PROTIST

TITLE: Piercing the veil: A novel amoebozoan (*Janelia veilia* n. gen. n. sp.) reveals deep clades within Discosea through phylogenomics

SHORT TITLE: *Janelia veilia* n. sp., a discosean amoeba with trailing veil

AUTHORS
Robert E. Jones[1,2], Quentin Blandenier[1], Felicity Kleitz-Singleton[1], Tristan C. Henderson[1], Nicholas W. Fry[1], Idan Banson[1], Jonah Nguyen[3], Alexander K. Tice[1,2,4], Matthew W. Brown[1,2,#]

[1.] Department of Biological Sciences, Mississippi State University, Mississippi State, MS, USA
[2.] Institute for Genomics, Biocomputing & Biotechnology, Mississippi State University, Mississippi State, MS, USA
[3.] Department of Comparative Biomedical Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS, USA
[4.] Department of Biological Sciences, Texas Tech University, Lubbock, TX, USA

#CORRESPONDING AUTHOR
Matthew W. Brown, Department of Biological Sciences, Mississippi State University, Mississippi State, MS, USA, matthew.brown@msstate.edu

ORCID
Robert E. Jones: 0000-0001-5227-4773
Quentin Blandenier: 0000-0002-4297-0262
Felicity Kleitz-Singleton: 0000-0003-3459-3345
Tristan Henderson: 0000-0003-0449-4266
Nicholas Fry: 0009-0002-8516-4719
Idan Banson:
Jonah Nguyen: 0009-0009-2793-7550
Alexander K. Tice: 0000-0002-3128-1867
Matthew W. Brown: 0000-0002-1254-0608

ABSTRACT (200 Words Max)
Three major groups of primarily amoeboid taxa are present across Amoebozoa: Discosea, Evosea, and Tubulinea. While each of these groups were thought to have morphologically unique traits and members, the morphologic boundaries between each group have recently blurred. For example, it is demonstrated that several taxa in each group display monopodial limax amoebae, a characteristic most often associated with Tubulinea. Here we describe a novel discosean amoeba isolated from a freshwater pond, *Janelia veilia* n. gen. n. sp. Its cells have variable morphologies, but often display monopodial limax amoebae, with a unique trailing structure that appears to be derived from cellular material. In some cases, cells have conical pseudopodia or pointed pseudopodia. Using phylogenomics, we find that this taxon branches as sister to the recently described discosean *Mycamoeba gemmipara* and the sporocarpic protosteloid amoeba *Microglomus paxillus*, forming an order-level group we term Mycamoebida. Mycamoebida is fully supported as sister to Dermamoebida, together forming a subclass we term Dermelia. SSU rRNA phylogenies show that *Janelia veilia* n. gen. n. sp. is molecularly unique from any known organism, but branches with high support in a clade containing *Mycamoeba gemmipara* and several environmental sequences suggesting a larger diverse clade within Discosea.

KEYWORDS: Amoebae, phylogenomics, protist, Discosea, taxonomy, morphological plasticity

1. INTRODUCTION

Across the Eukaryotic Tree of Life (EToL), amoeboid organisms have evolved in nearly every major supergroup, i.e., Stramenopiles, Heterolobosea, Rhizaria, Obazoa, but amoebae are most often attributed to Amoebozoa. Amoebozoa represents a highly diverse clade of organisms that primarily display amoeboid morphology in which cells generate movement and capture prey with extensions called pseudopodia. This

ancient supergroup is estimated to be around 1.5 billion years old dating back to the Calymmian period of the Mesoproterozoic era (Eme et al., 2014; Porfirio-Sousa et al., 2024). It is composed of three major clades named Discosea (Cavalier-Smith et al., 2004, *sensu* Smirnov et al. 2011), Evosea (Kang et al. 2017), and Tubulinea (Smirnov et al., 2005) each with their own set of diverse taxa and many notable members (Adl et al., 2019). For example, Tubulinea includes the iconic amoebozoan genus *Amoeba* as well as shelled, testate amoebae in Arcellinida. Evosea includes many slime molds placed in Myxogastria (sporocarpic) and Dictyostelia (sorocarpic), as well as the human pathogen *Entamoeba hystolytica* in the anaerobic group Archamoebae and is currently the only clade of Amoebozoa to include flagellated taxa. Finally, Discosea is an immense group containing genera like the opportunistic pathogen *Acanthamoeba*, the commonly observed *Vannella*, and the large and charismatic *Mayorella*.

Historically, there was believed to be a clear morphological and behavioral distinction between Tubulinea and the rest of Amoebozoa. But recent advances in our understanding of the tree of Amoebozoa and careful taxonomic and descriptive works have shown that the morphological boundaries between the three major groups are unclear. One such type of contradictory cellular morphology is that of limax (slug-shaped) monopodial amoebae, which are tubular in cross-section. A tubular cross-section was viewed as a synapomorphy to Tubulinea, but this character is now recognized to be present across Amoebozoa, ranging from *Entamoeba*, *Schoutedamoeba*, *Kanabo*, and *Parakanabo* within Evosea (Ptáčková et al., 2013; Wichelen et al., 2016; Fry et al., 2024) to *Janickina* and *Coronamoeba* found in Discosea (Volkova and Kudryavtsev 2021; Kudryavtsev et al., 2022). This indicates either several independent convergent evolutions towards this morphotype or a synapomorphic character to the whole Amoebozoa. Other behavioral and life cycle characteristics such as the sporocarpic life cycle (i.e., the ability for a cell to form a prostrate subaerial stalked spore-bearing fruiting body) occur broadly in both Evosea and Discosea (Shadwick et al., 2009, Tice et al., 2016, Kang et al., 2017, Tice et al., 2023).

Discosea is composed of a deep dichotomy between the class-level subclades Centramoebia and Flabellinia and is estimated to be approximately 1.25 billion years old, emerging during the Ectasian period of the Mesoproterozoic era (Porfirio-Sousa et al., 2024). Despite the long evolutionary history of the Discosea, there are relatively few known genera within the lineage, which is particularly true in the Flabellinia including only 24 recognized genera (Adl et al., 2019; Kudryastev et al., 2022; Tice et al., 2023). However, environmental SSU rDNA sequence data shows considerable diversity that has not been sampled and whose morphological identity is unknown (Tice et al., 2023; Kudryastev et al., 2022).

