be interpreted as a corrected value of phylotype richness, as also shown in (Tedersoo et al., 2014). This corrected value of phylotype richness will not be correlated to the number of reads anymore (Fig. sup methods 1B).

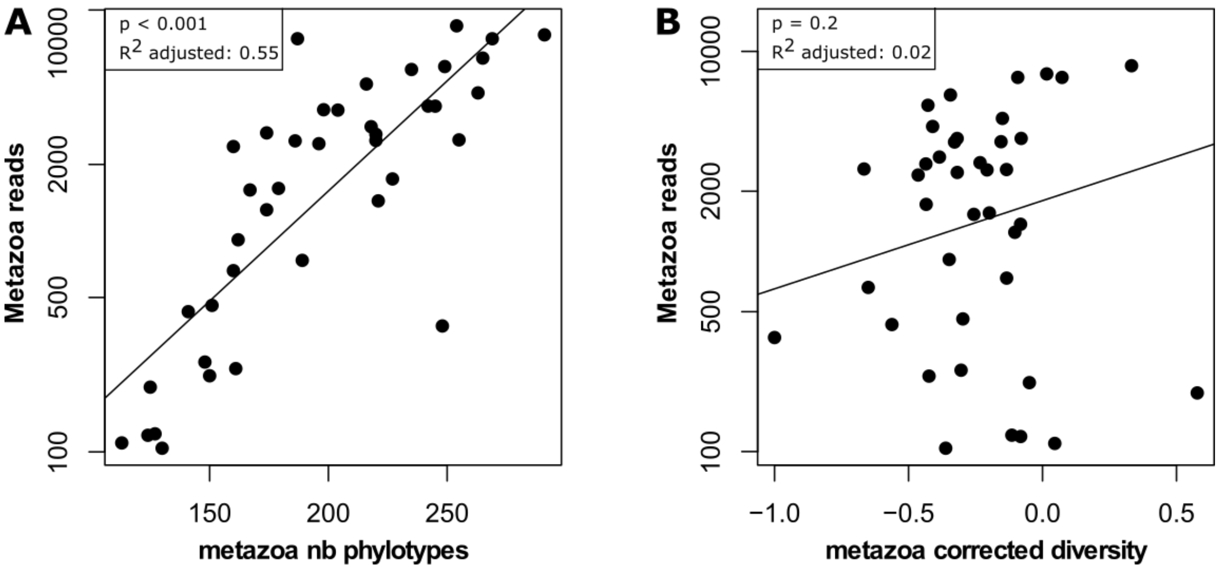
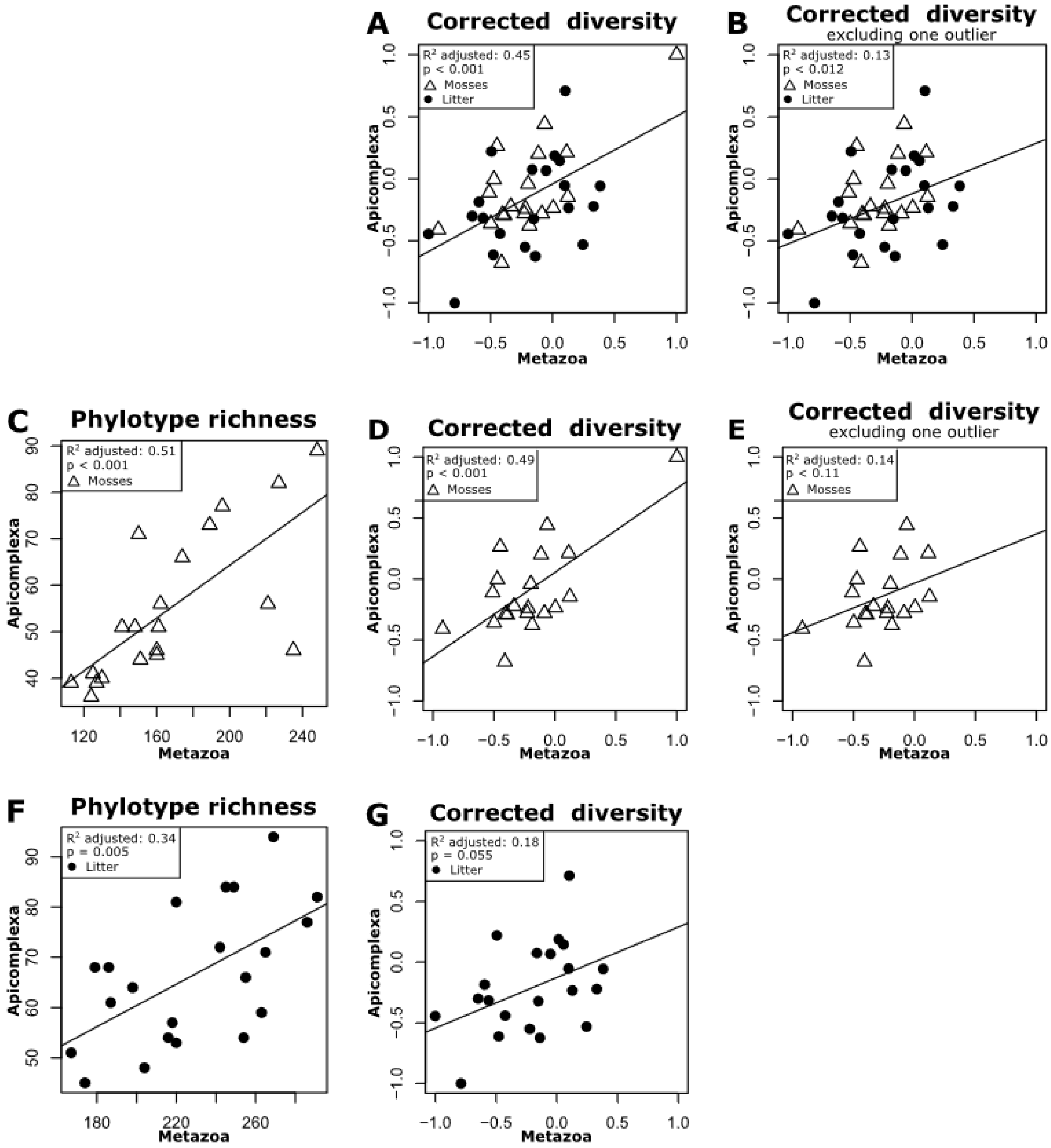


Fig. supp. methods 1. Relationship between the raw (A) and corrected (B) phylotype richness of Metazoa versus the number of Metazoa reads (V9 region of the 18S rRNA) from 41 soil litter and mosses samples collected in 11 alpine habitats in the Furka Pass region (Switzerland). The line shows the slope of the linear regression.

Reference for supplementary methods

Tedersoo L, Bahram M, Põlme S, Kõljalg U, Yorou NS, Wijesundera R, et al. (2014) Global diversity and geography of soil fungi. *Science* 346(6213): 1256688: doi:10.1126/science.1256688.



Supp. fig 1. Relationship between Metazoa and Apicomplexa corrected diversity (A, B, D, E & G) and phylotype richness (C & F) from 41 soil litter and moss samples collected in 11 alpine habitats in the Furka Pass region (Switzerland). The corrected diversity is based on the corrected phylotypes richness which is the normalized residuals of the number of phylotypes minus the predicted number of phylotypes based on the total number of reads in the same sample. This correction removes the potential bias of uneven sampling depth among samples. As one moss sample was characterised by a very high diversity in both metazoan and apicomplexan phylotypes, separate analyses were done excluding this samples (B & E).

Declaration of competing interest

The material in this manuscript is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration for Soil Biology & Biochemistry. The authors declare no competing financial interests.

Acknowledgement

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Chapter 10 - Generalized diversification of marine, freshwater and soil microbial eukaryotes during the Paleocene and Eocene: a preliminary work

Clément Duckert, Guillaume Lentendu, Enrique Lara, Edward A. D. Mitchell

Summary paragraph

The Paleocene-Eocene period (66 to 34 MY ago) is characterized by a global increase in temperature followed by a decrease in atmospheric CO2. This caused the Earth to transition from a greenhouse to icehouse climate with major impacts on the biosphere including a strong turnover of vertebrate, invertebrate, plant, and marine micro-eukaryote diversity. Here we show that these climatic changes also drove a radiation in soil euglyphid testate amoebae (Rhizaria: Euglyphida). This ubiquitous group of micro-eukaryotes build shells from self-secreted siliceous scales and play a key role in soil biogeochemical cycles by increasing silica turnover, ultimately resulting in higher silica supply to rivers and oceans. A time calibrated phylogeny revealed a significant increase in euglyphid diversification rate between 60 and 50 MY ago during a period when global temperature peaked and decreased several times. These changes mirror the evolution of diatoms in the ocean which are major CO_2 fixer. As the growth of these marine protists is limited by Si, any increase in Si supply to the ocean from terrestrial sources, such as the increase of soil Si cycling due to euglyphids could have contributed to lowering atmospheric CO_2 and thus to global cooling.

Introduction

The Paleocene-Eocene period, 66 to 34 million years (MY) ago is characterized by a global increase in temperature followed by a decrease in atmospheric CO_2 leading to Earth's transition from a greenhouse to an icehouse climate (Zachos et al. 2001). Major shifts in biota diversity and community composition in both continental and marine habitats have been directly linked to these global climatic changes. These shifts did not all present the same temporality: while the Paleocene-Eocene thermal maximum (55 MY) saw the appearance of several groups of modern animals (Perissodactyla, Artiodactyla and primates) in less than 200 KY (Gingerich 2006), the diversification and dispersion of C4 grasses starting during the Miocene was a gradual event spanning tens of million years (Jacobs et al. 1999).

This reshuffling of diversity is best documented for macro-organisms (i.e., plants and animals) and shell-producing marine micro-eukaryotes such as foraminiferans, radiolarians, diatoms and coccolithophores, which fossilize very well and have provided easily interpretable and continuous records (see Whidden and Jones 2012 as an example). Given the major changes occurring on continental biomes it is very likely that substantial changes also occurred among soil organisms during the Paleogene. However, most groups of soil micro-organisms are soft bodied and do not leave fossils. This makes it impossible to calibrate molecular clocks to date speciation events and assess if similar changes in diversity occurred among soil organisms during the Paleogene. Among soil microorganisms the only existing evidence is a radiation since the Oligocene for a rare biosphere soil diatom species complex, but it was linked to a change in ecological niche rather than a response to biogeoclimatic changes (Pinseel et al. 2020). It would thus be very useful to have a proxy for soil microorganisms of functional significance, with a good enough fossil record to calibrate a molecular clock.

Here we analysed the magnitude and timing of diversification rate for self-silicifying amoebae (Rhizaria: Euglyphida), a group of mostly terrestrial micro-organisms, but which also includes freshwater and marine taxa. We first compiled a reference alignment of every SSU rRNA gene sequence that could be assigned to euglyphids from GenBank and the Protist Ribosomal Reference (PR2) database (Guillou et al. 2012) to build a reference phylogenetic tree. This reference tree was then time-calibrated based on fossilized euglyphid scales that were uncovered in the Giraffe Pipe sediments and dated to 40 MY (Barber et al. 2013). To measure changes in diversification rate of euglyphids while also taking into account the extant diversity that is missing from our reference tree, we extracted the euglyphid SSU V4 sequences from Illumina metabarcoding datasets available on the Sequence Read Archive using a DNA probe designed specifically to capture euglyphids. This way we obtained 495’127 V4 short reads clustered in 3’730 operational taxonomic units (OTUs) from 50 bioprojects for a total of 1’761 samples, from soil, freshwater and marine ecosystems from all continents except Africa. We finally assigned a position on our reference tree for each of those OTUs in order to measure diversification rates.

