On the Biology, Diversity and Evolution of Nucleariid Amoebae (Amorphea, Obazoa, Opisthokonta

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On the Biology, Diversity and Evolution of Nucleariid Amoebae (Amorphea, Obazoa, Opisthokonta[1]

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Nucleariids are a small group of free-living heterotrophic amoebae. Although these organisms present a variety of cell sizes and cell coverings, they are mostly spherical cells with radiating filopodia, sometimes with several nuclei. *Nuclearia*, the genus that gives the name to the group, contains species that are opportunistic consumers of detritus, bacteria, and algae. The beautiful *Pompholyxophrys* is covered with endogenous siliceous pearls. *Lithocolla* covers itself with sand particles, or otherwise diatom frustules. The tiny *Parvularia* exclusively feeds on bacteria, and *Fonticula* is adapted to solid substrates and presents aggregative multicellular stages. Nucleariids belong to the Opisthokonta, which comprise animals, fungi, and their protist relatives, and form the earliest branch in the holomycotan clade (fungi and closest relatives). Hence, they are key for understanding the origin and diversification of Opisthokonta, an eukaryotic supergroup that contains organisms with different feeding modes, life-styles, and cell organizations. In this review, the reader will find an introduction to nucleariids, from their discovery in the 19th century until the most recent studies. It summarizes available information on their morphology, life history, cell organisation, ecology, diversity, systematics and evolution.

Key words: Amoeba; cristidiscoid; nucleariid; opisthokont; protist; rotosphaerid.

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Introduction

Nucleariids, formally known as Rotosphaerida (Rainer 1968), Nucleariidae (Cann and Page 1979; Patterson et al. 2000), or Cristidiscoidea (Page 1987), are non-flagellated, spherical or flat amoebae, with conspicuous nuclei and radiating filopodia (Galindo et al. 2019). Some are naked or only have a mucous coat. Others are covered with endogenous silica-based particles (idiosomes) or with exogenous particles (xenosomes). They are free-living (i.e., non-parasitic), phagotrophic organisms that thrive in water bodies around the world, mostly in freshwater, but also in marine settings, and even faeces. Depending on the species, they feed on unicellular small bacteria, large coccoid and filamentous cyanobacteria, or eukaryotic unicellular algae (such as diatoms).

This amoeboid lineage is important for understanding the evolution of the Opisthokonta supergroup (Adl et al. 2019). In particular, they are the earliest-branch in the Holomycota/Nucletmycea clade (Brown et al. 2009; Liu et al. 2009) that includes fungi, aphelids (Letcher and Powell 2019; Torruella et al. 2018), rozellids (Letcher and Powell 2018), and microsporidians (Bass et al. 2018) (see Fig. 1). At the same time, Holomycota are the sister lineage to Holozoa: animals and their relatives including choanoflagellates, filastereans, and ichthyosporeans (Ros-Rocher et al. 2021). Therefore, it is fundamental to include nucleariids in evolutionary studies dedicated to understanding any evolutionary event in Opisthokonta: e.g., the origin of multicellularity in fungi (Nagy et al. 2018) and animals (Ros-Rocher et al. 2021), the origin of osmotrophy in fungi (Richards and Talbot 2013) and teretosporeans (Torruella et al. 2015), or the symbiotic co-evolution of aquatic fungi and green algae that gave rise to terrestrial fungi and land plants (Magallón et al. 2018; Naranjo-Ortiz and Gabaldón 2019).

Nucleariids were discovered more than 150 years ago (Cienkowski 1865) but remain a poorly known lineage, probably because they do not impact human health, agriculture, or animal husbandry. However, the algivorous nature of some nucleariids may make them a relevant factor in the context of large-scale microalgae and cyanobacteria production, and can be of interest for environmental algal bloom bioremediation (Dirren et al. 2017).

Currently, five genera of filose amoebae are considered *bona fide* nucleariids. These are the uncovered *Nuclearia*, *Fonticula* and *Parvularia*, and the covered *Lithocolla* (with xenosomes) and *Pompholyxophrys* (with idiosomes) (Fig. 1, Table 1). At least another six morphologically described genera of filose amoebae are suspected to be nucleariids and await molecular confirmation: the *incertae sedis Vampyrellidium*, *Elaeorhanis*, *Pinaciophora*, *Rabdiophrys*, *Rabdiaster* and *Thomseniophora* (Table 2).

The identification, distinction and classification of amoebae has always been a difficult task because of their small cell size, their typically polymorphic nature, and the limited number of discriminating morphological characters (Patterson et al. 2000; Smirnov and Brown 2004). Amoeboid stages in the life history are common throughout eukaryotes, adding further complexity to the identification through morphology. Therefore, in the past, nucleariid amoebae have been connected to other rhizopods and heliozoan-like lineages, such as vampyrellids (Rhizaria; (Hess and Suthaus 2022)), radiolarians (Rhizaria; (Burki et al. 2016)), actinophryids (Stramenopiles; (Nikolaev et al. 2004)) and centrohelids (Haptista; (Burki et al. 2016)). Nevertheless, as in other protist groups, there have been substantial advances during the past 30 years. Thanks to electron microscopy and molecular sequencing, from phylogenies of the small ribosomal subunit RNA gene (SSU rRNA gene or 18S) up to concatenated phylogenomic analyses, researchers have started to clarify the systematics of these organisms. In the following sections we provide an overview of the biology of nucleariid amoebae. For further details, see the original sources cited below, as well as the following online valuable sources of images and descriptions: Microworld (F. Siemensma), Penard Laboratory (E. Völcker), Proyecto agua (A. Guillén), Protist Information Server (Y. Tsukii), and The world of Protozoa (M. Kawachi).

Morphology and Life History

Nucleariids are relatively simple cells compared to many other microbial eukaryotes. They are roundish, more or less vacuolated, with radiating filopodia, smooth plasma membrane and most species are embedded in some kind of mucous coat, with or without coverings (Figs 2, 3). Also, most species have a resting cyst during their life history, consisting of a round smooth cell covered by one or more layers of a thick, translucent material (Fig. 2C, D, 3D, J). They lack cytoplasmic microtubules, flagella (and the corresponding flagellar genetic toolkit (Galindo et al. 2021; Torruella et al. 2015)), any group-specific organelles, and extrusomes (membrane-bound structures to specifically secrete contents out of the cell).

Most nucleariid amoebae present simple life histories, as compared to many other opisthokont lineages with clearly distinct life stages (Dayel et al. 2011; Powell and Letcher 2018; Ros-Rocher et al. 2021; Torruella et al. 2018). The particularities of each species hamper generalisations, and therefore we describe each *bona fide* nucleariid genus in the following paragraphs. However, all of them are filose single cells, with one or few nuclei, that slowly feed and grow. Although it is expected that they are haplontic like most other opisthokonts, sexual reproduction, meiosis or changes of ploidy have not been studied. Most species divide by binary fission and some can encyst.