Here we report a novel discosean taxon, which we name *Janelia veilia* n. gen. n. sp., that displays a unique amoeboid morphology under light and scanning electron microscopy. Using transcriptomic data from this organism, new data from several other discosean taxa, and existing data from the breadth of Amoebozoa, we employed phylogenomics to get a clearer picture of the overall evolution of Amoebozoa. We further explored its phylogenetic placement in nuclear encoded small subunit (SSU) rRNA gene phylogenies, searching also environmental data to examine its and close relatives' distribution. Combining light microscopy with confocal immunohistochemical staining, scanning electron, and transmission electron microscopy we fully characterize this unique taxon and provide deep transcriptomic data from some of the poorly sampled diversity in Discosea, including the genera *Coronamoeba*, *Mayorella*, and *Vexillifera*. Our description here of *Janelia veilia* n. gen. n. sp., along with additional sampling of discosean genera, helps to elucidate the overall structure of Discosea.

2. RESULTS

Morphology of *Janelia veilia* n. gen. n. sp.

Our isolate HHMI3 is a small unique amoebozoan found in the benthos of a large freshwater pond on the campus of Janelia Research Campus operated by the Howard Hughes Medical Institute. The cells are most often found in culture as monotactic lanceolate (Fig. 1A) to lingulate amoebae sometimes appearing as monopodial limax amoebae often with a well-defined leading hyaloplasm forming a lobopodium (Fig. 1I-L). Occasionally we find cells with multiple leading pseudopodia (Fig. 1F). Sometimes the pseudopodia are conical in form during direction changes, roughly resembling those in the genus *Mayorella* (Fig. 1 B,C,D,F,G). In general, the amoebae display considerable plasticity in morphology (Suppl. Video 1, FigShare). The cells occasionally make finely pointed pseudopods on the lateral sides perpendicular to locomotive trajectory of the cell which appear to be used for gripping or prey capture rather than for forward

locomotion (Fig. 1E). The cells commonly produce a faint trailing structure, which we term a veil (Fig. 1A-D. The length of locomotive cells of our isolate HHMI3 ranged from 11.7 to 20.5 µm with an average of 15.2 µm. Our isolate was longer than it was wide with a breadth ratio of 1.8 – 4.1 (average 2.8). Cells are primarily uninucleate with an average nucleus diameter of 3.3 µm. Cysts ranged from 7.3 to 9.5 µm with an average of 8.5 µm. Nuclei were most conspicuous in cysts with a centrally located nucleolus (Fig. 1M,N).

Using confocal microscopy and immunohistochemistry, we stained DNA, actin, and microtubules (Fig. 2). *Janelia veilia* appears uninucleate (Fig. 2B) with actin ubiquitous through the cells (Fig. 2C) and microtubules appear to be localized near the nucleus (Fig. 2D,E). Most of our scanning electron microscopy (SEM) reveals an intricate trailing web-like structure composed of minute granules and threads or strands (Fig. 3A-D). We interpret this structure to be the wispy veil seen in light microscopy. Our first round of SEM preparation used vapor fixation, and the veil appeared dehydrated (Fig. 3A-C). So, we next prepared samples for SEM imaging in liquid fixative and found that the veil appeared to be in better condition (Fig. 3D). TEM reveals densely stained granular structures inside the nucleus near the periphery (Fig. 4).

Other discosean taxa.
In addition to *Janelia* we isolated and generated transcriptomes from several other discosean taxa including a novel *Mayorella* sp. (Biloxi) and *Vexillifera* sp. (Gen4) (Supplemental Fig. 1). We generated transcriptomes of a new strain of *Coronamoeba villafranca* and two additional *Mayorella* species from Culture Collection of Algae and Protozoa (Scotland, UK), *M. vespertilioides* CCAP 1547/10 and *M. gemmifera* CCAP 1547/8.

Phylogenomics.
Our phylogenomic analyses used 240 genes (75,380 amino acid sites) and recovered the three major lineages of Amoebozoa (Discosea, Evosea, and Tubulinea) with full support (Fig 5). Discosea is composed of Centramoebida and Flabellinia, the latter housing two fully supported major clades. *Janelia veilia* n. gen. n. sp. branches within Discosea sister to *Mycamoeba gemmipara*, and both form a sister clade to the two isolates of *Microglomus paxillus*, all with full bootstrap support (Fig. 5). This clade of *Janelia veilia* n. gen. n. sp, *Mycamoeba gemmipara*, and *Microglomus paxillus* branches sister to Dermamoebida sensu Kudryavtsev et. al 2022 also with full maximum likelihood bootstrap support (MLBS) (Fig. 5). All *Mayorella* taxa form a monophyletic fully supported clade within Dermamoebida, similar to the three *Vexillifera* cultures within Dactylopodida.

SSU rRNA gene phylogenetics.
In our SSU rRNA gene phylogenetic reconstruction, we utilized a dataset that was enriched in discosean taxa and related environmental sequences previously deposited on GenBank and from the long read data published in Jamy et al. 2020. *Janelia veilia* n. gen. n. sp. branches with full support as sister to an environmental sequence, Uncultured eukaryote AY835694, which originated from a biofilm from a marine anoxic environment of the Montreal Biodome's denitrification reactor (Laurin et al., 2008). This clade is a subclade that branches within a much larger highly supported (95% MLBS) clade that contains *Mycamoeba gemmipara*, the cultivated but morphologically uncharacterized amoebozoan isolates (amR1, amCP10 and amMP3), and their related environmental sequences as well as several other subclades made up of exclusively environmental sequences from soil and freshwater environments (Fig. 6). Here again, the newly obtained sequences of *Mayorella* form a robust monophyletic clade, including the *M. gemmifera* CCAP 1547/8 from the transcriptome which is identical to *M. gemmifera* EU719190 (Dykova et al., 2008). Finally, both sequences of *Coronamoeba villafranca* are virtually identical despite their independent isolation.

3. DISCUSSION

Our isolate HHMI3 herein named *Janelia veilia* n. gen. n. sp. is a novel discosean amoeba, notable for its highly variable morphology shifting from monopodial tubular to flat cells with conical and pointed subpseudopodia. Most interestingly, cells often exhibit a unique trailing structure that appears to be derived from cellular material (Fig. 1), but the exact origin and makeup of this trailing veil is still unclear. Due to its unique morphology, we suspected HHMI3 would be of phylogenetic interest to our overall goal of better understanding the macroevolutionary trends in Amoebozoa. Based on morphology alone it is difficult to estimate the phylogenetic position of this genus in the tree of Amoebozoa. As the trophic cells are most often tubular in form (cross-section), morphology would suggest a close affinity to tubulinid amoebae, such

as *Micriamoeba* (Atlan et al., 2012) or *Echinamoeba* (Baumgartner et al., 2003; Page, 1967; Page, 1975). However, recent discoveries have shown tubular amoebae exist in all major groups of Amoebozoa, making this an unreliable character for taxonomic placement (Ptáčková et al., 2013; Wichelen et al., 2016; Volkova and Kudryavtsev 2021; Kudryavtsev et al., 2022; Fry et al., 2024). Thus, once isolated we first generated RNAseq data. This allowed us to bypass any potential problems with amplifying the SSU rRNA gene of an uncharacterized organism (Brown et al., 2012) and provided the volume of sequence data necessary for large-scale phylogenomic analysis, including the SSU. We used these data for multigene phylogenomic analyses using PhyloFisher (Tice et al., 2021), as well as querying the SSU rRNA gene sequence from the transcriptome for single-gene phylogenetic analyses with other publicly available data.