Euglyphids are a diverse group of shelled amoeboid microeukaryotes. Their shell is made from self-secreted siliceous scales whose size and shape are species specific (Chapter 8, Revision of genus *Euglypha*). These scales are preserved in the fossil record and can be attributed with a high accuracy to extant lineages. Euglyphids are common in terrestrial and freshwater habitats. Together with other phagotrophic protists such as ciliates or flagellates or naked amoebae, testate amoebae play important roles in microbial food webs as predators of bacteria and other microbes including micro-metazoa (Geisen et al. 2018, Wilkinson and Mitchell 2010). Euglyphids stand out among soil microbial predators as one of the only groups to leave a fossil record and as such are a useful proxy for soil microbial communities in general.

Euglyphids contribute very significantly to silica cycling; annual biosilification by euglyphids was estimated to range from 10 to 227 kg SiO2 ha-1 yr-1 in temperate forest, which is within the range reported for higher plants (Aoki et al. 2007, Puppe et al. 2015). The biogeochemical cycles of carbon and silica are tightly linked over geological timescales as continental silica weathering is the main source of silica for silicifying phytoplankton, which in turn play a major role in ocean C sequestration through the carbon shunt (Suttle 2007).

Thus, the observed patterns for euglyphid diversification is indicative for major changes in the diversity and makeup of soil microbial communities during the Paleocene-Eocene period as well as a possible contributing cause of the global cooling through increased Si supply to freshwater and oceanic phytoplankton. One of the aims of this study is also to develop a workflow to study the diversification patterns of soil protists, organisms whose vast majority of species are not described and for which there are only few curated sequencing data available.

Method summary

Designing an *in-silico* probe to extract euglyphid DNA sequences from datasets

In order to recover Euglyphida 18S rDNA sequences from publicly available environmental sequencing projects, a novel bioinformatic approach was developed. First, an *in-silico* probe specific to Euglyphida targeting the 18S gene was designed using the PR2 database v 4.12.0 (Guillou et al., 2013): all Euglyphida references sequences were extracted to serve as target group, all other Rhizaria sequences except Cercozoa_X and Filosa-Imbricatea_X were used as the outgroup, as well as, in a second step, all Fungi sequences. Probes were searched in the size range from 15 to 35 nucleotid using the apolla tool (Lentendu, 2022). This tool lists and counts all k-mers of the desired length using Jellyfish v2.3.0 (Marçais and Kingsford, 2011), then keeps only k-mers specific to the target group, clusters k-mers of 1-nt differences using Swarm v3.0.0 (Mahé et al., 2015), resolves degenerate nucleotide code across all k-mers of each cluster to create one single probe per cluster and reports coverage of the probe in the target and outgroup clades. The longest probes with the best coverage of Euglyphida families and lowest coverage of outgroup clades were selected from the two runs with Rhizaria only or Rhizaria + Fungi as outgroup, and merged to create the final probe. The probe is 34-nt long and is located in the V4 region of the 18S rDNA (ACTRACTWCTGCGAAAGCATTCACCAAGGATRTC).

Gathering long sequences

To build a backbone phylogenetic tree of the order Euglyphida we first gathered all long (>800bp) SSU rRNA gene sequences available on GenBank that were tagged either as "SSU", "18S" or "small ribosomal subunit". We then extracted the sequences of euglyphids using the *in-silico* probe located in the V4 region which, after the removal of the few non-euglyphid or highly diverging sequences, yielded 480 unique sequences. This reference alignment, with the addition of sequences of four other Cercozoa (Sarcomonadea) to serve as the outgroup, was automatically aligned using MAFFT (Katoh and Standley 2013) as implemented on the CIPRES Portal (Miller et al. 2011) and edited to remove the ambiguously aligned positions using trimal (Capella-Gutiérrez et al. 2009) with parameters recommended by EukRef (Del Campo et al. 2018) for further phylogenetic reconstruction. The alignment was then checked visually for obvious alignment errors.

Building the time-calibrated tree

A first phylogenetic tree was built using RAxML v.8 (Stamatakis et al. 2014) with the GTRCAT model and 1000 bootstraps. We constrained the monophyly of the genus *Euglypha* as two nodes inside this genus were later time calibrated and the monophyly of this genus is supported by a consistent morphology between species. The topology of the resulting phylogenetic tree was then used as a constraint to generate a time-calibrated tree using MrBayes 3.2.7 (Ronquist et al. 2012) with the parameter GTR + GAMMA under a birth-death speciation process and a relaxed uncorrelated clock with a lognormal distribution of rates. This analysis was run using the time-calibration detailed below on four MCMC runs for 100x10^6 generations sampled every 5000 generations resulting in 80000 samples, of which 25% were discarded as the burn-in to build the consensus tree. Convergence of the four runs was checked using Tracer (Rambaud et al. 2018).

Time calibration of the reference tree

Several fossils of euglyphids have been documented, with the oldest found in the Giraffe Pipe sediments dated to around 40 MY (Barber et al. 2013). Some of these fossils present parietal scales elongated into sword-like spines that can be unambiguously assigned to the section *Acanthophorae* of the genus *Euglypha* and rhomboidal scales that can be assigned to section *Rhomboidalae* of the genus *Euglypha* as defined by Duckert et al. (in prep). We calibrated the *Acanthophorae* and *Rhomboidalae* nodes using the same calibration (lognormal distribution with an offset of 40 MY, mean age of 45 my and a standard deviation of 3 so that the 95% interval range from 41 to 51 MY). This calibration is distinct from that of González-Miguéns et al. (2022), who used the same fossils but misassigned them based on an erroneous interpretation of the morphology of *Euglypha cristata*, *E. tuberculata* and *E. acanthophora*. These taxa were furthermore erroneously assigned to the genus *Scutiglypha*, as each of them does not present the scutiform scales that characterize *Scutiglypha* (Foissner and Schiller 2001) and thus cannot be included in this genus. It is also distinct from the calibration used by Delay et al. (2016) who used the same fossil to date only the crown node of the genus *Euglypha*. We did not consider the fossils of *Cyphoderia* reported by Wagoner (1996) in our analysis as they cannot be reliably assigned to this genus. We also did not consider the fossil of *Trinema* reported by Schiller (2007) dated to be 31 MY old (Gaudant 2009), as we did observe euglyphid scales that could be attributed to the genus *Trinema* in the same Giraffe Pipe sediments (data not shown) but no precise node could be calibrated. We evaluated the relevance of these calibrations by performing the same analysis and calibrating only one node at a time. The age of the fossils gives more a minimum date than an estimation, and it is likely that these nodes are older than the fossils. However, several fossils of testate amoebae older than 40My were found but they did not contain any *Euglypha*. For this reason, we do not consider the nodes to be that much older than the calibration.

Extracting euglyphids from environmental sequencing datasets

We applied our *in-silico* probe on environmental sequencing datasets available in the Sequence Read Archive. A list of Bioproject accessions from short read archive was collected from NCBI, by searching attributes of Biosample accessions with the query '("target gene 18S"[Attribute] OR "target gene 18S rDNA"[Attribute]) AND "target subfragment V4"[Attribute]'. The list of accessions was complemented by a literature search with the same keywords. For all bioprojects, the "read_run" information were download from ENA SRA filereport API service. For simplicity, only projects sequenced on Illumina MiSeq machines were selected. The final list included 74 candidates Bioprojects totalling 3600 samples. For each Bioproject, a newly developed tool (probe_sra_marker; Lentendu, 2022) was used to extract all reads matching the designed probe and proceed them through a standard metabarcoding pipeline. Raw read files were downloaded with aspera connect v3.9.7 and paired reads matching the Euglyphida probe were extracted with cutadapt v2.10 (Martin, 2011). A set of commonly used metabarcoding primers was provided to infer the primer used. If a primer pair was detected consistently in R1 and R2 libraries for more than 10 % of raw reads, this pair was selected and used to remove primers from the 5'-end using cutadapt. Reads were pair-end assembled using pandaseq v2.11 with the "simple_bayesian" algorithm (Masella et al., 2012), then dereplicated and filtered for no N nucleotide using VSEARCH v2.13.6 (Rognes et al., 2016). Reads were clustered into OTUs using Swarm with the fastidious mode on and a maximum difference of one. The OTU representative sequence (i.e., the most abundant sequence) was used to search and remove chimera using UCHIME (Edgar et al., 2011) as implemented in VSEARCH, and to assign taxonomy against the full PR2 database using the "usearch_global" algorithm as implemented in VSEARCH. When multiple sequencing libraries were provided for a biosample, reads of OTUs were summed at the biosample level. Biosample metadata were recovered from the ENA SRA data API service in XML format and integrated into the OTU table.

This resulted in 15’912 OTUs (997’908 reads from 2’280 samples) of which 4’106 were first taxonomically assigned to the order Euglyphida. We retained only OTUs with at least 90% identity to at least one sequence of the reference alignment. This resulted in 3’810 OTUs (495’127 reads from 1’761 samples from 50 bioprojects), of which 3’730 were kept after removing the OTUs whose sequence could not be aligned with the reference alignment or were placed in the outgroup during subsequent analyses. Samples containing at least one OTU were distributed worldwide except in Africa and Central Asia, but were mostly collected in Europe or North America. Euglyphid OTUs were found in 422 (25%) terrestrial samples, 179 (10%) freshwater (water or sediments) samples, and in 1’115 (65%) marine samples (including estuarine (14) and intertidal (113) samples) (Fig. 1).

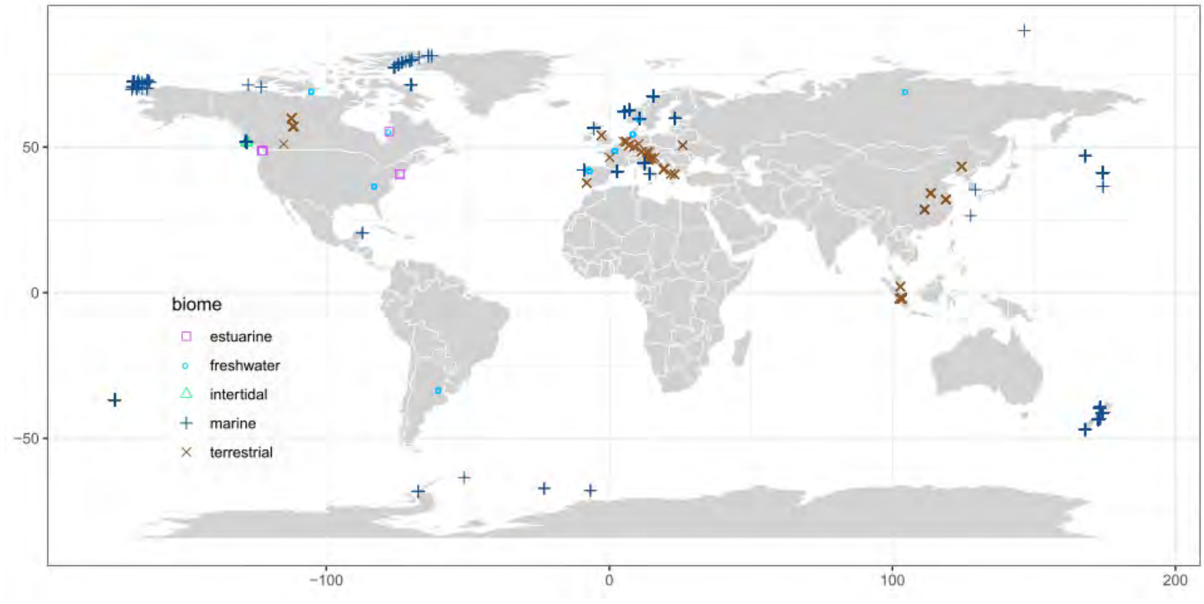

Figure 1: Location of samples retrieved from the Sequence Read Archive that contain at least one OTU assigned to the order Euglyphida after filtering. The symbols indicate the biome from which the sample was taken.