Species from the highly polymorphic genus *Nuclearia* (Fig. 2A-F) are the most often studied nucleariids. For a summary of the distinct forms of *Nuclearia*, see Figure 4. They are round when floating or crawling (from ~10 to ~60 μm in diameter), and flattened to elongated when attached to substrates (reaching ~100 μm), like when slowly grazing on filamentous cyanobacteria (Artari 1889; Blanc-Brude et al. 1955; Dirren et al. 2017; Galindo et al. 2019). It has been proposed that the spherical, floating cells with radiating filopodia could regulate their buoyancy (Dirren et al. 2017). Their colour also depends on their food consumption, presenting colourless, or green to orange digestive vacuoles, and even red lipid globules after the consumption of purple bacteria (Fig. 2B). Cells with two or more nuclei are commonly observed, especially after being fed (Fig. 2C, F). Multinucleated cells, as syncytia originated by multiple cells fusing (see figure S1 from Dirren et al. (2014)); or as coenocytes resulting from karyokinesis without cytokinesis (Fig. 2C, F and figure S3C-D in Galindo et al. (2019)) have been reported. Most species are often covered with a mucous coat, that can contain bacteria. Also, different strains from the same species can form cysts (Dirren and Posch 2016) or not (Yoshida et al. 2009) (see examples at Fig. 2C, D).

Pompholyxophrys species (Fig. 2G-I) have been observed and studied multiple times from fresh samples, but never in culture (Galindo et al. 2019). From these observations, they are always floating, nearly spherical cells (15 to 66 μm) (Roijackers and Siemensma 1988) with radiating, unbranched filopodia; see videos from Proyecto Agua (A. Guillén). Cells are tightly surrounded by spherical, ovoid, discoid or bone-shaped perforated silica pearls (depending on the morphospecies), embedded in a mucous coat, which might limit cell shape variability. As in *Nuclearia*, the cytoplasm is full of green, yellow, orange and red vacuoles depending on their food consumption, including lipid globules. It is unknown if any *Pompholyxophrys* species can encyst.

Lithocolla (Fig. 2J-L) cells have been rarely studied (Mikrjukov 1999; Penard 1904; Roijackers and

Table 1. Nucleariid genomic and transcriptomic data. All data from cultures and whole genome/transcriptome amplification could contain bacterial or algal contamination from their food.

Species	Sequencing data	Food	cultures
Fonticula alba	Genome GCF_000388065	*Klebsiella*	ATCC 38817
Fonticula-like SCN 57-25	Metagenome assembled genome GCA_001724245 (Kantor et al. 2015)	Unknown	Uncultured
Lithocolla globosa	SnP Metatranscriptome (Galindo and Torruella 2019)	*Navicula*, *Phaeodactylum*, *Isochrysis*	Available at Dalhousie University (Alastair Simpson)
Nuclearia delicatula	JP100 Metatranscriptome (Torruella et al. 2019)	Filamentous cyanobacteria e.g., *Oscillatoria*, *Escherichia coli*	Sciento JP100 is available, but CCAP 1552/6 is not available
Nuclearia moebiusi	CCAP 1552/4 Expressed sequence tags (Liu et al. 2009)	*E. coli*, yeast	CCAP 1552/4 and CCAP 1552/7 are not available
Nuclearia pattersoni	CCAP 1552/2 Expressed sequence tags (Liu et al. 2009); XT1 Whole transcriptome amplification (Galindo and Torruella 2019)	*Escherichia coli*; unknown for XT1	XT1, CCAP 1552/2, and CCAP 1552/8 are not available
Nuclearia thermophila	JP100 Metatranscriptome (Torruella et al. 2019) Metagenome in progress	Filamentous cyanobacteria e.g., *Oscillatoria*, *E. coli*, flour	Sciento JP100 and CCAP 1552/5 are available
Parvularia atlantis	Metatranscriptome (Torruella 2017) genome (Ocaña-Pallarès 2022)	Small bacteria e.g., *Halomonas, Alteromonas, Pseudomonas* and *Pseudoxanthomonas*	ATCC 50694
Pompholyxophrys punicea	Whole transcriptome and genome amplification (Galindo and Torruella 2019)	Unknown	Uncultured
Pompholyxophrys sp.	Whole transcriptome and genome amplification (Galindo and Torruella 2019)	Unknown	Uncultured

Siemensma 1988; Schulze 1874) and only once isolated and cultured (Galindo et al. 2019). Ranging from 10 to 50 μm, cells are tightly covered with exogenous material (xenosomes), which are embedded within the mucous envelope after phagocytosis. This material can be composed of small quartz grains, diatom frustules, or even chalk particles depending on the medium conditions. In culture, *Lithocolla globosa* can be observed uncovered flattened or a bit elongated like *Nuclearia*, but it is mostly spherical, and moves slowly (Galindo et al. 2019). Cells have multiple radiating filopodia, sometimes tapering and branching and the cytoplasm contains orange to red globules and greenish digestive vacuoles. The marine *L. globosa* does not have contractile vacuoles, and no cysts have been reported.

Fonticula alba (Fig. 3A-F) was isolated once from dog dung (Deasey and Olive 1981; Worley et al. 1979). It is a small amoeba, spherical to elongated (5 to 12 μm), with filose or monopodial lobose pseudopodia (Fig. 3A-C) that is adapted to solid substrate. It is the only known nucleariid species that presents distinct stages (see figure 7A in Toret et al. (2022)), additional to single cells and spherical cysts (Fig. 3D). The life history of this species includes two social stages that depend on bacterial cell density: collective association and sorogenesis. At some point in agar cultures, individual cells aggregate to produce a fruiting body, a “sorocarp” formation with a stalk (Fig. 3F). Stalk cells are sacrificed to form the elevated structure, while only the cells at the top will encyst and disperse in the form of oval spores (Fig. 3E) (Brown et al. 2009). Recently, a collective of cells with a dynamic leader–follower organisation has been described, when few bacteria are present in the medium. This consists of few to several tens of cells quickly moving towards a certain direction. One cell leads on the front, and the rest follow, with connections between cells that are supported internally by actin (Toret et al. 2022).

Finally, *Parvularia atlantis* (Fig. 3G-L) has only been isolated once (López-Escardó et al. 2018; Torruella et al. 2015). It is a tiny, filose amoeba, spherical when floating or flattened when attached to the substrate (3 to 5 μm), that feeds on small rod-shaped bacteria (Table 1). Sometimes, a single refractive vacuole is almost filling the entire cytoplasm (Fig. 2 in López-Escardó et al. (2018)). Both trophic and encysted cells have a mucous coat, and have one or two nuclei. In transmission electron microscopy (TEM) preparations, several cells have been observed within the same coat.

Cell Organisation

The ultrastructure of nucleariids has been studied in all *bona fide* genera, except for *Lithocolla*. It is worth noting that the *incertae sedis Vampyrellidium* has also been studied at the ultrastructural level (Patterson et al. 1987), which supported its