From our SSU rRNA gene phylogenetic analysis we found that *Janelia veilia* was affiliated with *Mycamoeba* within a well-supported clade (95% MLBS) with several environmental sequences from soils and freshwater (Fig. 6). However, *Microglomus* does not group directly with this clade, rather the genus is sister to *Dermamoeba* with no MLBS support. The branch length leading to *Microglomus* is extremely long and the sequences of *Microglomus paxillus* strains are very divergent. Therefore, the placement of this genus is not clear in SSU phylogenetics. Indeed, SSU rRNA gene phylogenies are of little utility to resolve deep evolutionary relationships within Amoebozoa due to the limited amount of phylogenetic signal available (Tekle et al., 2008). We continued our analyses using multigene phylogenomics to resolve some of these more difficult taxa, which has proven itself a worthy tool for examining relationships among Amoebozoa (Kang et al., 2017; Tekle et al., 2022).

In our phylogenomic analyses of Amoebozoa, *Janelia veilia* n. gen. n. sp. branches with full MLBS support with *Mycamoeba gemmipara,* a small amoeba, only up to 7 µm in length, and the only species of the genus *Mycamoeba* (Blandenier et al., 2017). Together *Janelia* and *Mycamoeba* branch with full MLBS as sister to *Microglomus paxilis*, a monotypic genus of protosteloid amoebae (Olive et al., 1983; Olive & Stoianovitch, 1977; Spiegel et al., 2017). The phylogenetic affinity of this organism to any other known protosteloid was unclear since its description (Spiegel et al. 2017), until it was found to be hosted within Flabellinia, as sister to *Mycamoeba* (Tice et al., 2023). Given the results of our phylogenomic analysis we conclude that *Mycamoeba*, *Janelia,* and *Microglomus* fall within a clade that we redefine as the new order Mycamoebida.

Mycamoeba, *Janelia,* and *Microglomus* all share a common feature identified in TEM studies. Each of these taxa have conspicuously electron dense nucleolar material around the periphery of the nucleus (Fig. 4). This was noted by Blandenier et al. (2017) in their description of *Mycamoeba* and can also be found in the few *Microglomus* TEM images available (most notably figure 18 of Olive et al. 1983). Mycamoebida taxa are mostly found in soils but can be found in freshwater as in *Janelia* and several environmental sequences (Fig. 6), and on bark in the case of *Microglomus*. This broad range of inhabited environments likely means there is a large amount of undiscovered diversity between the current genera in the novel order Mycamoebida.

During our culturing of *Trichosphaerium sieboldi* for an unrelated project, we isolated a novel strain of *Coronamoeba villafranca*, an amoebozoan genus within Discosea, belonging to the subclass Flabellinia (Kudryavtsev et al., 2022). As it was pertinent to this current work, we included this isolate in our discosean dataset. In our phylogenomic analyses *Coronamoeba* branches sister to the Dermamoebidae family, which consists of *Dermamoeba* and *Paradermamoeba*, with full bootstrap support (Fig. 5). The fully supported clade of Dermamoebidae + *Mayorella* + *Coronamoeba* + *Mycamoeba* + *Microglomus* + *Janelia* is a clade of extreme morphological variability. We are defining this clade as “Dermelia”. While there is no known morphological synapomorphy linking these taxa, phylogenomic analyses clearly demonstrate this grouping that is now only possible to observe given the new data provided here.

“Dermelia” is a highly variable clade with exceptional diversity in form. Locomotive cell lengths range from 7 µm, *Mycamoeba gemmipara* (Blandenier et al., 2017), to 75 µm, *Dermamoeba algensis* (Smirnov et al., 2011). The disc-like structures of *Coronamoeba’s* cell coat are topped with unique crown-like structures (Kudryavtsev et al., 2022). *Janelia veilia* n. gen. n. sp. displays a unique trailing “veil” that appears to be derived from cellular material. *Mycamoeba gemmipara* exhibits a unique life cycle where trophozoites form walled coccoid stages that grow through successive budding giving a very fungal like appearance, particularly on agar surfaces (Blandenier et al., 2017). Members of “Dermelia” also occupy a wide range of

ecological habitats. *Janelia veilia* n. gen. n. sp. and *Dermamoeba algensis* were both isolated from freshwater ponds (Smirnov et al., 2011), but *Coronamoeba villafranca* and several *Mayorella* are marine or live in brackish waters (Dykova et al., 2008, Kudryavtsev et al., 2022). *Microglomus paxillus* has been found on dead twigs of orange tree or from the bark of *Casuarina* or *Juniperus* trees (Olive et al. 1983, Tice et al., 2023). Finally, *Mycamoeba gemmipara* was found in soil from a coniferous forest near Neuchâtel (Switzerland) but seems to have aquatic relatives (Blandenier et al., 2017) (Fig. 6). *Dermamoeba algensis* is primarily algivorous, several species of *Mayorella* are largely opportunistic, preying on fungi, algae or other eukaryotic cells (Dykova et al., 2008), but most other members of "Dermelia" are typically bacterivorous. Hence, "Dermelia" houses a vast diversity in terms of size, cell coat structure, life cycle, ecological habitats, and prey, that remain to be more characterized.

Discosea is composed of Centramoebida and Flabellinia, within Flabellinia there are two fully supported major clades, the previously mentioned "Dermelia" and another we are calling "Thecavania". "Thecavania" is a portmanteau of *Thecamoeba* and *Vannella* comprised of Dactylopodida + Vannellida + Thecamoebida as well as the *incertae sedis* genera *Stygamoeba* and *Vermistella*. We do not recover Stygamoebida (as shown in Tekle et al., 2022). The bifurcation of Thecavania and Dermelia represents roughly the same amount of evolutionary depth and breadth as (ca. 1,100 – 1,200 mya) (Porfirio-Sousa et al., 2024). Here we present the superorders Dermelia and Thecavania, the new order Mycamoebida, the new family Mycamoebidae, as well as the new taxon *Janelia veilia* n. gen. n. sp.