Phylogenetic tree including the short reads

To assess if major clades in the Euglyphida were missing from our reference alignment and were represented only by short reads, we built a phylogenetic tree using sequences from the reference alignment and the short read OTUs (hereafter only referred to as OTUs). The monophyly of the genus *Euglypha* and the two nodes that were time-calibrated was also constrained.

Phylogenetic placement of the short reads and diversification analyses

The reference tree was pruned using the R package Phytool (Revell 2012) by collapsing every clade younger than 10 MY old into single terminal branches. This was done to enhance the performance of the phylogenetic placement method which can yield weaker results if OTUs are placed on a phylogenetic tree with tips that are too closely related (Stamatakis et al. 2014). These OTUs were then aligned on our reference alignment using Papara (Berger and Stamatakis 2012) and placed, along the long sequences corresponding to the pruned branches, on the pruned tree using the evolutionary placement algorithm (EPA) as implemented in RAxML v.8 (Stamatakis et al. 2014). The results of the EPA analysis were summarized using GAPPA (Czech et al. 2020), and the OTUs were kept on their most likely placement. After removing the outgroup, shifts in diversification rate in the pruned tree were analysed using BAMM (Rabosky et al. 2018). This analysis can account for species missing from phylogenetic trees (here the OTUs), but only for predefined clades. To account for the OTUs placed on a non-terminal branch of the tree, the descending branches must be virtually collapsed into a clade, then the OTUs of each branch are summed up and attributed to the whole clade. There is thus a trade-off between forming clades that are young enough to accurately represent the topology of the original tree and recent diversification events, but also old enough to include most OTUs. Here we formed clades based on three thresholds, at 35, 50 and 65 MY. The same analysis was also performed using the original time-calibrated tree without accounting for missing species. Extinction events were investigated using the R package TESS (Höhna et al. 2016), however this analysis does not consider missing species and is based only on the topology of the original time-calibrated phylogenetic tree.

Sensitivity analysis

We evaluated the impact of the assumptions we made by performing the dating and diversification analyses using different combinations of tree topologies, fossil calibrations, evolution models and strategies to place the OTUs on our phylogenetic tree. We tested the impact of the birth-death evolution model by performing the dating and diversification analyses on a time calibrated tree generated under the alternative uniform (birth only) model as implemented in MrBayes. We also tested the impact of the fossil calibration by performing the molecular clock analysis with only one of the two nodes calibrated.

Results and Discussion

Diversity and diversification patterns of the order Euglyphida

The Euglyphidae and Trinematidae, two families with mostly small and terrestrial species, appear to be significantly more diverse than the other groups of euglyphids. Interestingly, only a few OTUs were placed within environmental clades compared to the groups that include taxa that have been characterized (Fig. 2). The phylogenetic tree built using the whole dataset reveals that the reference alignment covers most of the phylogenetic diversity of the Euglyphida (Fig. 3). Nonetheless, we found two family-level clades (referred to as EEC-5 and EEC-6), one placed at the base of the Paulinellidae (65 OTUs) and one at the base of the environmental clade EEC-2 as defined by Lara et al. (2016) (67 OTUs) that do not include any long sequences.

Our analysis date the crown node of the order Euglyphida to be 184 MY (95% interval: 131-292) which is younger than what has been found by Berney and Pawlowski (2006), the only study giving an estimate for the appearance of the euglyphids using different fossil calibrations. As a point of comparison, they estimated the split between the Paulinellidae and Euglyphidae to be 207 MY (95% interval: 123-316) while we retrieve a date of 172 MY (95% interval: 122-238). However, their study was not focused on the Euglyphida and concerned the whole eukaryotic tree of life, and among the several points of calibration that they used, the closest to the euglyphids was only at the base of the Rhizaria. This node is much older than the Euglyphida and was estimated to be 614 MY old, and this discrepancy might be explained by the remoteness of their calibration point. This is nonetheless only a difference of 17% which, given the paucity of exploitable points of calibration, we consider relatively small.

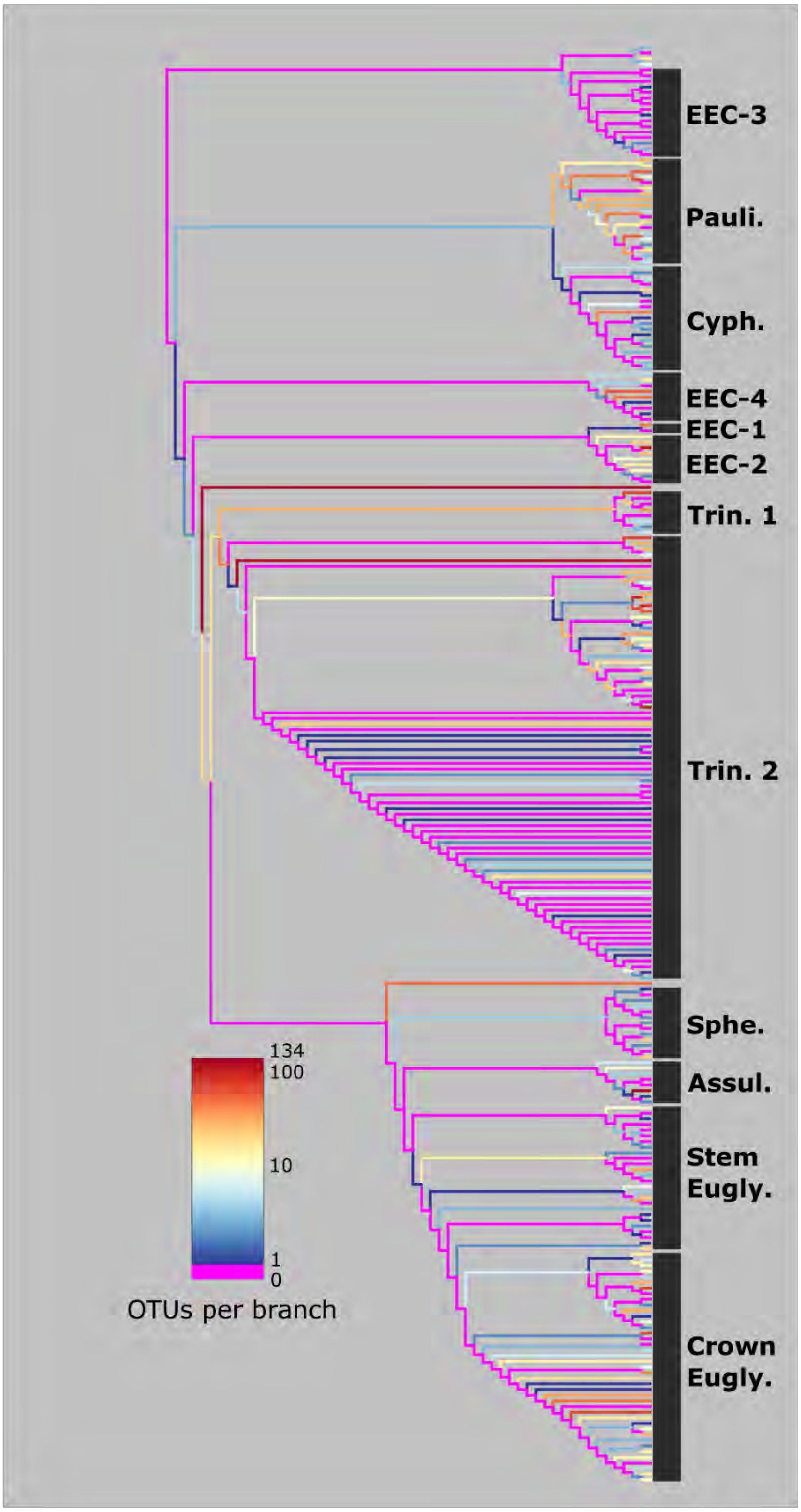

Figure 2: Doodle phylogenetic tree of the Euglyphida. The colour of each branch represents the number of OTUs that have been placed by RaxML EPA on that branch. The main taxonomic groups are indicated with EEC-1, 2, and 4 referring to the environmental clades defined by Lara et al. (2016), Cyph. to the Cyphoderiidae, Pauli. to the Paulinellidae, Sphe. to the Sphenoderiidae, Assul. to the Assulinidae, Eugly. to the Euglyphidae and Trin. referring to the Trinematidae.

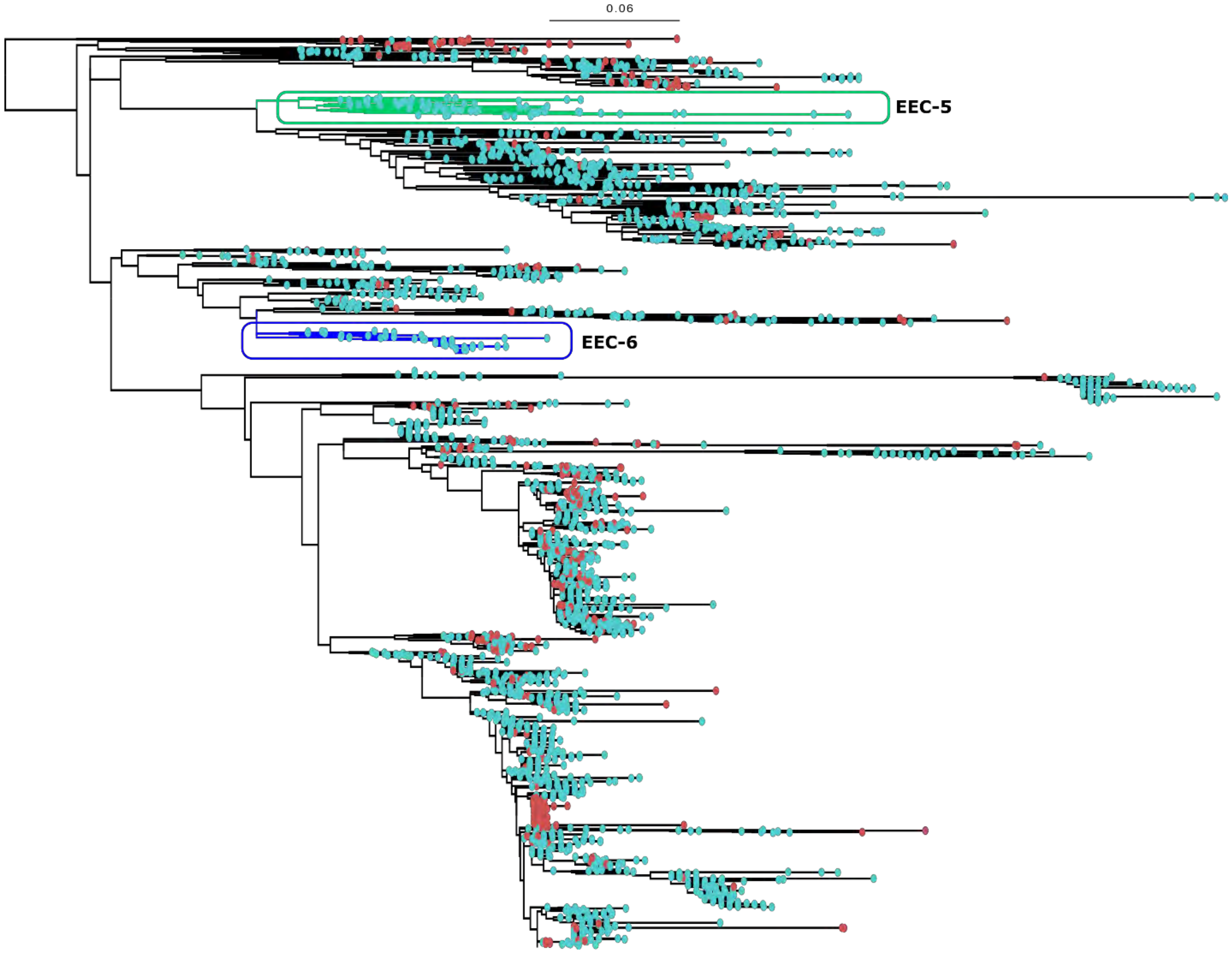

Figure 3: Maximum likelihood phylogenetic tree of the full dataset (reference alignment and short read OTUs) showing a good mixing of short and long sequences. The long sequences are represented by red dots, and the OTUs are represented by light blue dots. Two major clades composed only of OTUs (referred to as EEC-5 and 6) are highlighted in green and dark blue.