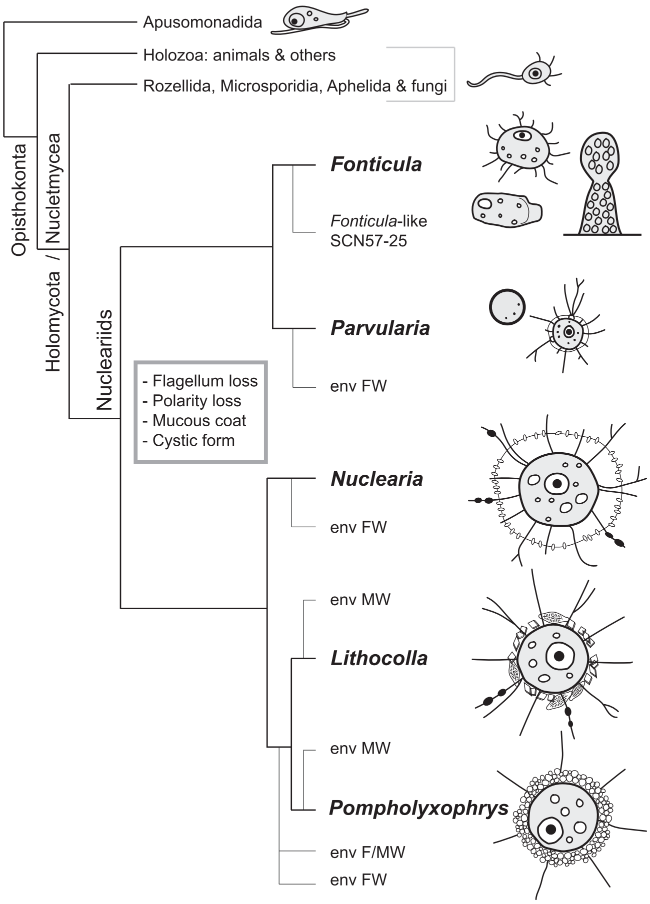

Figure 1. Nucleariid phylogenetic context. Cladogram showing nucleariids and their sister lineages; branches not to scale. Apusomonadida are bacterivorous biflagellates, the sister lineage to Opisthokonta (Heiss et al. 2017). Holozoa (animals and related protists) and all Holomycota besides nucleariids (Aphelida, Rozellida, Microsporidia and fungi) are ancestrally uniflagellated phagotrophs (Ros-Rocher et al. 2021). The last common ancestor of nucleariids already lost the opisthokont flagellum, lost the cell polarity and had a mucous coat. The phylogenetic resolution of the five *bona fide* nucleariid genera is based on phylogenomics, including the position where environmental clades (env) may be placed based on the SSU rRNA gene (Galindo et al. 2019).

Table 2. Taxonomic names of *incertae sedis*, filose amoebae suspected to be nucleariids. Classification of silica-scaled genera is based on Nicholls (2013). FW = Freshwater; MW = Marine water.

Genus	Habitat	Coverings	Comments
Elaeorhanis Greeff, 1873	FW/MW	Sand grains and diatoms frustules	3 species (Frenzel 1897; Greeff 1869; Mikrjukov 1999). Some authors relate it to *Lithocolla* (Mikrjukov 1999), but others to *Diplophrys* (Stramenopiles) (F. Siemensma)
Pinaciophora Greeff, 1873	FW/MW	Perforated plate scales	3 species morphologically related to *Pompholyxophrys* (Bessudova et al. 2022; Greeff 1869; Wee and Millie 1983). The most abundant genus of the silica-scaled rotosphaerids, with a worldwide distribution (Esteban et al. 2007)
Rabdiaster Mikrjukov, 1999	MW	Solid plate and spine scales	5 species (Mikrjukov 1999; Nicholls 2013). *R. multicostata* was moved from *Rabdiophrys* (Nicholls 2013), and from *Pinaciophora* (Roijackers and Siemensma 1988)
Rabdiophrys Rainer, 1968	FW/MW	Hollow plate and spine scales	3 species recognised (Mikrjukov 1999), several other species were moved from *Pinaciophora* (Roijackers and Siemensma 1988), later moved to *Thomseniophora* (Nicholls 2013)
Thomseniophora Nicholls, 2013	MW	Perforated plate and spine scales	16 species formerly assigned to *Pinaciophora* and *Rabdiophrys*. An alternative classification with three genera (*Thomseniophora*, *Turriplaca* and *Eiffelospina*) has also been proposed (Cavalier-Smith and Chao 2012)
Vampyrellidium Zopf, 1885	FW	Uncovered	2 species (Patterson et al. 1987; Surek and Melkonian 1980; Zopf 1885). Similar to *Nuclearia*, but have a perinuclear striated band, cytoplasmic microtubules, and many filopodia. Feed by penetrating algal cell walls with a specialised, sucking pseudopodium

classification as a nucleariid, although it showed cytoplasmic microtubules. Ultrastructural analyses have been done on a single strain of *Fonticula* (Deasey and Olive 1981), *Parvularia* (López-Escardó et al. 2018) and *Pompholyxophrys* (Patterson 1985), and on multiple *Nuclearia* species: in *N. simplex* (Mignot and Savoie 1979), *N. moebiusi* (Patterson 1983), *N. pattersoni* (Dyková et al. 2003), *N. thermophila* (Yoshida et al. 2009) and multiple new *Nuclearia* strains (Dirren and Posch 2016). Based on these studies and optical observations, simplified schematic models of the five *bona fide* nucleariid genera are depicted in Figure 5. The following paragraphs describe the cell organisation from the centre of the cell to the exterior.

All studied species are easily recognised by their prominent vesicular nucleus, sometimes with several nuclei present in one cell. Nuclei, whether central or eccentric, have a prominent central nucleolus in all nucleariids, except for *Parvularia,* which can also have peripheral nucleolar material (López-Escardó et al. 2018); and *Fonticula*, which has an indistinct nucleolus (Brown et al. 2009; Deasey and Olive 1981; Worley et al. 1979). Karyokinesis, which performs a closed mitosis as in fungi, has only been observed in *Nuclearia* (Patterson 1983) and *Fonticula* (Deasey and Olive 1981). Also, microtubules have only been observed inside the metaphasic nucleus (Patterson 1983).

Their cytoplasm contains multiple vacuoles, which can occupy most of the cell body of the tiny *Parvularia*. The most conspicuous are the food vesicles (green, orange or red) in algivorous species, the contractile vacuole of freshwater species, and lipid globules (Fig. 3L). All studied specimens contain mitochondria with lamellar (flat) or discoidal cristae. Some *Nuclearia* contain symbiosomes (Cann and Page 1979). These endosymbiotic bacteria are from distinct proteobacteria lineages and *Rickettsia*, as proven by *in situ* hybridization and molecular phylogenetics (Dirren et al. 2014; Dirren and Posch 2016; Dyková et al. 2003); the latter were also detected in single-cell genomes of *Pompholyxophrys* (Galindo et al. 2019). Another important