4. CONCLUSIONS

Here we describe *Janelia veilia* n. gen. n. sp., as well as provide novel transcriptomic data from other discosean taxa. *Janelia veilia* displays a trailing veil, which, to our knowledge, is a completely unique morphology to amoebae. Our detailed morphological and phylogenomic analyses place *Janelia veilia* within the new order Mycamoebida, alongside *Mycamoeba* and *Microglomus*.

Recently, numerous examples of novel organisms have violated morphology-based taxonomic understanding in Amoebozoa (Ptáčková et al., 2013; Wichelen et al., 2016; Volkova and Kudryavtsev 2021; Kudryavtsev et al., 2022; Fry et al., 2024). It is now necessary to use molecular data to confidently and accurately place a novel amoeba. Traditionally, the SSU rRNA gene has been used for species identity in Amoebozoa and in most other protist groups, but this gene can be problematic to amplify and result in sampling bias (Brown et al., 2012; Tekle et al., 2008). Additionally, it provides insufficient phylogenetic signal to evaluate deep taxonomic relationships (Tekle et al., 2008). To alleviate these problems, we took a transcriptomic route. This approach gives the volume of molecular data necessary to perform phylogenomic analyses using hundreds of genes, including the SSU rRNA (Kang et al., 2017; Tekle et al., 2022). With this amount of data, we overcome issues with organisms that were previously difficult to place, i.e., *Microglomus,* and gain sufficient phylogenomic signal to make fully supported claims on deep taxonomic relationships.

The high morphological and habitat variability among these few described genera in the novel order Mycamoebida, combined with the environmental sequences that fall into this group, indicates that this order likely contains a vast amount of undescribed diversity. There is still a wealth of discoveries to be made in this group and groups like this, demonstrating the value in continued sampling and isolation of unknown organisms. Describing and characterizing species and clades is of primary importance to furthering ecological, evolutionary, and biogeographic studies.

5. TAXONOMIC SUMMARY

ZooBank registration number of the present work is urn:lsid:zoobank.org:pub: XXXXXXXXXXXXXXXXXXXXXX

* Eukaryota (Chatton, 1925) Whittaker & Margulis, 1978 (DOMAIN)

** Amorphea Adl et al. 2012 (KINGDOM)

*** Phylum Amoebozoa Lühe, 1913 emend. Cavalier-Smith, 1998

**** Class Discosea Cavalier-Smith et al., 2004

***** Subclass Flabellinia Smirnov et al., 2005 sensu Kang et al., 2017

****** Superorder Thecavania Jones et al.
The least inclusive clade defined by containing the genera *Thecamoeba* + *Vannella* + *Vexillifera*
Zoobank ID: urn:lsid:zoobank.org:act:XXXXXXXXXXXXXXXXXXXXXXX

****** Superorder **Dermelia Jones et al.**
The least inclusive clade defined by containing the genera *Dermamoeba* + *Paradermamoeba* + *Mayorella* + *Coronamoeba* + *Mycamoeba* + *Microglomus* + *Janelia* n. gen.
Zoobank ID: urn:lsid:zoobank.org:act:XXXXXXXXXXXXXXXXXXXXXXX

******* Order **Mycamoebida Jones et al. n. order**
The least inclusive clade defined by containing the genera *Mycamoeba* + *Microglomus* + *Janelia* n. gen.
Zoobank ID: urn:lsid:zoobank.org:act:XXXXXXXXXXXXXXXXXXXXXXX

******** Family **Mycamoebidae Jones et al. n. fam.**
The least inclusive clade defined by containing the genera *Mycamoeba* + *Janelia* n. gen.
Zoobank ID: urn:lsid:zoobank.org:act:XXXXXXXXXXXXXXXXXXXXXXX

********* Genus ***Janelia* Jones et Brown n. gen.**
ZooBank ID: urn:lsid:zoobank.org:act:XXXXXXXXXX

Diagnosis. Amoebae most often limax-shaped with a monopodial morphology (generally tubular shape) usually with a well-defined leading hyaloplasm forming a lobopodium in locomotion that is roughly equal to the width of the cell. Stationary cells or cells changing direction may be very plastic in form from polypodial with several lobopodia or cells with both lobopodia and finely pointed pseudopodia along the lateral sides of the cells. Cells may be slightly wrinkled along the sides, and they usually display a trailing veil-like structure of unknown origin. Often this veil is inconspicuous and requires careful observation to be seen. The veil is usually as long as the cell body. Occasionally, cells may display a broad leading pseudopodium that is wider than the rest of the cell body. Amoeboid cells are greater in length than breadth, most often without a clearly defined uroid. When uroid is present, it is either pointed or bulbous. Cells are primarily uninucleate. Nuclei are ellipsoidal to circular with densely staining nucleolar material around the periphery in transmission electron microscopy. Cysts round with central nucleus and a conspicuous central nucleolus. Contractile vacuoles may be inconspicuous, large singular in the rear of a locomotive cell, or small and numerous central or in the rear of the cell in locomotion.
Type species. *J. veilia*
Etymology. Named after the location of the type isolate HHMI3, the Janelia Research Campus. *Janelia* is feminine.

***Janelia veilia* Jones et Brown n. sp.**
ZooBank ID: urn:lsid:zoobank.org:act:XXXXXXXXXXXXXXXXXXXXXXX
Diagnosis. Cells typical of the genus as described above. Length in locomotion 11.7-20.5 µm (average 15.2 µm, SD = 1.9, n = 130), width in locomotion 3.7-9.3 µm (average 5.6 µm, SD = 0.9, n = 130), length to breadth ratio 1.8-4.1 (average 2.8), nucleus diameter at widest point 3.9-2.5 µm (average 3.3 µm, SD = 0.4, n = 31). Nucleus ellipsoid to circular. A single nucleus per cell observed. Single central nucleolus is usually not obvious, but when observed circular to oval (ca. ½ the diameter of nucleus). Nuclei display conspicuously condensed electron dense nucleolar material around the periphery in Transmission Electron Microscopy (TEM). Cells often with a trailing veil of cellular material near equal length of the cell. Cysts 7.3-9.5 µm in diameter (average = 8.5 µm, SD = 0.5, n = 20). Primarily a bacterivore.
Type location. Strain HHMI3 of *Janelia veilia* n. sp. was obtained from a freshwater pond (39.071551, -77.464584) on the Janelia Research Campus of Howard Hughes Medical Institute in Ashburn, Virginia, USA.