The distinct thresholds (35, 50 and 65 MY) used to form the virtual clades did impact the results, as the location of the shifts in net diversification rate were not the same depending on the threshold (Fig. 4). As expected, a threshold of 35 MY excludes more OTUs than the older thresholds (394 OTUs) and results in younger shifts. This is because BAMM cannot account for missing species outside of predefined clades, in this case OTUs placed on internal branches, which makes younger clades artificially present more speciation events than their parental branches. However, the same analysis using clades based on a threshold of 50 and 65 MY performed better, and excluded only 282 and 209 OTUs from the analysis, respectively, of which 132 form clades that are at least 100 MY old and could not be included anyway. These two thresholds retrieved similar results, with most shifts being placed on the same branch or in proximity, and indicate a major increase in diversification rate in up to six lineages between 64 and 37 MY before present, with five of them occurring between 64 and 49 MY before present. Interestingly, our results suggest that this increase in diversification rate did not only involve terrestrial groups, but also freshwater and marine clades (the Cyphoderiidae and the Paulinellidae). The analyses also retrieved a shift on the branch leading to Euglyphina as defined by Kosakyan et al. (2016) around 120 MY ago. These dates should be considered as lower estimates as these diversification events could be older than our results suggest, due to the node that we calibrated being significantly older than the fossils for example. It is thus unlikely that these events are younger.

It must be noted that the information provided by the OTUs is essential to get a clear signal. The analyses performed without accounting for missing species do not retrieve the same shifts in net diversification rate (data not shown) while the lineage accumulation and diversification rate through time based only on the reference alignment do not show a significant increase (Fig. 5).

Biological interpretation

Speciation rate has been shown to accelerate in response to environmental changes (Gavrilets 2000). As such the frequent transitions from warm and humid to cold and dry climates during the early Cenozoic, and the resulting changes in vegetation cover, might have contributed to the diversification of the euglyphids. Three major climatic events occurred around the period of diversification of the Euglyphida: the mid-Paleocene biotic event (MPBE, 59 MY), the Paleocene-Eocene thermal maximum (PETM, 55 MY) and the Early Eocene Climatic Optimum (EECO, 53 to 49 MY). The MPBE, PETM and EECO are characterized by changes in temperature and pluviometry that have been accompanied by massive turnover in flora and fauna, but also in marine micro-organisms. As examples, the MPBE correlates with the appearance of many species of large benthic foraminifera lineages whose diversity peaked during the PETM, declined sharply, and peaked again during the EECO (Whidden and Jones 2012). The PETM also correlates with the appearance of many of the modern mammal lineages, despite them being phylogenetically distant (Gingerich 2006).

Also, the PETM is characterized by a humid climate, increased plant productivity and silicate weathering (Browen et al. 2004), a compound that is essential to most euglyphids to build their shell. The increase in dissolved silica, either due to increased rock weathering or the expansion of grassland during the Miocene, has been proposed as a contributing factor to the diversification of marine and freshwater diatoms (Cermeno et al. 2014, Kidder and Gierlowski-Kordesh 2005). Thus, the remobilization of silica during the PETM might have contributed to the diversification of euglyphids. Moreover, the diversification of a group or highly efficient silicifying soil protist may represent a significant contribution to the overall budget of Si supply to oceans from river systems. Thus, as silicate weathering is a major cause of climate cooling through the capture of atmospheric CO2 (Yang et al. 2020), euglyphids and other terrestrial self-silicifying organism could have played a role in the Earth’s cooling after the EECO.

Interestingly, the fact that most environmental clades (ECC-1, 2, 4, the stem Paulinellidae and the stem Cyphoderiidae) did not show an increase in their net diversification rate might support this interpretation. Potentially, these clades cannot be assigned to described taxa because they do not look like canonical euglyphids, i.e., they might not have a silica shell. As such they might not participate in the biogeochemical cycle of silicon and were not significantly impacted by an increase in dissolved silica. Several species of euglyphids that do not form silica shells have been recently described (Tarnawski and Lara 2015, Howe et al. 2011, Dumack et al. 2021). It must be noted that among those, *Micropyxidiella edaphonis* and *Ovulinata parva* are members of lineages in the stem Paulinellidae for which we retrieved a significantly lower diversification rate than the genus *Paulinella* whose species have a silica test.

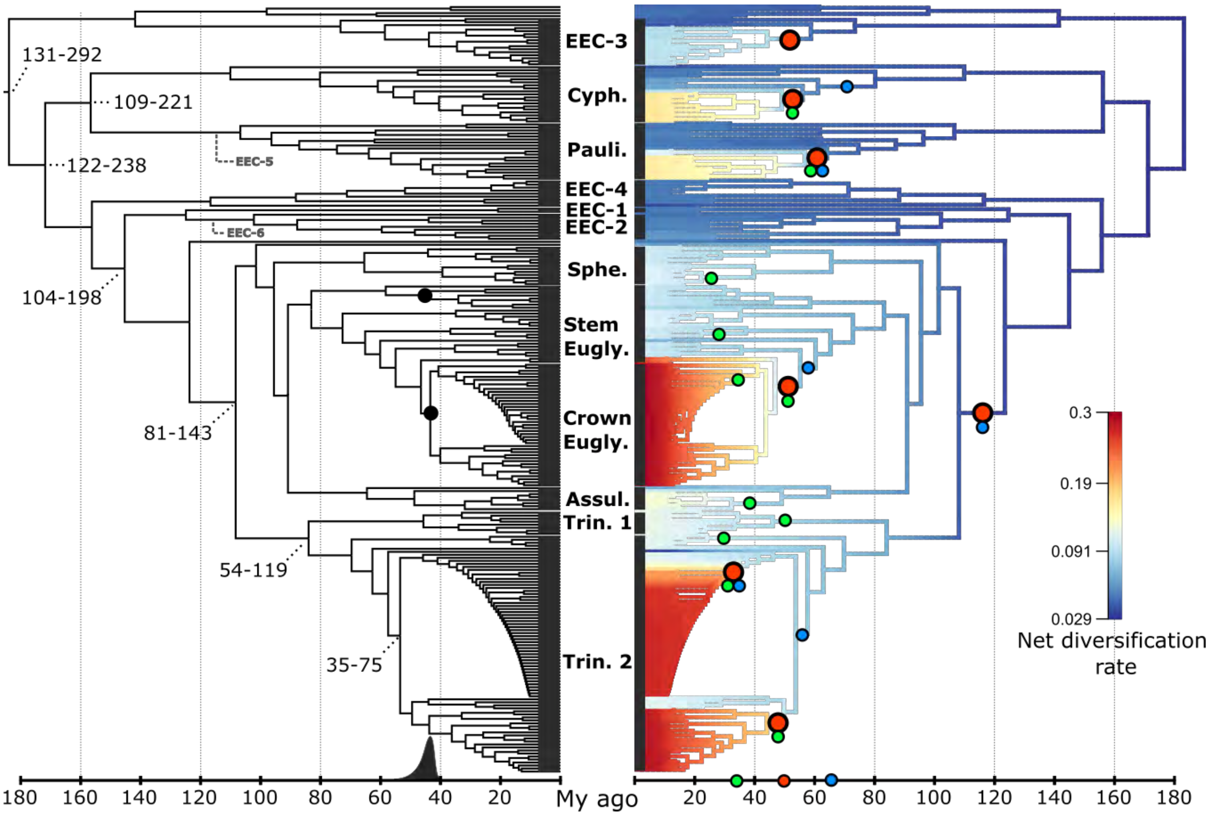

Figure 4: Time calibrated tree and diversification rate of the order Euglyphida after terminal branches younger than 10 MY were collapsed. Left: the 95% confidence interval of major splits are indicated next to the nodes; black dots indicate the nodes that were calibrated based on the Giraffe Pipe fossils, and the probability distribution used for the calibration is shown on the time axis; the positions of the new environmental clades, EEC-5 and EEC-6, are indicated on the tree. Right: The colours of the branches of the tree indicate the diversification rate retrieved by BAMM; the coloured dots on the tree indicate the location of the shifts in diversification rate, and the colour of the dot indicates the strategy used to define the clades in which the OTUs were summed; green dots are shifts retrieved by BAMM using clades defined based on a 35 MY threshold, red indicates a 50 MY threshold, and blue a 65 MY threshold. Each threshold is indicated on the time scale. The diversification rate indicated on the tree is based on the 50 MY threshold. The main taxonomic groups are indicated in the middle with EEC-1, 2, and 4 referring to the environmental clades defined by Lara et al. (2016), Cyph. to the Cyphoderiidae, Pauli. to the Paulinellidae, Sphe. to the Sphenoderiidae, Assul. to the Assulinidae, Eugly. to the Euglyphidae and Trin. referring to the Trinematidae.