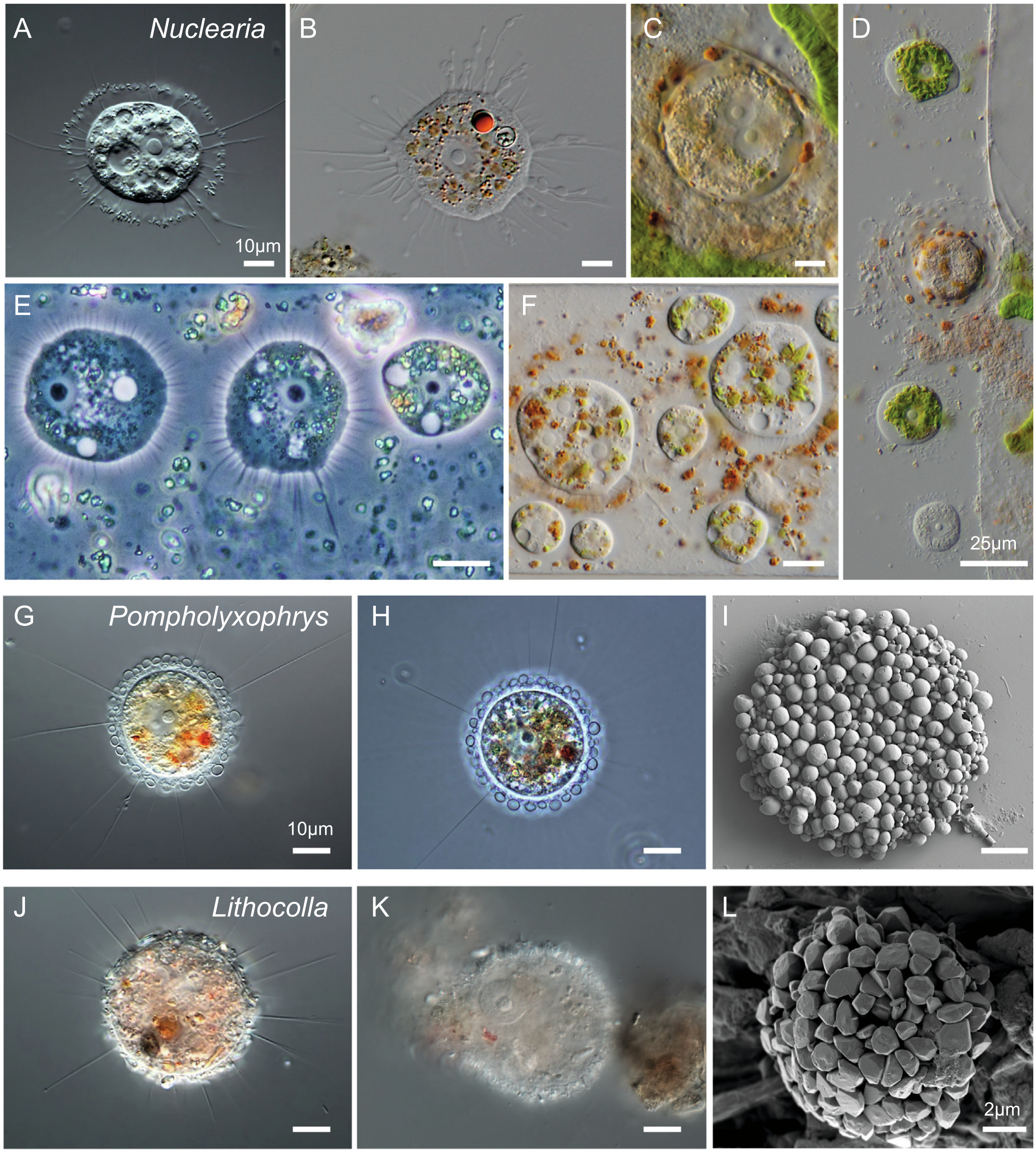

Figure 2. Light and electron micrographs of *Nuclearia* (**A-F**), *Pompholyxophrys* (**G-I**) and *Lithocolla* (**J-L**) cells from freshwater natural samples. All light micrographs are under x100 magnification (except D under x63) with differential interference contrast (DIC), except E and H that are phase contrast. H and K are scanning electron microscopy images focusing on the coverings of scaled amoebae. **A** is a *Nuclearia* floating cell covered with a mucous coat with ectosymbiotic bacteria. **B** is a slightly flattened cell without the mucous coat, with a red vesicle from engulfed purple bacteria, and obvious knobbed filopodia. **C** and **D** show cysts, with a thicker translucent coat, with excreted brownish material. **D** and **E** show unfed, empty cells with numerous radiating filopodia, and cells filled with greenish digestive vacuoles and few to no filopodia. **F** shows multiple feeding cells, including huge multinucleated cells: coenocytes. **G** and **H** are *Pompholyxophrys* cells with reddish digestive vacuoles and long and unbranched filopodia. **I** shows the external silica-based pearls covering the whole cell. **J** and **K** are freshwater *Lithocolla* cells covered with exogenous material. **L** shows a detailed whole cell covered with sand particles. Scale bars represent 10 µm in all images, except when stated differently.

organelle is the perinuclear Golgi apparatus, which contains numerous dictyosomes. Although it has not been observed in *Parvularia* or *Lithocolla*, this organelle is likely where the extracellular mucilage is produced. Indeed, in *Fonticula*, the Golgi has been observed to produce the material that cements aggregated cells into the stalk of the multicellular fruiting body (Fig. 3F) (Deasey and Olive 1981).