Type material. The type culture (HHMI3) is deposited in a metabolically inactive state in the Culture Collection of Algae and Protozoa (CCAP). This culture is also considered the hapantotype (name-bearing type) of the species, under article 73.3 of the International Code of Zoological Nomenclature (ICZN, 1999).
Gene Sequence data. The nearly complete SSU-rRNA gene of the type isolate (HHMI3) is deposited on GenBank under accession XXXXXXXXX.
Etymology. For the specific epithet, we chose “veilia” referring to the often-observed trailing veil that many amoebae display. The suffix “-ia” was added to noun veil into a more naturally flowing word with genus name Janelia.

6. MATERIALS AND METHODS

6.1 Isolation and culturing of *Janelia veilia*. Originally, 20 mL of water with a small amount of upper benthos sediments was collected from a freshwater pond (39.071551, -77.464584) in front of the Howard Hughes Medical Institute Janelia Research Campus (Virginia, USA). The sample was brought into the lab after ca. 2 days of transport with the container sealed but opened and resealed every 12 hours to maintain oxygen levels. Once in the lab the tube with water and sediments was shook for roughly 5 seconds and four drops (ca. 40µL each) of this suspension were put onto a sterile non-nutrient spring water agar 100mm Petri dish (1L Deer-Park spring water and 15g agar) separated by roughly 2 cm of space between each drop. After the agar surface absorbed the liquid drops, the plate was sealed using Parafilm M (Heathrow Scientific, Vernon Hills, IL, USA), inverted, and left on the benchtop at room temperature under normal light conditions. Daily observations of the plate were conducted using a 10x objective on a compound microscope, with a primary focus on the periphery of the dried soil solution droplet. Single amoeba cells were isolated five days after plating, employing a 30-gauge platinum wire crozier-loop sterilized through an ethanol flame. The platinum loop was used to drag cells through the meniscus formed by contacting the loop with the agar surface, transferring them from one plate area to another. These cells were then transferred onto spring water agar, along with a streak of *E. coli* (MG1655) as a food source, establishing a clonal culture through this methodology. Cultures were maintained by serial passage as above monthly.

6.2 Isolation and culturing of other investigated taxa

6.2.1 *Mayorella* spp. *Mayorella* sp. (Biloxi) originated from ca. 60 mL of sediments (small rocks, silt, and coarse sand), plant material (i.e. fallen leaves and roots from aquatic plants) and brackish water collected in February 2021 in the side of a boat ramp in an estuary in Biloxi (Bayou Laporte, Mississippi, USA; 30.410711, -88.954368). The sample was brought to the lab and left on a shelf until January 2022 where it was shaken for roughly 5 seconds and ca. 20ml of liquid was poured in an empty Petri dish and observed under an inverted microscope (Zeiss Axiovert 135). Several naked amoeba cells were found thriving and five of them were isolated and cultivated in 10mL of Crystal Geyser® Alpine Spring Water® (CG Roxane, USA) and one sterilized (via autoclaving) rye berry in a 25cm^2 vented tissue culture flask. This species thrives in eutrophic conditions with fungal and algae contaminations that appeared to be prey for this *Mayorella* isolate. Under these cultivation conditions the culture was very stable over several months. After about five weeks, the density of amoebae became significant (i.e., several hundred *Mayorella* cells), along with other eukaryotic and prokaryotic taxa. From that point, we isolated five amoebae from the original culture and cultivated this species in a fresh tissue culture flask with a sterile rye berry. We were also able to cultivate this isolate without any other eukaryotic contaminants in a media composed of 1 ml of 802 media and 19 ml of Fiji water, but this culture was less stable and did not last more than ca. 1 month. *Mayorella gemmifera* (1547/**8**) and *M. vespertilioides* (1547/10) were purchased from the Culture Collection of Algae and Protozoa (CCAP, Scotland, United Kingdom). The cultures were provided in tissue culture flasks, and after arrival, the cells were passed to fresh vented tissue culture flasks and maintained in the conditions and media suggested by CCAP. Cultures were maintained by serial passage monthly.

6.2.2 *Coronamoeba villafranca.* The isolate TriC1A1 was collected as a contaminate from the CCAP culture 1585/2 labeled as *Trichosphaerium sieboldi*. *Trichosphaerium sieboldi* was maintained in 25 cm^3 vented tissue culture flask in 10mL of sterile N75S (New Cereal Leaf 75% Seawater media, 1.0L of 75% natural seawater boiled with 1.0g powdered cerophyll (Carolina Biological Supply, USA) for 5 minutes, filtered through a coffee filter and sterilized via autoclave). From this culture a small amoeba (*Coronamoeba villafranca*) was isolated by picking a cell using a fine glass pipette and moved to sterile N75S under the same culture conditions as used to maintain the original culture. Cultures were maintained by serial passage monthly.

6.2.3 *Vexillifera* sp. Gen4. Isolate Gen4 was collected in July 2019 from a small (ca. 10x10X10mm) freshwater puddle formed from a dripping water line behind the Harned Hall Biological Sciences building on Mississippi State University campus (33.455701, -88.787987). The collected water was brought into the lab and five drops (ca. 40µL each) of water were placed on two wMY agar plates each. The plates were observed daily using a 10x objective on a compound microscope. After three days single amoeba cells were isolated by employing a 30-gauge platinum wire crozier-loop sterilized through an ethanol flame. The platinum loop was used to drag cells through the meniscus formed by contacting the loop with the agar surface, transferring them from one plate area to another. These cells were then transferred onto wMY agar, along with a streak of *E. coli* (MG1655) as a food source, establishing a clonal culture. Cultures were maintained by serial passage monthly.

6.3 Light Microscopy.

Both agar and liquid culture slides were used for detailed observations. Agar culture slides were prepared as in Brown et al. (2012) by melting a small block (approximately 4mm^3) of spring water agar onto glass slides, covered with a coverslip. This process involved gently heating the bottom of a glass slide over a Bunsen burner flame, causing the agar to melt and form a layer between the slide and the coverslip. After cooling for 10 minutes at room temperature, the coverslip was slid off, revealing the thin agar surface. Subsequently, the agar was inoculated with amoebae and cysts, and a new coverslip, along with sterile spring water, was applied. For liquid slides, cells on agar were suspended in liquid culture medium and then transferred to a glass slide or taken from a liquid culture if growing in liquid media and placed onto a glass slide with a 1.5H coverslip. After 15 minutes, cells were examined using differential interference contrast (DIC) on a Zeiss Axioskop 2 Plus upright compound microscope (Carl Zeiss Microimaging, Thornwood, NJ, USA) under a 40x Plan-NeoFluar (NA 0.75) connected to a Canon (Huntington, NY, USA) CMOS digital camera (EOS R, 30.3MP full frame mirrorless) controlled by Canon EOS Utility software for Macintosh. Morphometric data using cell measurements were acquired using ImageJ software (http://imagej.nih.gov/ij/) and the Scale Bar Tools for Microscopes utility (http://image.bio.methods.free.fr/ImageJ/?Scale-Bar-Tools-for-Microscopes.html).