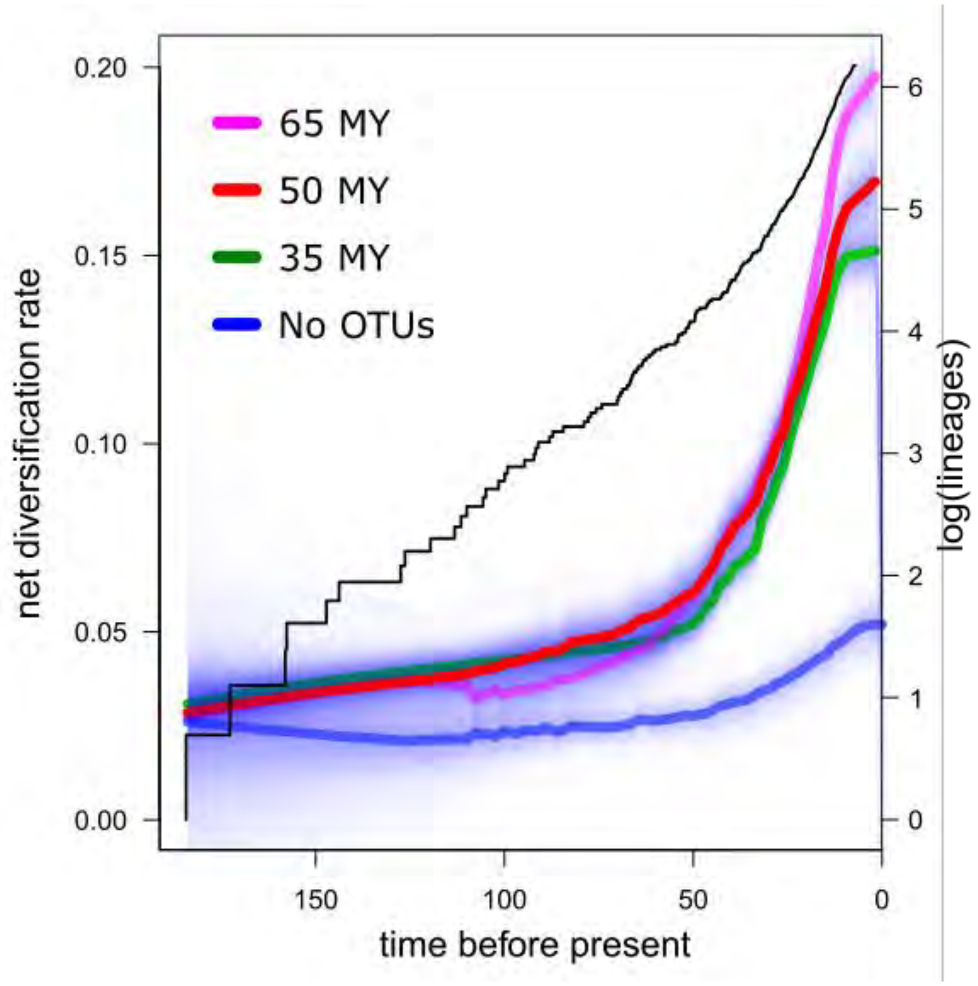

Figure 5: Lineage accumulation and net diversification rate through time of the order Euglyphida. The lineage accumulation through time is based on the topology of the non-pruned time-calibrated phylogenetic tree. The diversification rate through time is represented by the coloured lines, and the colours indicate the date of the threshold used to define the clades in which the OTUs were summed.

While a synchronous increase in the net diversification rate of several lineages can be explained by the exploitation of a new niche, it could also reflect a major extinction event. Rather than observing an increase in the net diversification rate, we might be observing a constant rate in the lineages that survived the extinction (Crisp and Cook 2009). We did however not find evidence for a mass extinction event that would have affected the euglyphids (data not shown), but the analyses were performed using only the reference alignment constituted of the long sequences, which accounts for only 480 taxa, whereas the full dataset including OTUs comprises 4’210 taxa. Possibly, in the same way the full dataset was needed to retrieve a clear signal in the diversification analyses, a phylogenetic tree built including the OTUs might be needed to find evidence for a mass extinction. However, such phylogenetic trees composed mostly of short sequences might not possess enough phylogenetic signals to yield accurate results and result in an inaccurate topology and estimate of the mutation rate, which could produce speciation events with erroneous dates.

Future work

While the age of split between the Paulinellidae and Euglyphidae is close to what was found by Berney and Pawloswki (2006), the 95% confidence interval is very large, spanning more than 100 MY. Other calibration points, in the form of minimum and maximum age constraints, are needed to offer a clearer picture of the radiation events within the Euglyphida. This would also help link them to one of the mass extinction or major climatic events that happened during this period.

The samples in which at least one OTU of euglyphid was found are highly imbalanced in terms of habitats, with freshwater samples (179) being far less represented than terrestrial (422) or marine samples (1’115). To account for this, it might be interesting to give more weight to the freshwater and terrestrial samples during the phylogenetic placement of the OTUs and reprocess the diversification analysis to check how this imbalance impacts the results. Nonetheless, as our analyses retrieved the diversification of clades from all habitats (terrestrial, freshwater and marine), it is unlikely that it will significantly impact the results.

Further sensitivity analyses should be performed to ensure that these results are not artifacts due to bias in the analyses. The internal nodes of the phylogenetic tree of Euglyphida are mostly unsupported in any of our phylogenetic trees, including the crown node of the genus *Euglypha*, and as such the topology of our tree might not correspond to the actual evolution of the euglyphids. The topology recovered in our phylogenetic reconstruction remains the one that best represents our reference alignment, but to fully evaluate the impact of the incertitude of the topology on the dating and diversification analyses, they should be performed on phylogenetic trees with distinct topologies. Additionally, it might be interesting to perform these analyses on a dataset excluding partially or entirely the long sequences of the *Trinema*-2 subclade in which, suspiciously, only few OTUs were placed and which is also much younger (37 MY) than the other group (64 to 49 MY) that radiated. Sequences from this subclade all come from a single study, and their diversity might be inflated by sequencing errors.

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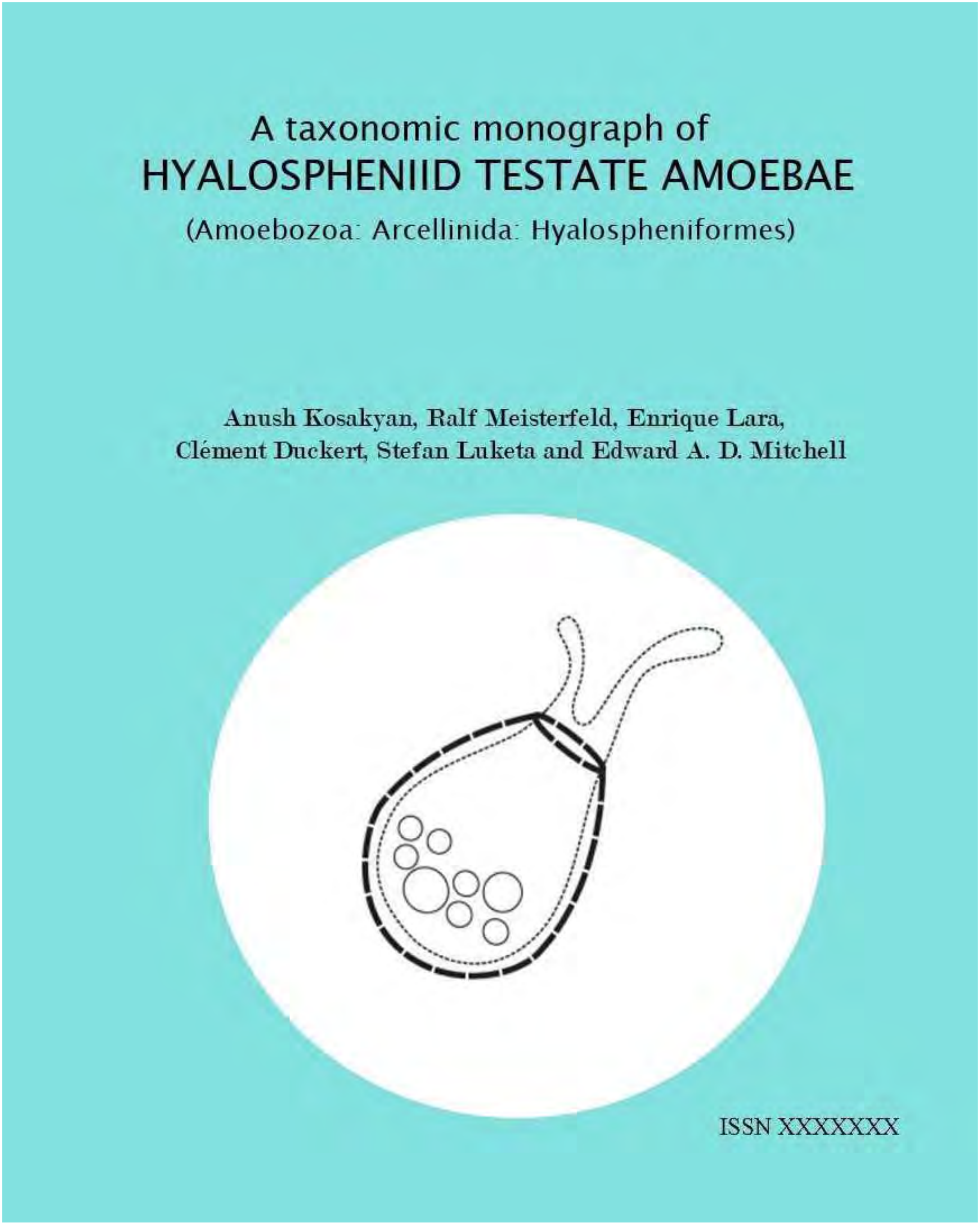

A taxonomic monograph of
HYALOSPHENIID TESTATE AMOEBAE
(Amoebozoa: Arcellinida: Hyalospheniformes)

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Clément Duckert, Stefan Luketa and Edward A. D. Mitchell

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ORIGINAL ARTICLE

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Dispersal limitations and historical factors determine the biogeography of specialized terrestrial protists

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Abstract

Recent studies show that soil eukaryotic diversity is immense and dominated by micro-organisms. However, it is unclear to what extent the processes that shape the distribution of diversity in plants and animals also apply to micro-organisms. Major diversification events in multicellular organisms have often been attributed to long-term climatic and geological processes, but the impact of such processes on protist diversity has received much less attention as their distribution has often been

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We would like to dedicate this paper to co-author Richard Payne. Richard was a member of a group of 8 climbers caught in an avalanche in the Himalayas at the end of May.

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The testate amoebae of New Zealand: A checklist, identification key and assessment of biogeographic patterns

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Abstract

New Zealand (NZ) is a well-known hotspot of biodiversity and endemism for macroscopic organisms, but its microbial diversity is comparatively poorly documented. We assembled all records on NZ testate amoebae published since the early 20th century and present a comprehensive taxonomic checklist for NZ. Testate amoebae are reported from six major habitat types across both the North and South Islands of NZ, but the sampling effort is ecologically and geographically biased in favour of wetlands and the South Island. As a result, 93% of all 128 morphotypes recorded in NZ occur in wetlands, 28% are restricted to the South Island, and diversity is greater at higher latitudes. Around 50% of morphotypes have a broad latitudinal distribution across the NZ mainland, whereas 15% have narrow latitudinal ranges. Future research should aim to broaden the geographical and ecological ranges. We predict that our list of NZ testate amoebae will expand substantially with future work, and that the latitudinal diversity gradient will be inverted. We also introduce an interactive, fully illustrated, online Lucid key for the rapid identification of NZ testate amoebae. As many morphospecies are cosmopolitan, this key provides a useful tool for testate amoebae identification in other parts of the world.