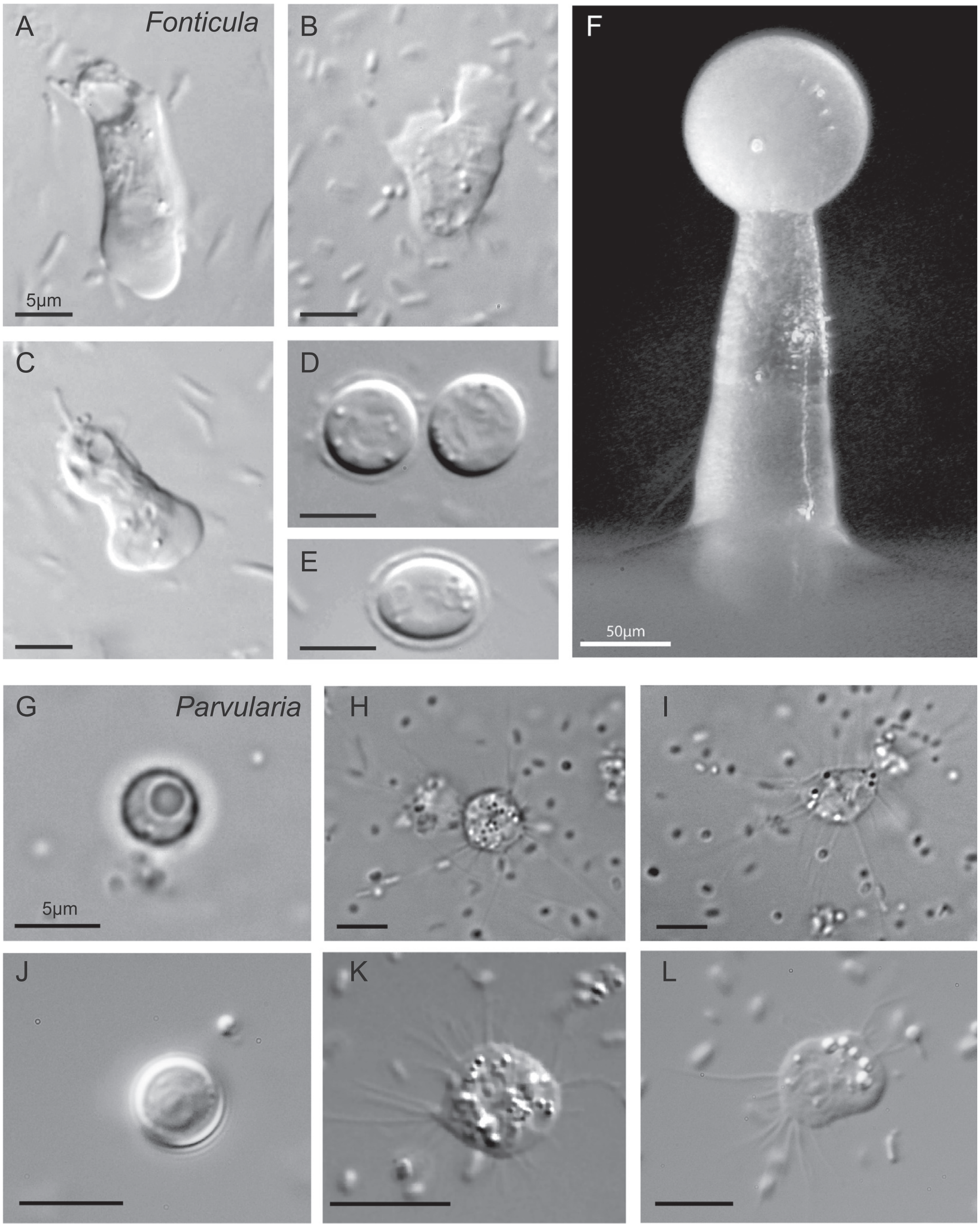

Figure 3. Light micrographs of *Fonticula alba* (**A-F**), courtesy of Matthew W. Brown and Alexander K. Tice; and of *Parvularia atlantis* (**G-L**). **A-E** are cultured *Fonticula* cells in a liquid medium under x100 magnification with differential interference contrast (DIC). **A-C** are lobose and filose amoebae. **D** are cysts. **E** is an oval spore. **F** is a multicellular fruiting body on a cut agar block, under reflected light at x10 magnification. **G-I** are cultured *Parvularia* cells in a liquid medium under x100 magnification with phase contrast, and **J-L** with DIC. **H** and **I** are floating and elongated filose amoebae. **K** and **L** are flattened amoeboid filose forms. **G** and **J** are cysts. Scale bars represent 5 µm in all images except F, representing 50 µm.

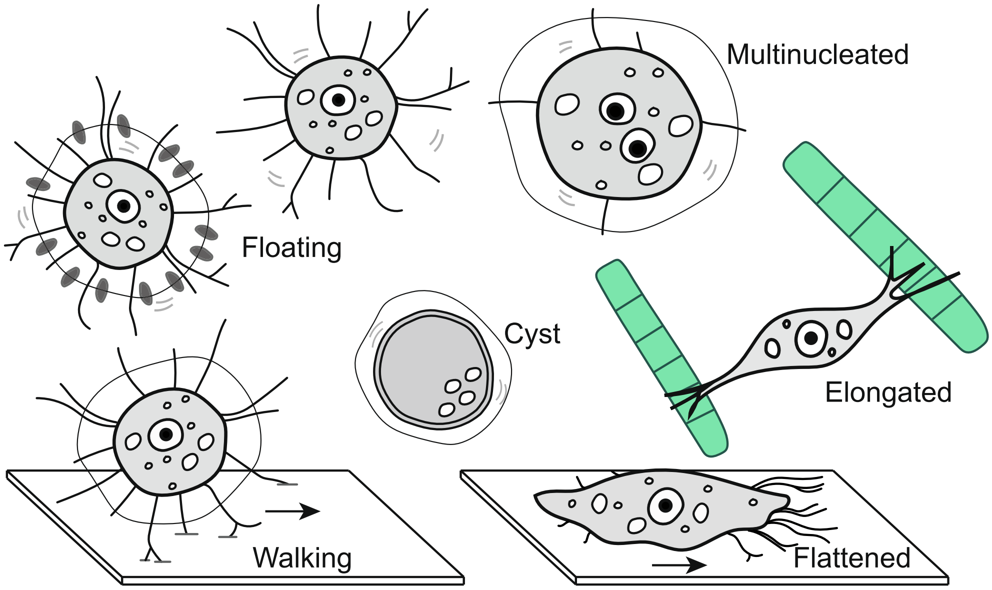

Figure 4. Morphological variety of *Nuclearia*, showing floating, walking, flattened, multinucleated, elongated and cyst forms.

The enigmatic mucous coat, sometimes called glycocalyx (Dirren et al. 2017), that embeds most nucleariid cells, can be present or not in the same organism depending on the conditions. The mucous coat seems to be made of fibrous material that runs parallel to the cell membrane. It can have one or two layers and often harbours bacterial ectosymbionts, another distinctive characteristic of *Nuclearia* (Fig. 2A, D) (Artari 1889; Cienkowski 1865; Dirren and Posch 2016). In covered species, such as *Lithocolla*, the mucous coat contains xenosomes, but in *Pompholyxophrys*, the cell produces siliceous pearls (idiosomes) within vesicles that migrate to the exterior of the cell, where they get trapped in the mucous coat (Patterson 1983).

Finally, also in the cell periphery, multiple characteristic thin hyaline pseudopodia are found. These filopodia originate from any point of the cell surface. They are sometimes branching, or tapering when attached to substrates, and have knobs of cytoplasm for elongation and retraction (never observed in *Pompholyxophrys*), but are never stiff, anastomosing or reticulating (Figs 2–4). Several ultrastructural analyses have confirmed that they are not supported by microtubules nor bear extrusomes as in heliozoan-like organisms (Deasey and Olive 1981; López-Escardó et al. 2018; Patterson 1983, 1985).

Ecology and Diversity

Most nucleariid amoebae live in freshwater environments, but are also found in marine settings (*Lithocolla*) and even faeces (*Fonticula*) (Table 1). Distribution and diversity information is available from isolation and fresh sample observations (e.g., Penard (1904)), sampling of scales/coverings visualised with electron microscopy (e.g., Bessudova et al. (2022)), and molecular metabarcoding analyses (e.g., del Campo et al. (2015)). Such studies are fundamental for understanding the biology and taxonomy of protists. From an ecological point of view, nucleariids are free-living predators: small cells like *Parvularia* and *Fonticula* feed on small bacteria and large cells such as *Nuclearia*, *Pompholyxophrys,* and *Lithocolla* feed also on detritus and unicellular eukaryotic algae (see Nucleariids in the Lab section). While their ecological role has not been studied in context, all nucleariids are slow-paced grazers (Dirren et al. 2017), probably growing in response to the availability of their food sources, for example after algal blooms.

Nuclearia contains a dozen morphologically described species (Yoshida et al. 2009), but only four are confirmed molecularly, *N. pattersoni* (Dyková et al. 2003), *N. thermophila* (Yoshida et al. 2009), *N. delicatula* and *N. moebiusi* (Dirren