6.4 RNA isolation, transcriptome sequencing, Assembly, Proteome prediction.

For *Janelia veilia* HHMI3 and *Vexillifera* sp. Gen4, two culture plates per culture of high amoebae density were flooded with 3 mL of liquid wMY and scraped to suspend the cells, whereas for *Coronamoeba villafranca* TriC1A1, four 25cm^2 culture flasks of densely grown amoebae were shaken vigorously for ca. 30 seconds to detach cells from the flask surface. The suspended cells of these three taxa were then centrifuged at 200*g* for 3 min to remove the agar from the suspension. The supernatant of the two former taxa as well as poor of the later one was transferred to a fresh 1.5 mL tube and centrifuged at 1000*g* for 15 min. The supernatant was discarded, and the cell pellet was resuspended in 300 µl of Lysis Buffer. RNA was extracted using a Direct-zol RNA MicroPrep kit (Zymo Research) following the manufacturer's recommended protocol. The lysed cell mixture was passed through a Zymo-Spin IC Column at 10,000g, then went through two rounds of washing and centrifugation at 10,000*g*. Cell contents in the spin column were washed with 400 µl of Direct-zol RNA PreWash followed by 700 µl of RNA Wash Buffer, then eluted in 52 µl of nuclease-free water.

Following RNA extraction, we used a NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs) to select and purify eukaryotic mRNA by following the manufacturer's recommended protocols. Briefly, NEBNext Magnetic Oligo d(T) Beads were treated with RNA binding buffer and 50 µl of these treated beads were then mixed with 50 µl of the purified RNA. The mixture was incubated at 72˚C for 5 min to denature the RNA and facilitate the binding of poly-A RNA to the beads, then allowed to incubate at room temperature for 10 min with mixing to allow the poly-A RNA to bind to the beads. The beads were then pelleted on a magnetic stand and washed twice with 200 µl of Wash Buffer to remove unbound RNA. Afterwards, we added 50 µl of Tris Buffer and incubated first at 80˚C for 2 min, and then at 25˚C to elute the poly-A RNA from the beads. Then by re-pelleting the beads, the supernatant contained the desired mRNA which was transferred to a clean tube.

For the three *Mayorella* cultures, we used instead a single-cell transcriptomics approach as described in Onsbring et al. (2020). Briefly, ca. 20-30 cells from each taxa were isolated with a P10 pipette and washed

3 times by transferring them into clean water to ensure that no eukaryotic contaminants remain. The cells were then left overnight to digest their prey. The next day, 5 cells of each culture were isolated independently in the minimal volume possible, but bellow than 0.5 µl, and placed in a clean PCR tube. We added 2.3 µl of a lysis mix composed of Superase-In (Thermo Fisher) and TritonX100 in each tube and performed 6 cycles of Freezing-thaw between a lead block at -80°C and warm water.

Purified mRNA from the three whole culture extraction, as well as the 15 single-cells from the three *Mayorella* spp, were reverse transcribed to cDNA following Smart-seq2 protocols (Picelli et al., 2014). The resulting cDNA libraries were prepared for sequencing on the Illumina platform using a Nextera XT DNA Library Preparation Kit following manufacturer protocols. The Nextera XT libraries were pooled, together with other libraries from organisms for unrelated studies, and sequenced on either a Illumina HiSeq 4000 (150bp Paired-end) instrument at Génome Québec (Montréal, Canada) or HiSeqX (150bp paired-end) instrument at Psomagen (Rockville, Maryland, USA). Trinity v2.8.5 (Haas et al., 2013) was used to remove adaptors, primer sequences, and low-quality bases with the "trimmomatic" option (Bolger et al., 2014) and to assemble the remaining sequences. The following trimming parameters were used: "ILLUMINACLIP:2:30:10 SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25". Protein sequences were then predicted from the assemblies using TransDecoder v5.5.0 (https://github.com/TransDecoder/TransDecoder/).

6.5 Phylogenomics.

From the predicted protein sequences identified from our new transcriptomes, we queried for 240 housekeeping genes using PhyloFisher v1.2.13 (Tice et al., 2021) as detailed in Jones et al. (2024). PhyloFisher was used to identify probable orthologs and discard probable paralogs using default parameters. Examination of the calls as ortholog proteins, unknown contaminants, and paralogy was further assessed by manual parsing of single gene trees using the ParaSorter software included with PhyloFisher (https://github.com/TheBrownLab/PhyloFisher). The curated PhyloFisher dataset includes taxa representing and sampled across the known breadth of eukaryotes diversity. Once each protein sequence tree was parsed, we collected all Amoebozoan taxa along with our new transcriptomes and outgroup Obazoa taxa to generate a dataset of 240 orthologs. The genes were then processed fully to generate new single gene alignments of amoebozoan, and the outgroup data contained in PhyloFisher using the default PhyloFisher protocol (see Tice et al. 2021). The processed ortholog alignments were concatenated with orthologs from the PhyloFisher database into a multigene supermatrix covering 75,380 amino acid sites and 97 taxa. This matrix was used as input to generate a phylogenomic tree using IQTree2 (Minh et al., 2020). First, a starting tree was inferred under the LG+G4+C20 site heterogeneous model of evolution. Using this tree as a guide, we inferred another tree under the LG+G4+C60 site heterogeneous model of evolution, collecting posterior mean site frequencies (PMSF) inferred from the dataset to use for non-parametric real bootstrap replication under the LG+G4+C60+PMSF model of evolution. Using the PSMF model we inferred a final maximum likelihood (ML) tree with 100 real bootstrap replicates.