Keywords: Testate amoebae; New Zealand; Checklist; Identification key; Biogeography

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Introduction

Testate amoebae are a polyphyletic group belonging to three deeply divergent lineages: Amoebozoa (lobose testate amoebae) (Nikolaev et al., 2005) and the SAR clades (filose testate amoebae) Rhizaria (Bhattacharya et al., 1995; Dumack et al., 2016) and Stramenopiles (Gomaa et al., 2014). They represent one of the most diverse and abundant

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Phylogenetic divergence within the Arcellinida (Amoebozoa) is congruent with test size and metabolism type

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Abstract

Arcellinida (lobose testate amoebae) are abundant and diverse in many ecosystems, especially in moist to aquatic environments. Molecular phylogeny has shown that overall test morphology (e.g., spherical or elongate) is generally conserved in Arcellinida lineages, but the taxonomic value of other traits (e.g., size, ornamentation, mixotrophy/heterotrophy metabolism type) has not been systematically evaluated. Morphological and physiological traits that correspond to genetic differences likely represent adaptive traits of ecological significance. We combined high-resolution phylogenetics (NAD9-NAD7 genes) and advanced morphometrics to assess the phylogenetic signal of morphological traits of a group of elongate *Difflugia* species (Arcellinida). The phylogenetic analyses revealed two clades which could be reliably separated by test size and the presence/absence of mixotrophy. Differences in test size may reflect trophic level, with smaller organisms occupying lower trophic levels. In addition to having larger tests, elongate mixotrophic *Difflugia* are characterised by wide, flat bases and an inflation of the lower two thirds of their test. These morphological traits may provide additional volume for endosymbionts and/or increased surface area to aid light transmission. Our results showcase greater diversity within the elongate *Difflugia* and highlight morphological traits of ecological and evolutionary significance.

Keywords: *Difflugia*; Centroid size; Geometric morphometrics; Molecular barcoding; Morphometry; NADH gene complex

Introduction

Arcellinida Kent, 1880 are an order of testate (shelled) Amoebozoa, characterized by broad and thick (lobose) pseudopods used for feeding and locomotion. Like other testate amoebae they enclose their cell body within a test, the morphology and composition of which can be used to identify species (Meisterfeld et al. 2002). Several features make Arcellinida valuable ecological indicators: (1) they occur in great abundances in moist habitats, from forest soils to peatlands to lakes and even brackish waters (Mitchell et al. 2008; Ogden and Hedley 1980); (2) a relatively fast reproduction rate and a large scope of morphological plasticity

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Assessing the ecological value of small testate amoebae (<45 μm) in New Zealand peatlands

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Abstract

Methodological advances are essential for robust ecological research. Quantitative reconstructions of environmental conditions using testate amoebae rely on sound taxonomy. While the taxonomy of large species is relatively well resolved, this is not the case for most small taxa (typically <45 μm long). In New Zealand, peatlands contain a diversity of both cosmopolitan and characteristic large southern endemic taxa, but also have a high abundance of small taxa. The latter are often lumped into morphotypes reducing their value as ecological indicators. In this study, we demonstrate how (a) lumping small taxa versus splitting them into unique types, and (b) including or excluding them from community analysis influenced their ecological inference. We assessed testate amoeba composition in six peat bogs from New Zealand, three that were moderately-to-highly impacted, and three that were non-impacted. Environmental variables were measured at each sampling site and the surface testate amoeba community patterns and community-environment relationships compared. We found a clear division between impacted and non-impacted sites. Several distinct small taxa were more strongly related to water-table depth and conductivity, while the larger taxa were more correlated to pH. These results show that improved taxonomic resolution of small taxa can provide more informed environmental assessment.

Keywords: Bogs; Human impact; New Zealand; Taxonomy; Testate amoebae; Wetlands

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Introduction

Testate amoebae are a polyphyletic assemblage of free-living single-celled shelled eukaryotes (Mitchell et al., 2008). Molecular phylogenetic studies based on ribosomal RNA and protein gene sequences show that these organisms belong to the three supergroups Amoebozoa (Nikolaev et al., 2005), Stramenopiles (Gomaa et al., 2014), and Rhizaria (Bhattacharya et al., 1995; Dumack et al., 2016). They are highly diverse, ubiquitous in soil, litter, mosses, lakes, rivers and brackish water environments (Amesbury et al., 2017; Barnett et al., 2017; Charman, 1997; Charman et al., 2007; Fernández et al., 2015; Koenig et al., 2018; Royles et al., 2016; Swindles et al., 2015), and they represent one of the most abundant and diverse groups of terrestrial protists.

Testate amoebae are increasingly being used as models for microbial biogeography (Lara et al., 2016; Mazei et al., 2018; Smith et al., 2007), as indicators of ecological integrity

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ORIGINAL ARTICLE

Mycamoeba gemmipara nov. gen., nov. sp., the First Cultured Member of the Environmental Dermamoebidae Clade LKM74 and its Unusual Life Cycle

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Keywords
Budding; Discosea; eukaryotic diversity; fungus; high throughput sequencing; Longamoebia; ribosomal genes; serial dilution; yeast.

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ABSTRACT

Since the first environmental DNA surveys, entire groups of sequences called "environmental clades" did not have any cultured representative. LKM74 is an amoebozoan clade affiliated to Dermamoebidae, whose presence is pervasively reported in soil and freshwater. We obtained an isolate from soil that we assigned to LKM74 by molecular phylogeny, close related to freshwater clones. We described *Mycamoeba gemmipara* based on observations made with light- and transmission electron microscopy. It is an extremely small amoeba with typical lingulate shape. Unlike other Dermamoebidae, it lacked ornamentation on its cell membrane, and condensed chromatin formed characteristic patterns in the nucleus. *M. gemmipara* displayed a unique life cycle: trophozoites formed walled coccoid stages which grew through successive buddings and developed into branched structures holding cysts. These structures, measuring hundreds of micrometres, are built as the exclusive product of osmotrophic feeding. To demonstrate that *M. gemmipara* is a genuine soil inhabitant, we screened its presence in an environmental soil DNA diversity survey performed on an experimental setup where pig cadavers were left to decompose in soils to follow changes in eukaryotic communities. *Mycamoeba gemmipara* was present in all samples, although related reads were uncommon underneath the cadaver.

OUR vision of protist diversity has been radically challenged since the introduction of observation-independent environmental DNA surveys. Large and deep-branching groups of eukaryotes have been discovered with classical cloning/sequencing strategies, thus overtaking our estimations on eukaryotic environmental diversity. Unsuspected alveolate clades appeared to be extremely diverse in marine systems (López-García et al. 2001), and were found later to be exclusively composed by parasitoids (Guillou et al. 2008). Likewise, many new stramenopile lineages were discovered in the early 2000s in marine systems (Massana et al. 2004). These organisms, which include nowadays 25 different lineages spread all across the tree of stramenopiles, were found to be the most diverse and numerous bacterivores in the sunlit part of oceans (Massana et al. 2014), and thus vesting them with a prominent role in the oceanic microbial loop (Azam et al. 1983). In deeper waters, a particular group of excavates, the diplonemids revealed an immense diversity (Lara et al. 2009; Lukes et al. 2015). New massive sequencing technologies also revealed a large diversity in opisthokonts (del Campo et al. 2015).

Other systems like soils, however, have been by far not as deeply studied as the ocean. The high prevalence of fungal, plant, and metazoan sequences has been for a long time a major hindrance for studies on soil protist diversity (Lesaulnier et al. 2008). Still, previously unsuspected deep branching clades have also been discovered in soils, like the Opisthosporidia (also known as Rozellomycota) (Karpov et al. 2013; Lara et al. 2010). Recently, the development of high throughput sequencing has allowed obtaining high numbers of phylotypes, showing

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Protist taxonomic and functional diversity in soil, freshwater and marine ecosystems

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ABSTRACT

Protists dominate eukaryotic diversity and play key functional roles in all ecosystems, particularly by catalyzing carbon and nutrient cycling. To date, however, a comparative analysis of their taxonomic and functional diversity that compares the major ecosystems on Earth (soil, freshwater and marine systems) is missing. Here, we present a comparison of protist diversity based on standardized high throughput 18S rRNA gene sequencing of soil, freshwater and marine environmental DNA. Soil and freshwater protist communities were more similar to each other than to marine protist communities, with virtually no overlap of Operational Taxonomic Units (OTUs) between terrestrial and marine habitats. Soil protists showed higher γ diversity than aquatic samples. Differences in taxonomic composition of the communities led to changes in a functional diversity among ecosystems, as expressed in relative abundance of consumers, phototrophs and parasites. Phototrophs (eukaryotic algae) dominated freshwater systems (49% of the sequences) and consumers soil and marine ecosystems (59% and 48%, respectively). The individual functional groups were composed of ecosystem - specific taxonomic groups.

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Deconstructing *Difflugia*: The tangled evolution of lobose testate amoebae shells (Amoebozoa: Arcellinida) illustrates the importance of convergent evolution in protist phylogeny

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A B S T R A C T

Protists, the micro-eukaryotes that are neither plants, animals nor fungi build up the greatest part of eukaryotic diversity on Earth. Yet, their evolutionary histories and patterns are still mostly ignored, and their complexity overlooked. Protists are often assumed to keep stable morphologies for long periods of time (morphological stasis). In this work, we test this paradigm taking Arcellinida testate amoebae as a model. We build a taxon-rich phylogeny based on two mitochondrial (COI and NADH) and one nuclear (SSU) gene, and reconstruct morphological evolution among clades. In addition, we prove the existence of mitochondrial mRNA editing for the COI gene. The trees show a lack of conservatism of shell outlines within the main clades, as well as a widespread occurrence of morphological convergences between far-related taxa. Our results refute, therefore, a widespread morphological stasis, which may be an artefact resulting from low taxon coverage. As a corollary, we also revise the groups systematics, notably by emending the large and highly polyphyletic genus *Difflugia*. These results lead, amongst others, to the erection of a new infraorder Cylindrothecina, as well as two new genera *Cylindrifflugia* and *Golemanskia*.

1. Introduction

Dobzhansky's famous sentence (Dobzhansky, 1973) "Nothing in Biology Makes Sense Except in the Light of Evolution" reflects the importance of understanding the evolutionary history of organisms to make inferences on their biology. These evolutionary patterns can be inferred by unravelling the phylogenetic relationships between groups, based on the comparative analysis of independent homologous characters (De Luca et al., 2019; Gorospe et al., 2020; Peters et al., 2017; Zumel et al., 2021). Furthermore, the effect of selective pressures may lead to predictable patterns along these phylogenies. For instance, positive selection may favour a particular trait in a specific environment, typically leading to homoplastic evolution of similar morphological adaptations in far-related organisms across the phylogenetic tree (Losos et al., 1998; Ruedi and Mayer, 2001). On the other hand, stabilizing selection may promote the conservation of a morphological phenotype for long periods of evolutionary time (stasis) (Muñoz et al., 2014; Szudarek-Trepto et al., 2021). The effects of these evolutionary forces has been largely observed in the field and experimentally tested in the laboratory, mostly on large organisms such as plants and animals (Blount et al., 2018). However, most of eukaryotic diversity is of microscopic size and classified as protists (Adl et al., 2019); for this reason, protists must imperatively be taken into account in any generalization patterns about the evolution of eukaryotic life.

Abbreviations: COI, Cytochrome Oxidase subunit I; NADH, nicotinamide adenine dinucleotide dehydrogenase; SSU, SSU rRNA gene.