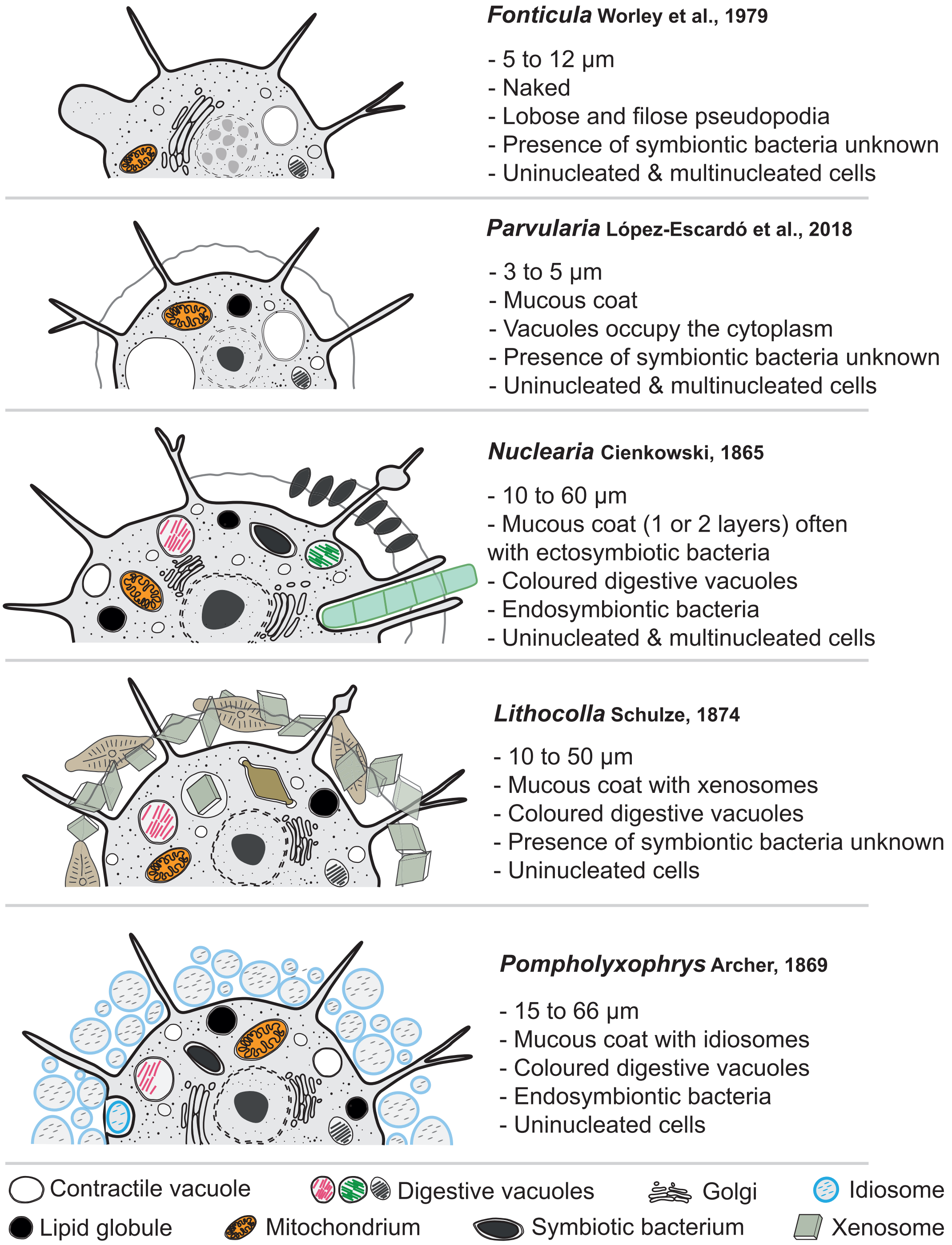

Figure 5. Cell organisation. Diagrams summarising the main cellular features of the five *bona fide* nucleariid genera; proportions not at scale.

and Posch 2016). All these species seem able to feed on a wide range of food sources, including yeast cells (Patterson 1983), flour granules (Yoshida et al. 2009), purple bacteria, small protists (E.V. observations), various filamentous cyanobacteria, and possibly chlorophyte algae (Cann 1986). Recently, it has been proposed that bacterial ectosymbionts help *Nuclearia* with cyanobacterial degradation and toxicity resistance (Dirren et al. 2017). In this regard, *Nuclearia* may be ecologically important, for both their capacity to consume many food sources and their wide environmental range, which may be comparable to the successful saprotrophic function of aquatic fungi (Galindo et al.

2019). *Nuclearia* have not been compared with other predators of filamentous cyanobacteria, experiments should elucidate if they are outcompeted by much larger and faster ciliates, which have specialized feeding-structures like the cytopharynx (Hausmann 2002).

Nuclearia species diversity is supported by the various SSU rRNA metabarcoding analyses recovering *Nuclearia* operational taxonomic units (OTUs) (Galindo et al. 2019) from aerobic and microaerophilic settings such as pristine peat bogs, city lakes, drinking water, waste waters, and decomposing matter. Based on these SSU rRNA surveys, it seems there are still many uncharacterised *Nuclearia* species that may even be novel genera, considering their genetic distance, such as strains NZ, D1 and A1 (Dirren and Posch 2016). Moreover, *Vampyrellidium* (Patterson et al. 1987; Surek and Melkonian 1980; Zopf 1885) would likely be phylogenetically close to *Nuclearia* if it is ever to be molecularly characterised (Table 2). It is worth noting that the largest known genetic diversity of *Nuclearia* has been found in lake Zurich (Dirren and Posch 2016), which has a level of genetic diversity comparable to the diversity found worldwide (Galindo et al., 2019). If confirmed with further sampling, this would lend support to the 'everything is everywhere' hypothesis, which states that protists are not governed by the same biogeography rules as larger organisms (Finlay 2002), but this matter requires more dedicated studies. In addition, two *N. pattersoni* isolates have been found in fish gills (Dyková et al. 2003) and a fresh tadpole dropping (Galindo et al. 2019), besides *Fonticula* being isolated from dog dung (see below). Whether *Nuclearia pattersoni* or *Fonticula* affect metazoans is still unknown, but some species of free-living amoebae (e.g., *Acanthamoeba*, *Balamuthia*, *Naegleria* or *Sappinia*) are opportunistic pathogens of animals (Visvesvara et al. 2007).

The small *Fonticula alba* and *Parvularia atlantis* have only been isolated once. *Fonticula* (Worley et al. 1979) has no other SSU rRNA environmental sequence represented in metabarcoding surveys. There is a metagenome assembled genome, "*Fonticula*-like SCN 57-25″ (Table 1), which originated from a lab-scale bioreactor used to study cyanide and thiocyanate contaminated wastewater in gold ore processing (Kantor et al. 2015). Although this assembly lacks the SSU rRNA, its close relationship to *Fonticula* has been confirmed using phylogenomics (Galindo et al. 2019). *Parvularia* (López-Escardó et al. 2018) has few closely associated SSU rRNA freshwater environmental sequences that could represent new genera, based on their genetic distance (Galindo et al. 2019). The low genetic diversity in metabarcoding studies of *Parvularia* and *Fonticula*, as well as *Pompholyxophrys*, (see below), could be explained by undersampling of freshwater and soils, but could be also due in part to primer bias during SSU rRNA gene amplification.

The diversity of covered nucleariids is mostly known from morphological studies of fresh samples, never from cultures. *Pompholyxophrys*, with seven described morphospecies based on the shape of their silica pearls, may be seasonal and more restricted to clear freshwater bodies, wet *Sphagnum* moss, and 'pristine' peat bogs (Bessudova et al. 2022; Leonov 2012; Roijackers and Siemensma 1988; Wujek 2015). Recently, several single cells of *Pompholyxophrys* were isolated (Galindo et al. 2019). They were identified as *P. punicea* through morphology, but when characterised molecularly, three distinct SSU rRNA gene sequences were revealed. This suggests that, like *Nuclearia*, *Pompholyxophrys* could have pseudocryptic species (Mann and Evans 2008), and that morphological characterisation needs to be coupled with genetic sequencing to confirm species identification; a fundamental task to understand nucleariid diversity.