6.6 SSU rRNA Gene Phylogenetics.

The SSU rRNA gene sequences of *Janelia veilia*, *Coronamoeba villafranca* an amoeboid contaminant of CCAP 1585/2, *Mayorella gemmifera* CCAP 1547/8, *Mayorella* sp. Bioloxi, *Mayorella vespertilioides* CCAP 1547/10 and *Vexillifera* sp. Gen4 were bioinformatically obtained from each of the corresponding transcriptomes using the function BLASTN and a SSU database as query. These new sequences were included into a dataset with 55 other Discosea taxa and with 3 Variosea taxa as outgroups sourced from GenBank (Clark et al., 2016). To infer a phylogenetic tree from these data we first explored the environmental long read SSU rDNA dataset of Jamy et al. 2020 to identify where in the environment our taxa of interest that are closely related to HHMI3 occur. The Jamy et al. 2020 data was collected from Bioproject PRJEB25197 from https://github.com/Pbdas/long-reads. The data was clustered using VSEARCH v2.28.1 (Rognes et al. 2016) [vsearch --cluster_fast long_read.18S.otus.fasta --id 0.95 --strand both --threads 8 --clusters cluster]. From these clusters, a table of presence per environmental type grouped as soil, freshwater, and marine was built allowing for a percentage per environment out of 100%. A BLAST (Camacho 2009) database was made from the clustered nucleotide data and searched for homology using BLASTN queried with a nucleotide file consisting of HHMI3, *Mycamoeba*, *Coronamoeba*, and *Microglomus* SSU rRNA sequences. All blast hits above an e-value threshold of 1e-200 were collected and combined with a seed alignment of Discosea SSU rRNA sequences. All collected and seed sequences were aligned

using MAFFT with the AUTO option (Katoh and Standley, 2013). Alignments were trimmed of poorly/ambiguously aligned sites with TRIMAL (Capella-Gutiérrez et al. 2009) utilizing a gap threshold of 0.90. A preliminary tree was inferred with FastTree under the GTR+GAMMA model of evolution. The tree was visualized in Figtree (https://github.com/rambaut/figtree/releases) and the clade consisting of cluster sequences branching with sequences in the seed alignment of Discosea were collected, while the other clusters were removed. The remaining (30 clusters) and seed alignment listed above were aligned and trimmed as described above. A final phylogenetic tree was inferred using Maximum Likelihood through RAxML V. 8.2.12 (Stamatakis, 2014) with the GTR+CAT model of nucleotide substitution using the rapid hill climbing search algorithm. Topological support for this phylogeny was assessed with 1,000 real bootstrap replicates under the same model of evolution as the final dataset tree inference.

6.7 Confocal Microscopy.

Cytoskeleton fluorescence staining of *Janelia veilia* was prepared as in Brown (2021). A block of agar was cut from an area of the culture in which cell growth was very dense. The block was then placed upside down on a chamber culture slide (Lab-Tek™ II Chamber Slide - Thermo Fisher Scientific – 154461). 500µL of liquid wMY was added to the chamber slide and allowed to sit for 15 minutes. The agar block was then removed and the liquid wMY aspirated with a 1mL transfer pipette. Cells were then fixed by gently adding 1mL of -80°C methanol to the chamber slide and incubated at room temperature for 2 minutes. The liquid was then aspirated. The chamber slide surface was rinsed by adding 500µL of PBS and allowing it to gently flow down onto the glass surface. After 3 minutes, the liquid was removed, and this rinse process was repeated twice more. 500µL of Serum Blocking Buffer was added and allowed to incubate for 10 minutes at room temperature. 500µL of 1:500 primary antibody [monoclonal Anti-α-Tubulin antibody produced in mouse clone B-5-1-2] was added and allowed to incubate for 30 minutes at room temperature. 2 drops, ca. 100 µL, of ActinGreen 488nm ReadyProbes Reagent (Thermo Fisher Scientific | R37110) were added and allowed to incubate for 30 minutes at room temperature. The liquid in the chamber slide was gently aspirated. The chamber slide was rinsed three times as previously described. 500µL of 1:1000 secondary antibody [Goat anti-Mouse IgG (H&L) Secondary Antibody, Alexa 594 | Thermo Fisher Scientific | A11032] was added and allowed to incubate for 15 minutes at room temperature while shielded from light. Two drops of ActinGreen 488nm ReadyProbes Reagent (Thermo Fisher Scientific | R37110) were added and allowed to incubate for 10 minutes at room temperature. The chamber slide was rinsed twice as previously described. The culture slide chamber sides were removed with the included side removal tool. The sample was then mounted using a drop of Fluoromount-G (Thermo Fisher Scientific | 00-4958-02) and a clean 1.5H cover slip (22mm^2) was placed on the slide. The edges were then sealed with transparent nail lacquer and allowed to let dry for 15 minutes. Cells were visualized with an inverted confocal microscope (Leica TCS SPE-II) equipped with four solid state lasers (405, 488, 532/561, 635 nm excitation), under an Advanced Correction System (ACS) 63x-Oil (NA 1.30) objective controlled by the LAS X Leica software.

6.8 Scanning Electron Microscopy (SEM).

For SEM we performed both vapor and liquid fixation. For vapor fixation, amoeboid cells of *Janelia veilia* isolate HHMI3 were grown on wMY agar plates until they reached a dense culture stage. A drop of sterile liquid wMY was added to a coverslip, then a small (5 mm^2) block of agar was cut from an area of high amoebae density and placed cells-down in the liquid on the coverslip. Amoebae were allowed to transfer from the agar block to the surface of the coverslip for thirty minutes. The agar block was then removed, and the coverslip was inverted inside a closed center-well culture plate over 3 drops of 4% osmium tetroxide for 15 min inside of a fume hood to vapor fix the amoebae cells. Afterward, the coverslip was rinsed with sterile water four times, then placed face up. Cells on the coverslip were dehydrated through ethanol-water series (25%, 50%, 75%, 100%), each step lasting 15 minutes. Cells on the coverslip were then dried with a Tousimis Autosamdri-931 Multi-Application Critical Point Dryer (Tousimis, Rockville, MD, USA) using 100% ethanol and liquid carbon dioxide. The coverslip was affixed to metal SEM stubs with carbon tape and coated with 20 nm of platinum in an EMS150T Turbo-Pumped Sputter Coater (Electron Microscopy Sciences, Hatfield, PA, USA). Cells on the coverslip were observed using a JEOL 6500 10-kV SEM (JEOL USA, Peabody, MA, USA) at the Institute for Imaging & Analytical Technologies, Starkville, MS.

For liquid fixation, amoeboid cells of *Janelia veilia* isolate HHMI3 were grown directly on a glass coverslip placed on top of the agar of a wMY agar plate. Amoeboid cells were passed directly onto the glass coverslip from a previous high density culture plate by cutting a small (5 mm^2) block of agar from the prior culture

plate and inverting it, cells-down, onto the glass coverslip. The glass coverslip was previously streaked with *E. coli* MG1655 using a sterile cotton swab to encourage the amoebae to crawl out from under the agar block onto the surface of the glass and were allowed to do so over several days. This ensured that the amoebae maintained typical locomotion on a surface appropriate for fixation. After the amoebae had spread across the glass coverslip, the agar block was removed, and the coverslip was transferred to a fixative solution of 2.5% glutaraldehyde and 1% osmium tetroxide (200µL 25% glutaraldehyde, 500µL 4% osmium tetroxide, 500µL 0.2M cacodylate buffer, 800µL spring water) on ice. Cells were allowed to fix for 30 minutes. The coverslip with fixed cells was then removed from the fixative solution and rinsed three times with spring water. Cells were then dehydrated through ethanol-water series (50%, 70%, 80%, 85%, 90%, 95%, 100%), each step lasting 15 minutes. Cells on the coverslips were then dried with a Tousimis Autosamdri-931 Multi-Application Critical Point Dryer using 100% ethanol and liquid carbon dioxide. Coverslips were affixed to metal SEM stubs with carbon tape and coated with 20 nm of platinum in an EMS150T Turbo-Pumped Sputter Coater. Cells were then observed using a JEOL 6500 10-kV SEM (Institute for Imaging & Analytical Technologies, Starkville, MS).