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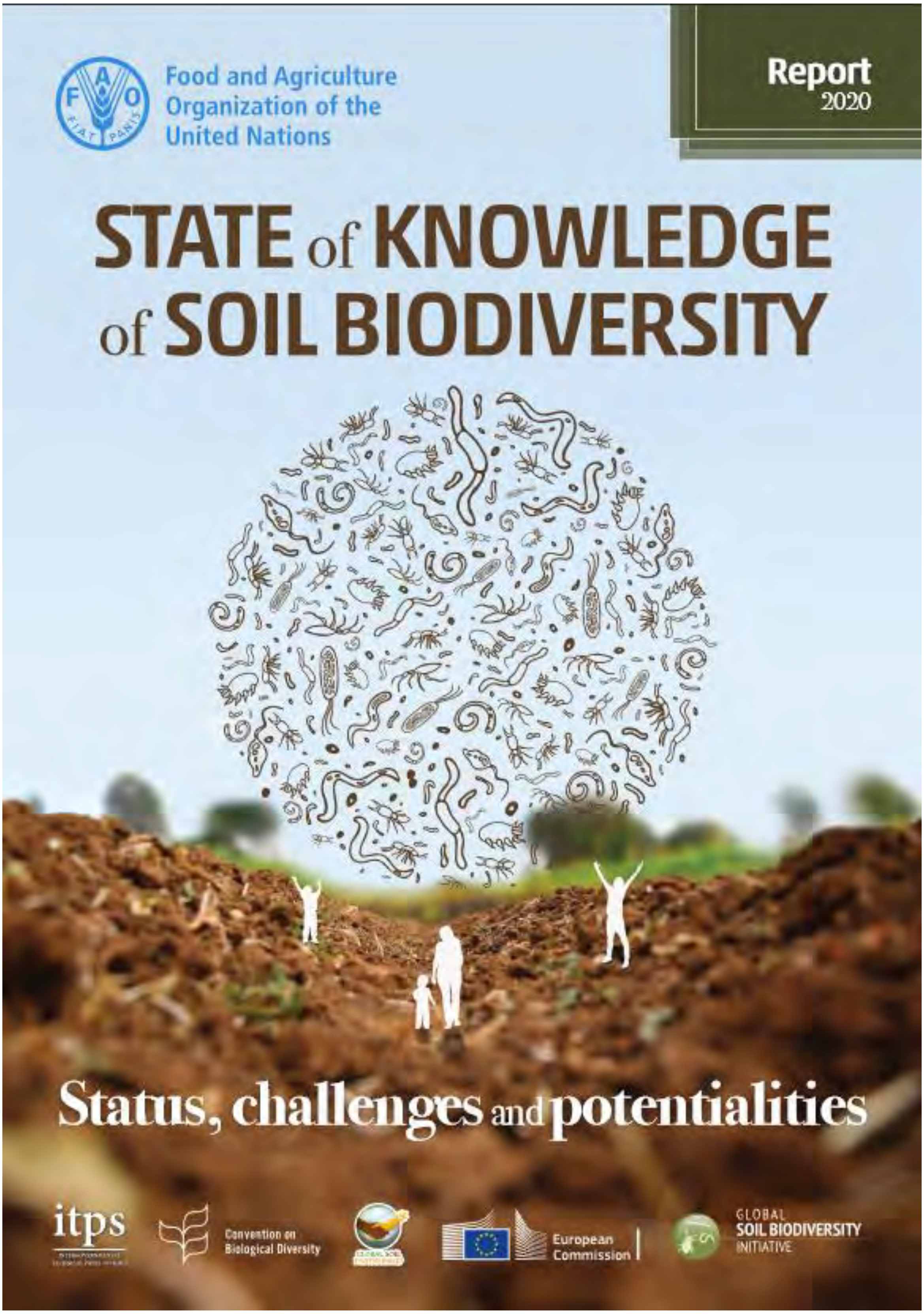

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Chapter 11 – Discussion

Rather than discussing the results of my chapters, I chose to use the experience I acquired during my work to discuss more general matters that presented themselves in my chapters or appendices and are interesting to bring up. In this optic, I will discuss the role of taxonomists towards end-users and the need for taxonomic revisions, parataxonomy in protistology, and taxa delineation and nomenclature issues.

Towards taxonomic consistency

Reliable data, reproducibility and comparability between studies are essential to ensure sound results, but also for the advancement of science as a whole (Gotfryd and Hansell 1985). As such taxonomic consistency (i.e., the consistency of taxon identification), and by extension taxonomic work, should be a pre-requisite in biodiversity science. However, lacunar (incomplete) and bad (misapplied) taxonomy are still major impediments, with identification errors being one of the main sources of variability in ecological studies, be they focused on macro-organisms (see Scott and Hallam 2002 for an example on vegetation) or protists. However, this issue has bigger implications for protists: in studies using diatoms (arguably one of the most studied groups of protists) to assess the quality of freshwaters, identification errors were the main source of variability, accounting for up to 25% of the variation of the final quality index (Prygiel et al. 2002). There are at least two factors that explain taxonomic inconsistency, the first being rooted in the perception of taxonomy by the end-users (mostly ecologists) and other scientists, the other being rooted in the state of the taxonomy itself.

The first factor is that good taxonomy (in the sense of correctly identifying taxa) is not valued, and most ecological studies, even those focused on macro-organisms, do not include taxonomists, reference any identification keys, or present proofs of correct taxonomic identifications (Bortolus 2008). Furthermore, taxonomic work is often criticized as only descriptive or even non-scientific and fails to attract citations and fundings, which limits the resources available to improve taxonomic knowledge (Ebach et al. 2011). Unfortunately, this is also the case in protistology, where taxonomy is sometimes considered more as an annoyance than a necessary tool (based on the accumulation of personal anecdotes). A quick look into the original publications used by Amesbury et al. (2016), a major meta-study on the usage of testate amoebae in peatland paleoecology, yields findings similar to those of Bortolus (2008): most of these publications do cite identification keys or taxonomic literature, but they are however mostly inadequate, and only a few show pictures of at least some taxa, and none provide pictures for every taxon. These “taxonomic impediments” have already been reviewed extensively elsewhere (see Ebach et al. 2011) and are out of the scope of this discussion, however they have a direct impact on the current state of protist taxonomy and should be acknowledged.

The second factor explaining taxonomic inconsistency is the deficient state of the taxonomy of soil protists. The issues discussed here are relevant to any poorly studied taxonomic groups (Wiliams et al. 2007), however due to their microscopic size and often lack of recognizable morphological traits, these issues are likely to impact more the taxonomy of soil protists. The re-investigation of a species that was described before the appearance of modern tools (e.g., electron microscopy and DNA sequencing) will often reveal it to be an aggregate of distinct species. While some older descriptions show clear incompetence (Thessen et al. 2012), their general lack of precision mostly reflects the technical limitations of the time, and important morphological features of protists were often invisible to even the most diligent taxonomists. As a result, the taxonomy of many protist groups is still in its infancy despite being studied for centuries, and it is only now that we are starting to grasp the vastness of their diversity. Thus, the majority of species still remains to be described, even in well-studied taxonomic groups. Moreover, a lot of the old and inadequate taxonomic literature has not been revised and still serves as references in ecological studies. It then should not come as a surprise that protists are frequently misidentified even by experts.

There are several actions that should be taken to address taxonomic inconsistency. Obviously, the description of new species is necessary to present a more accurate picture of the diversity. But also, it is necessary to better understand the range of inter- and intraspecific variation and to identify the traits onto which species description and identification should be based. This work can also be greatly facilitated by new molecular tools. Thanks to environmental sequencing, we now know where to look to find taxonomic novelty and how much is missing from species catalogues. Already, many clades are known only from environmental sequences and await a formal description (Pawlowski 2013). Furthermore, with Fluorescence In Situ Hybridization (FISH) we have the means to find a species in a sample based on its DNA barcode. As an example, in the order Euglyphida, this approach led to the discovery of *Micropyxidiella edaphonis*, a terrestrial member of the Paulinellidae which previously only included marine or freshwater species (Tarnawski and Lara 2015).

While the description of new species is necessary, taxonomic work must also include the revision of older taxa as without a curation of the taxonomy, new species will be merely added on top of a shaky base. Taxonomic revisions are essential in protistology, as many old descriptions are too vague to offer more information than a general outline, and the generalized absence of type material prevents any clarification of the identity of the targeted taxon (Patterson and Larsen 1992). Furthermore, the sanitation of lists of species names has a practical utility as it is also beneficiary to the end-users by removing names that are ambiguous and thus cannot be associated with reliable data (Larsen and Patterson 1990). However, taxonomic revisions and the invalidation of older species are rarely done, possibly because it feels like it is not worth the effort needed to deal with obscure nomenclatural rules and the requirement to thoroughly investigate a hardly accessible literature, leading old names to still pollute databases (Boscaro et al. 2014). Also, this work is often not rewarded as it is considered as having poor citation potential and is mostly relegated to low impact factor journals (Ebach et al. 2011, Cotterill and Foissner 2009). During our taxonomic revision of the genus *Euglypha*, we followed a simple moto to decide the validity of a taxon: “Taxa based on descriptions that do not refer to the discriminatory characteristics currently used to distinguish species cannot acquire an unambiguous identity” (Larsen and Patterson 1990). Nonetheless, rather than invalidating dubious taxa, our first idea was to designate neotypes at least for the well-known names that often appear in the ecological literature (such as *Euglypha ciliata* or *E. rotunda*) to settle their identity, as in my opinion, well-known names should be kept (see Duckert et al. 2018 and Duckert et al. 2020 on *Alabasta* (*Nebela*) *militaris*). However, we decided against this idea when we realised that the new, arbitrary identities behind those names would have to compete with often more than a century of taxonomic records, which was unlikely to succeed. Furthermore, settling the identity of a name does not eliminate all the erroneous ecological and biogeographic data that will still be associated with that name. Thus, because any attempts to salvage those names would have caused more confusion than benefits, we chose to invalidate every taxon that did not follow our simple criterion, resulting in the validation of only eight names pertaining to *Euglypha* out of 170 (including synonyms and dubious affiliation). While this is a massive shake up of the taxonomy of this genus, this will benefit both taxonomists and ecologists, as future taxonomic work can now build upon a sound base and ecological data can be assigned more reliably to these organisms.

Finally, it is important to tackle bad taxonomy by preventing its appearance and its propagation when encountered. An easy solution is to make it mandatory that publications based on species composition be accompanied by proofs of correct identification such as pictures or vouchers, and to demand that a trained taxonomist be consulted (Bortolus 2008, Kercher et al. 2003). Furthermore, when taxonomic errors are encountered, they should be corrected even if they do not impact the findings of the publication (the challenge being to not make it look like taxonomic pedantry, see Duckert 2020 and Bian et al. 2020). Also, identification and taxonomic assignation depend on the resources available (Kociolek and Spaulding 2000), and thus, taxonomic inconsistency and bad taxonomy will persist for as long as we lack an easily accessible, centralized, and user-friendly taxonomic literature (Ebach et al. 2011). As such, the online identification keys that are currently being developed for several taxonomic groups (see diatoms.org on Northern America diatoms, and arcella.nl on testate amoebae) are thus invaluable and might be the most important taxonomic resources available for the future. Also, phylogeny-based taxonomy is currently changing the name and affiliation of many species, and online taxonomic resources have the benefit of being easily updatable and can reflect the advances in taxonomy faster than paper publications.