Lithocolla diversity is even less studied. The marine *L. globosa* has been reported a few times from coastal waters (Galindo et al. 2019; Page and Siemensma 1991; Schulze 1874). Accordingly, the only strain ever cultured (Galindo et al. 2019) was placed within a clade of environmental SSU rRNA sequences from European coastal waters (del Campo et al. 2015) (Fig. 1). Another species, *L. flavescens*, has been described once from a freshwater lake (Penard 1904), but without molecular evidence it is difficult to confirm whether this organism truly belongs to the same genus (Fig. 2J-L). In addition, the *incertae sedis* organism *Elaeorhanis* has been recorded occasionally from marine and freshwater sites (Frenzel 1897; Greeff 1873; Mikrjukov 1999). *Elaeorhanis* is a filose amoebae with a large nucleus and radial filopodia, that is covered with diatom frustules (Table 2). Only molecular studies could tell if these morphological similarities with *Lithocolla* are of phylogenetic meaning.

Still, there are at least four genera of silica-scaled filose amoebae that could be part of the nucleariid diversity: *Pinaciophora*, *Rabdiophrys*, *Rabdiaster,* and *Thomseniophora* (Table 2). During the expansion of electron microscopy, starting from the last quarter of the past century, scientists have characterised siliceous scales associated with silica-covered nucleariids from marine and freshwater environments, mostly without observing whole cells (Bessudova et al. 2022; Croome et al. 1987; Esteban et al. 2007; Nicholls and Dürrschmidt 1985; Thomsen 1978; Wujek 2015). However, as these studies are not always based on the observation of living cells, the results may have certain limitations. It is possible that scales are being transported from other environments (e.g., coastal water samples could contain material washed in from freshwater systems). In this regard, microscopy-based biodiversity surveys of whole cells have been vital in overcoming these issues (Leonov 2012; Roijackers and Siemensma 1988; Tong et al. 1997), although modern studies should be linked with molecular characterisations, perhaps using single cell genomics (Galindo et al. 2019). This issue of the environmental source of scales is shared with metabarcoding studies (Santoferrara et al. 2020). In this case, DNA samples could contain material of nucleariid cysts coming from elsewhere (Amaral-Zettler et al. 2002; Arroyo et al. 2018; Couradeau et al. 2011; Heger et al. 2018; Lalla et al. 2021). This issue can be solved by probing the RNA of metabolising cells (del Campo et al. 2015). Overall, the genetic diversity of nucleariid barcodes points to at least five uncultured clades (Galindo et al. 2019), perhaps this is where the *incertae sedis* scaled filose amoebae will find their phylogenetic placement. Particularly, there is an environmental clade containing both marine and freshwater sequences, which correlates with the nature of the scale distribution of *incertae sedis Rabdiophrys* and *Pinaciophora* (e.g., Esteban et al. (2007)) (Table 2).

Nucleariids in the Lab

Culturing strains in laboratory conditions is the ideal situation in which to study protists. In culture conditions, slow nucleariids can be studied using time-lapse techniques to observe all their different forms (this is the case for *Nuclearia*, Fig. 5). Although single cells from fresh samples provide important information; e.g., *Pompholyxophrys* (Galindo et al. 2019; Patterson 1985), whole cultures can be grown to obtain high quality sequencing data (Table 1) and perform thorough electron microscopy studies (see Cell Organisation section and Fig. 4). Yet, we advise to keep some cell isolates frozen for whole genome/transcriptome amplification, which can assist in case cultures cannot be maintained for long periods.

To culture nucleariids, first one must look at environmental samples similar to the ones where the desired species has been described, or where environmental OTUs have been detected (see Ecology and Diversity section). Once nucleariids are observed in the sample, it is recommended to isolate single cells, using traditional suction with a glass pipette or a micromanipulator. As heterotrophic organisms, nucleariids require other organisms to feed on, usually present in the same sample where nucleariids are found. It would be recommended to isolate more than one prey to ensure culturing. Although prey trials have not been systematically done for most nucleariids, they can be maintained with various preys. Except for the uncultured *Pompholyxophrys*, observed to feed on diatoms (Patterson 1985), detritus and eukaryotic algae (E. V. personal observations), other nucleariids are easy to maintain in culture. All cultured nucleariid species can be maintained at 14 °C, but grow optimally at 25–27 °C, in aerobic conditions.

When food is abundant, *Nuclearia*, *Parvularia, and Lithocolla* cultures can easily last for more than a month, simply requiring some aeration and agitation every week to increase the culture viability for several months up to years (G.T. personal experience). *Nuclearia* grow well with the cyanobacterium *Planktothrix* (e.g., Dirren et al. (2017)) and *Oscillatoria* (Galindo et al. 2019) in BG-11 medium. *Lithocolla* can feed on *Navicula*, or *Phaeodactylum* diatoms and *Isochrysis* haptophytes in F/2-Si medium (Galindo et al. 2019). *Parvularia*, likely can feed on any kind of small *Klebsiella*-like bacteria in ATCC802 or cereal grass media (López-Escardó et al. 2018). *Fonticula alba* is the only species that grow better on agar medium in slight variations of glucose, peptone or tryptone, and yeast extract with *Klebsiella* (e.g., Toret et al. (2022)), it grows poorly on liquid medium always attached to the substrate (Worley et al. 1979). For long term conservation, small species as *Fonticula* and *Parvularia* are easily cryopreserved, but *Nuclearia* cultures often die. A detailed summary of the availability of nucleariid cultures, food, and sequencing data is in Table 1.

Systematics and Evolution

A Short Story on Nuclariid Systematics

By the end of the 19th century, most 'putative' nucleariid species were described, and classified together with other naked or covered filose amoebae; see the seminal book on heliozoans by Penard (1904). By that time, naturalists observing fresh or enriched samples with classical light microscopy had described the genera *Nuclearia*, *Pompholyxophrys*, *Lithocolla*, and *incertae sedis Elaeorhanis*, *Pinaciophora*, and *Vampyrellidium* (Table 2). Moving to the second half of the 20th century, Rainer coined the group Rotosphaeridia, within heliozoans, to include non-flagellated, scaled, filose amoebae lacking axopodia (pseudopodia supported by microtubules); i.e., *Pompholyxophrys*, *Pinaciophora*, *Lithocolla*, and *Rabdiophrys* (Rainer 1968). Cann and Page established the family Nucleariidae including *Nuclearia*, *Gobiella* (*incertae sedis* (Hess and Suthaus 2022)), and *Nucleosphaerium* (syn. *Nuclearia* (Cann 1986; Patterson 1984)) (Cann and Page 1979). Later, Patterson and colleagues performed TEM of *Nuclearia* (Patterson 1983), *Pompholyxophrys* (Patterson 1985) and a *Vampyrellidium* species (Patterson et al. 1987). Based on these ultrastructural analyses, Page established the order Cristidiscoidida, within class Filosea, to include both families Pompholyxophryidae and Nucleariidae because of the disc-shaped mitochondrial cristae (Page 1987). Meanwhile, Patterson proposed the family Nucleariidae to include *Nuclearia*, *Vampyrellidium* and *Pompholyxophrys* together with other silica-covered amoebae (*Pinaciophora* and *Rabdiophrys*) (Patterson et al. 2000; Patterson 1999).