Parataxonomy for the end user

To be fair, a third cause of taxonomic inconsistency must be invoked: it is especially difficult to identify protists species. Due to the state of the taxonomy and the general difficulty to identify them, protists are rarely identified to the species level in routine work and are instead treated into broad morphotypes (see Amaro et al. 2015, Anderson et al. 2013 and Nolte et al. 2010 as examples). Lumping organisms into morphotypes unavoidably bias estimate of their diversity, as species can be split or lumped together depending on their morphological variability (Krell 2004). This can also impact taxonomic groups unevenly, depending on their species richness, the soundness of their taxonomy, and how easy it is to discriminate their species (Derraik et al. 2002, Abadie et al. 2008). However, this parataxonomic classification, here in the sense of lumping morphologically similar organisms into morphotypes regardless of taxonomy (Oliver and Beattie 1993), is useful in protistology, and possibly even needed, because most species could likely not be correctly identified even if their taxonomy was solved. Furthermore, there is most of the times no right answer when it comes to species identification, as most species are still to be described (Pawlowski et al. 2012). Also working within a parataxonomic framework presents the advantage of yielding much more data because a precise taxonomic identification (and thus taxonomic expertise) is not required and can be done faster by more people (Krell 2004), which can be precious when resources are limited (Derraik et al. 2002). However, the parataxonomy of protists is unregulated, which in some cases can lead to the same problems as a lacunar taxonomy: confusion, consistency issues and low comparability between studies because how morphotypes and their names should be formed are not well defined.

Furthermore, this usage of parataxonomy is often not acknowledged in protistology, as broad morphotypes are often referred to as “species” (and not “morphospecies” or, more accurately, “morphotypes”) even if they clearly include organisms that are morphologically, ecologically, or genetically too divergent to constitute a single species. Also, protists are increasingly shown to have limited geographical distribution, and as such it is likely that a species reported from distant locations, even on the same continent (Singer et al. 2019), is an aggregate rather than a true species. This blurs the limit between taxonomy (i.e., working with curated taxa) and parataxonomy (i.e., working with broad morphotypes) as it is often not clear if a name is used to refer to an actual taxon or an aggregate of vaguely similar organisms that have been force-fitted under the same name. It must also be noted that this usage of parataxonomy is fundamentally different from taxonomy based on a morphological species concept (i.e., a “morphospecies”) or low-resolution taxonomy, as both treats taxa that are based on objective criteria while parataxonomy is subjective - I would even argue that the term “morphospecies” is misleading and should not be used: either a species based on morphology fits the current standards to be considered as valid (and is thus rightly a “species”), or it does not and is only a collection of morphologically similar but distinct organisms ("a morphotype"); this might be considered as only a matter of semantics, but isn't taxonomy just semantics applied to organisms?

In the case of the usage of testate amoebae in peatland ecology, it is often impossible to work at the species level because most species are not described, those that were have been poorly so (most older "species" should be considered as mere morphotypes until proven otherwise), the limits between species are not clear, and the identification literature is mostly old, scattered, and hardly reflects our current understanding of the diversity of testate amoebae (see the introduction to this thesis, Chapter 1, 2, 3 and 8). Because actual taxonomy is too incomplete and can be consulted only with great difficulty, parataxonomy *de facto* is the system of classification of testate amoebae in routine work, as ecologists must sort the morphological variation of the organisms encountered in the field into broad morphotypes (revealed by the terms "type" or "cf.") rather than well-defined taxa. However, given the lack of agreed upon standards this approach suffers from several shortcomings with clear downsides. While the bigger and more conspicuous morphotypes likely show more consistency between studies, the smaller morphotypes and those that present only few morphological traits can be treated haphazardly.

As a concrete example the name *Euglypha rotunda* (invalidated in Chapter 8) has been shown to hide several lineages that are morphologically distinct and are not monophyletic, and thus should be considered as an artificial group (Wylezich et al. 2002) - in our revision of the genus *Euglypha*, we could even assign more than 20 distinct species to this name. Furthermore, no identification key gives a reliable description of this morphotype. As such, when *E. rotunda* (or *E.* cf. *rotunda* or *E. rotunda*-type) appears in ecological relevés it should be interpreted in terms of general morphological traits (*Euglypha* spp., test small to medium, no spines, (see Charman et al. 2000)) rather than as a real taxon. However, this definition overlaps completely with that of *Euglypha laevis* (invalidated) and partially with that of *E. tuberculata* (invalidated) which is roughly defined as a large *E. rotunda* (see Charman et al. 2000), and which of these names will be reported depends more on personal preferences than objective criteria. Because these names are not associated with a clear description and their usage is not consistent, this clearly hinders the comparability between studies (see Amesbury et al. 2016, a meta-analysis where the *Euglypha* taxa were split into two groups in a process that appears random). While this example was chosen because I am more familiar with testate amoebae, these issues involve most protist taxa, albeit to varying degrees. Lastly, as the reproducibility of the results within and across observers cannot be guaranteed without a clear description of each morphotype, this approach can be criticized as being non-scientific (Krell 2004, Abadie et al. 2008, Vink et al. 2012).

Nonetheless despite these shortcomings, the usage of broad morphotypes reveals the inadequacy of the current taxonomy and the need for a user-friendly method to sort the morphological variation of the organisms encountered on the field. Identification of protists to the species level cannot be reasonably expected most of the time, even in well documented groups such as diatoms (Williams 2020, Cantonati et al. 2018) and ciliates (Lynn 2008), and might not be needed (see Beattie and Oliver 1994, and Pik et al. 2009 where taxonomic sufficiency is discussed). Moreover, the current taxonomic efforts pale in comparison to the diversity of protists yet to be described, and even if all species were reliably described, it would take a long time before they can be associated with reliable ecological data. We thus need a curated artificial classification that defines ecologically consistent units, with several level of resolution, that is adapted for the end-user, and whose nomenclature is regulated. Broad morphotypes should be defined, with the help of taxonomists, based on easily observable morphological traits (i.e., visible in optic microscopy in routine work) that do not let room for much interpretation. Their nomenclature should be regulated to ensure comparability between studies and in a way that makes it clear that they refer to morphotypes, and not real taxa. We give an example in our revision of the genus *Euglypha* in which we split it into sections that were defined with precise morphological traits (i.e., their identification should not rely on personal interpretation) and can be easily identified. Furthermore, while these sections were named based on well-known (but invalid) morphotypes, they clearly do not refer to actual species, avoiding any possible confusion.

This approach can be seen as intermediate between focusing on taxonomy and using a trait-based approach, as the sections were based on discrete morphological traits. A “trait-based” approach is based on the principle that species present traits that are adapted to the habitat, and thus these “functional traits” might be ecologically relevant and as informative as the community composition to infer environmental conditions (Fournier et al. 2012). Functional traits of testate amoebae are increasingly used in peatlands ecology, as they are less prone to observation errors and taxonomic bias (Marcisz et al. 2020) and can offer a better insight into environmental processes than community composition, as the functional traits are a direct response to an ecological factor (Marcisz et al. 2016). The hope is that the gain in comparability and consistency between studies that this system bring might render euglyphids relevant in ecological studies using testate amoebae as bioindicators. However, the soundness (i.e., the species vs. morphotypes relation) of this approach will have to be tested when more taxonomic data are available. In a similar way, diatoms analysts already use “voucher floras”, collections of specimen pictures grouped into morphological Operational Taxonomic Units (mOTUs), to serve as a basis for identification within or between studies (Bishop et al. 2017). These voucher floras are done beforehand by experts based on samples from the region that will be the focus of the study and are then handled to analysts for the routine work, and only at the end of study are mOTUs assigned taxonomic names (if possible). Voucher floras have the advantage of being transparent records of the study (Alers-Garcia et al. 2021) and have been shown to significantly reduce analysts bias and to increase comparability between studies (Tyree et al. 2020). Also, this approach prevents force-fitting ambiguous specimens to a name (Alers-Garcia et al. 2021), which in turn greatly limits the pollution of databases with unreliable observations.

Delimiting taxa based on morphology, molecules… and nomenclature.

Delineating species remains one of the biggest issues in the taxonomy of protists, as it is often not clear how to interpret the morphological or genetical variations (i.e., what traits should be considered and how much they should differ). Despite the many species concepts that have been proposed, none has been universally accepted to delineate protists, and the best candidate (the biological species concept) was often rejected because most protists are presumed to not reproduce sexually or their reproductive isolation cannot be practically tested. Here we basically applied the “pragmatical species concept”, where species are defined as “*clusters of organisms which passed a threshold of evolutionary divergence*” (Seifert 2014) and looked at two types of evidence (morphology and molecules) to define this threshold. In Chapter 8, the combination of morphological and molecular data showed that the intraspecific morphological variability was low and that morphological differences were generally accompanied by genetic variation, allowing us to define the required criteria to delineate species. Furthermore, defining the baseline of intraspecific variation allowed establishing that clear morphological differences strongly signal distinct species, meaning that species (at least for the genus *Euglypha*) can be described based on morphology alone if the criteria are met. This approach nonetheless demands that each clade be studied separately, has the traits that should be observed and the required threshold have to be re-evaluated between taxonomic groups.

While we have shown that species *can* be delineated based on morphology alone (i.e., if the baseline of intraspecific morphological variation has been established), I would advocate against splitting higher ranks without extensive molecular data. Splitting genera based on morphology alone is very likely to render the original genus polyphyletic or paraphyletic, requiring either to define many more genera (Kociolek and Williams 2015) or to later invalidate the new genus, creating taxonomical and nomenclatural issues that must be addressed later. During this thesis, we encountered the issue twice. First, when we wanted to erect a new genus to accommodate *Apodera angatakere* but resisted when we understood that this would create a nomenclatural nightmare if more species of *Apodera* where to be described (which is unavoidable), likely requiring to erect many monospecific genera. Second, when we invalidated the genus *Scutiglypha*, as keeping it would have required splitting the genus *Euglypha* in at least three genera (which is impractical). It was also in part for this reason that we decided to treat *Hyalosphenia papilio paynei* as a subspecies, as describing it as a species would have required to split *H. papilio* in at least four distinct species that would have to be named. Recently, several genera of testate amoebae were invalidated because they were based on morphological traits that might be relevant to discriminate species but not for higher-rank taxonomy (e.g., *Mediolus* (see Gomaa et al. 2017), *Antarcella* (see Gonzales-Miguens 2022), *Scutiglypha* (Chapter 8)) or because of nomenclature issues (*Armipyxis* (see arcella.nl by Siemensma 2022)). This is problematic for the end-user as well, as it leads to names that (albeit temporarily) competes with the previously prevailing appellation of a taxon and participates to the inflation of available names, adding confusion where there is already plenty.

Conclusion

During this thesis I aimed to review and improve the taxonomy of protists to allow using them reliably in research, using testate amoebae as a model group. Thanks to their conspicuous shell, testate amoebae are excellent model organisms. Focusing on this group to explore the taxonomic history and the diversity patterns of soil protists is basically playing with easy mode activated and allowed focusing on the research itself rather than dealing with the technical difficulties that comes with studying other protists. I covered, in my opinion, all the necessary steps to improve the taxonomy of protists and demonstrated how the by-products of taxonomy (e.g., taxa delineation, phylogenetic reconstruction and definition of synapomorphies) can be used to research the diversity and evolution of protists. More precisely, I re-evaluated the taxonomy and emended the description of single species using DNA barcoding and phylogenetic reconstruction, addressed their taxonomic history and nomenclature conundrum, described new taxa, revised entirely a family, redefined the taxonomic framework that should direct future work and proposed a lower, user-friendly taxonomic resolution for routine screening, tackled the propagation of incorrect taxonomy and used by-products of taxonomy combined with HTS or fossil data to assess the diversity and evolution patterns of protists. The taxonomy of testate amoebae and many other protists is currently at the crossroad between lacunar (i.e., incomplete) and bad (i.e., plain wrong) taxonomy, but we currently have the tools and the knowledge to address this. And one day it will stop looking like an episode of *Whose line is it anyway?* where everything’s made up and the names don’t matter.

References for the Introduction and Discussion

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