However, none of these classifications included *Fonticula*, since it was originally regarded as an acrasid (Worley et al. 1979) or a slime mould (Deasey and Olive 1981). In a reclassification of the Protozoa kingdom in 18 phyla, Cavalier-Smith created the subclass Cristidiscoidia (Cavalier-Smith 1993), including orders Nucleariida (with *Nuclearia* and *Pompholyxophrys*) and Fonticulida (with *Fonticula*). Later he considered scaled rotosphaerids (including *Pompholyxophrys*) as cercozoans (Cavalier-Smith and Chao 2012). Mikrjukov emended Cavalier-Smith's Cristidiscoidida as a synonym of Rainer's Rotosphaerida, arguing time priority for Rotosphaerida. He also argued that the name did not reflect the morphology of the organisms and could be confused with Discicristata, a group of excavates (Mikrjukov 1999). However, Mikrjukov included *Belonocystis* inside Rotosphaerida (Mikrjukov 1999), whereas *Belonocystis* is most likely an amoebozoan (Adl et al. 2019; Klimov and Zlatogursky 2016), and *Micronuclearia* (Mikrjukov and Mylnikov 2001) is currently related to *Rigifila* (Yabuki et al. 2013) and likely belongs within the CRuMs clade (Brown et al. 2018).

With the advent of molecular phylogeny, it was found that *Nuclearia* is related to fungi, based on the SSU rRNA gene (Amaral-Zettler et al. 2001; Medina et al. 2003) and six housekeeping genes (Steenkamp et al. 2006). After this grouping was confirmed by concatenated phylogenomic methods, the clade names Nucletmycea (Brown et al. 2009) and Holomycota (Liu et al. 2009) were coined to comprise nucleariids (*Nuclearia* and *Fonticula*) and fungi. Only recently, *Parvularia* (López-Escardó et al. 2018; Torruella et al. 2015) was included with *Lithocolla*, and *Pompholyxophrys*, in one phylogenomic analysis (Galindo et al. 2019), proving that covered and uncovered nucleariids are indeed evolutionary siblings.

Since then, most molecular studies have been using Cristidiscoidea sensu Cavalier-Smith, only including *Nuclearia* or environmental sequences (Amaral-Zettler et al. 2002; Arroyo et al. 2018; del Campo et al. 2015; Couradeau et al. 2011; Heger et al. 2018; Torruella et al. 2015). Meanwhile, Rotosphaerida sensu Mikrjukov has been used during the study of scale-bearing filose amoebae from a morphological point of view (Bessudova et al. 2022; Croome et al. 1987; Esteban et al. 2007; Nicholls and Dürrschmidt 1985; Roijackers and Siemensma 1988; Thomsen 1978; Wujek 2015), and more recently to include both uncovered and covered filose amoebae (Adl et al. 2019; Galindo et al. 2019).

Overall, the history of the systematics of filose amoebae is full of examples of problems resulting from misidentifications and incongruence between morphological descriptions and molecular phylogeny. This is the case of the paraphyly of *Nuclearia simplex* (Dirren and Posch 2016); the original description of *Fonticula* as an acrasid slime mold (Worley et al. 1979), the original identifications of *Parvularia* (López-Escardó et al. 2018), and *Capsaspora owczarzaki* (Holozoa) (Amaral-Zettler et al. 2001; Hertel et al. 2002) as *Nuclearia* species; or the sequencing of three distinct *Pompholyxophrys* SSU rRNA sequences from the same sample while aiming at one morphotype (Galindo et al. 2019). This is similar to the history of silica-scaled *incertae sedis* taxonomy that was based on the morphology of their coverings. For example, species initially classified as *Rabdiophrys* by Roijackers and Siemensma (1988), were reclassified in *Pinaciophora* by Mikrjukov (1999). Then Nicholls

(2013) combined those species with perforated plate and spine scales in *Thomseniophora*. Meanwhile, for the same species, *Turriplaca*, and *Eiffelospina* were proposed also to accommodate the morphological differences between scales (Cavalier-Smith and Chao 2012; F. Siemensma). Unfortunately, without molecular validation, these taxonomies are still preliminary (Table 2).

A Hypothetical Evolutionary Scenario for the Diversification of Nucleariids

It has been proposed that the last common ancestor of opisthokonts was a phagotrophic polarised cell with a single posterior flagellum (Fig. 1) (Cavalier-Smith and Chao 1995), in contrast to their biflagellate sister lineages, such as Apusomonadida (Heiss et al. 2017). This hypothesis is supported because the cell type with a posterior flagellum is found throughout the lineage: in Chytridiomycota fungi (Letcher and Powell 2014), Aphelida (Letcher and Powell 2019) and *Rozella* (Letcher and Powell 2018) in Holomycota, and in *Syssomonas*, *Pigoraptor* (Hehenberger et al. 2017), *Tunicaraptor* (Tikhonenkov et al. 2020) and choanoflagellates (Karpov 2016) in Holozoa.

In the recent phylogenomic analysis of the five nucleariid genera, the monophyly and internal relationships within the nucleariids were robustly recovered. The small *Fonticula* and *Parvularia*, both exclusively bacterivorous, branched together, followed by the larger *Nuclearia*, and then the covered *Lithocolla* and *Pompholyxophrys* (Fig. 1). From this phylogenetic backbone, the origin and diversification of nucleariids has been hypothesised. In this parsimonious scenario, the last common ancestor of the nucleariids would have been a small, freshwater, non-flagellate, filose amoeba that was able to secrete a mucous coat, an organism probably close to a current *Parvularia* or *Nuclearia* cell (Fig. 5) (Galindo et al. 2019).

This nucleariid ancestor would have diversified to the current five main genera, although the particular order and mechanisms are unknown. In this regard, *Nuclearia* acquired the ability to use many food sources, even toxic cyanobacteria (Dirren et al. 2014). At some point, some species acquired endosymbionts, like *Nuclearia* (Dirren and Posch 2016) and *Pompholyxophrys* (Galindo et al. 2019). An uncovered nucleariid ancestor must have acquired the ability to cover itself with exogenous material as in *Lithocolla*, or as in *Pompholyxophrys*, to secrete its own endogenous siliceous pearls. *Fonticula* would have specialised in living on solid substrates, innovating aggregative social behaviours such as collectives and fruiting bodies (Toret et al. 2022).

Future Prospects

With only five *bona fide* genera (Fig. 1, Table 1), at least six morphology-based genera to confirm (Table 2), and at least five exclusively environmental, molecular clades (Galindo et al. 2019), the nucleariids is still a poorly studied group.

Isolation, culturing and molecular analyses of the *incertae sedis* filose amoebae suspected to be nucleariids would be of great importance. As it may be challenging or even impossible to maintain some species in culture, single cell techniques can provide both morphological and behavioural data, coupled with nucleic acid extraction and amplification for molecular analyses to establish proper phylogenetic relationships.

Meanwhile, to keep tackling nucleariid diversity, in addition to morphological surveys, researchers must mine metabarcoding and metagenomics studies, which might be in Sequence Read Archive databases. This could reveal further environmental information on the rare organisms such as the particularly divergent *Fonticula alba*.

From an ecological stand point, genera like *Nuclearia* should be studied in terms of their rate of predation and growth to confirm if their ecological role is as important as expected based on abundance, and observed growth in isolated cultures.

Also, the most efficient way to understand the genetic functions of non-model eukaryotes is through comparative genomics. In this regard, nucleariid transcriptomics and genomics data (Table 1) have to be processed for decontamination and functional annotation, and then implemented in phylogenetic-informed functional categories. With sufficiently characterised species, researchers will be able to confidently reconstruct the last nucleariid common ancestor, and be able to compare it with other eukaryotes so as to infer the evolutionary changes that gave rise to the current forms of life.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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