6.9 Transmission Electron Microscopy (TEM).

For TEM, amoeboid and cyst cells of *Janelia veilia* isolate HHMI3 were grown on wMY agar plates until they reached a dense culture stage. Plates were then flooded with spring water and cells were scraped from the surface of the agar with a rubber scraper. 50 µL of the cell suspension was transferred to a 2 mL microcentrifuge tube. Cells were fixed with 2 mL of half strength Karnovsky's solution (2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1M sodium cacodylate buffer). Cells were fixed for 1 h, centrifuged at 2000xG for 1 min, and washed three times in 0.1M sodium cacodylate buffer, centrifuging between washes. After rinsing, cells were postfixed in 1% (v/v) OsO4 for 1 h. Post fixative was washed off with three 0.1M sodium cacodylate buffer washes, centrifuging between washes. Cells were dehydrated through graded ethanol series. Each step was 15 min and samples were centrifuged after each step—30%, 50%, 70%, 80%, 85%, 95%, and 100%. The 100% ethanol was gently pipetted off, and cells were changed into a 1:1 ratio of propylene oxide and 100% ethanol for 5 min. This was pipetted off, and cells were changed into a 1:1 ratio of propylene oxide and Spurr's resin. Cells were infiltrated overnight on a rotating disc. The next day, cells were changed into Spurr's epoxy resin and infiltrated on a rotating disc for 3 h. The epoxy resin was changed out for fresh Spurr's epoxy resin and was polymerized at 55°C overnight. Once polymerized, the 2 mL microcentrifuge tube was cut away, and sections (70-80 nm) were cut with diamond knife on a Reichert Jung Ultracut E ultramicrotome (Reichert-Jung, Buffalo, NY, USA). Sections were stained with 2% uranyl acetate for 25 minutes and washed three times in DI water. Sections were observed using a JOEL 2100 TEM at 200 kV (Institute for Imaging & Analytical Technologies, Starkville, MS).

FUNDING

This work was supported by the National Science Foundation (NSF) Division of Environmental Biology (DEB) through the grant 2100888 awarded to Matthew W. Brown.

ACKNOWLEDGEMENTS

This sample was collected during the *New Opportunities to Study Origins of the Eukaryotic Cell* meeting held at the Howard Hughes Medical Institute (HHMI) Janelia Research Campus. We thank the organizers of the meeting for the invitation to MWB. We would also like to thank Paige Iammarino and Dr. Rooban Venkatesh K. G. Thirumalai from the Institute for Imaging & Analytical Technologies at Mississippi State University for their help with SEM and TEM preparation and imaging.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Jones. Isolation, culturing, Gene tree parsing, manuscript first draft, reviewing and writing
Brown. first draft, Gene tree parsing, experimental design, manuscript reviewing and writing
Kleitz-Singleton. Isolation, culturing, Gene tree parsing, manuscript reviewing and writing
Tice. Culturing, Gene tree parsing, manuscript reviewing and writing
Blandenier. Isolation, Culturing, RNAseq, Gene tree parsing, manuscript first draft, reviewing and writing
Banson. Morphometrics, Microscopy, Gene tree parsing, SEM, reviewing and writing
Henderson. Culturing, morphometrics, Microscopy, Gene tree parsing, reviewing and writing
Fry. Culturing, SEM, Gene tree parsing, manuscript reviewing and writing
Nguyen. TEM

DATA AVAILABILITY

All molecular data associated with this manuscript are available on FigShare ({URL to be provided}). This includes transcriptome assemblies, predicted proteomes, alignments (trimmed and untrimmed), as well as phylogenetic trees.

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.

REFERENCES

Adl, S. M., Bass, D., Lane, C. E., Lukeš, J., Schoch, C. L., Smirnov, A., Agatha, S., Berney, C., Brown, M. W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., Campo, J. del, Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., … Zhang, Q. (2019). Revisions to the Classification, Nomenclature, and Diversity of Eukaryotes. *Journal of Eukaryotic Microbiology*, *66*(1), 4–119. https://doi.org/10.1111/jeu.12691

Atlan, D., Coupat-Goutaland, B., Risler, A., Reyrolle, M., Souchon, M., Briolay, J., Jarraud, S., Doublet, P., & Pélandakis, M. (2012). *Micriamoeba tesseris* nov. gen. nov. sp.: a new taxon of free-living small-sized Amoebae non-permissive to virulent Legionellae. *Protist*, *163*(6), 888–902. https://doi.org/10.1016/j.protis.2012.04.006

Baumgartner, M., Yapi, A., Gröbner-Ferreira, R., & Stetter, K. O. (2003). Cultivation and properties of Echinamoeba thermarum n. sp., an extremely thermophilic amoeba thriving in hot springs. *Extremophiles: life under extreme conditions*, *7*(4), 267–274. https://doi.org/10.1007/s00792-003-0319-6

Blandenier, Q., Seppey, C. V. W., Singer, D., Vlimant, M., Simon, A., Duckert, C., & Lara, E. (2017). *Mycamoeba gemmipara* nov. Gen., nov. Sp., the First Cultured Member of the Environmental Dermamoebidae Clade LKM74 and its Unusual Life Cycle. *Journal of Eukaryotic Microbiology*, *64*(2), 257–265. https://doi.org/10.1111/jeu.12357

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Brown, M. W. (2021). Effective and efficient cytoskeleton (actin and microtubules) fluorescence staining of adherent eukaryotic cells. *Protocols.io*. https://dx.doi.org/10.17504/protocols.io.8wkhxcw

Brown, M.W., Silberman, J.D., Spiegel, F.W. (2012). A contemporary evaluation of the acrasids (Acrasidae, Heterolobosea, Excavata). *European Journal of Protistology*. 48(2): 103-123.

Brown, M.W., Kolisko, M., Silberman, J.D., Roger, A.J. (2012). Aggregative Multicellularity Evolved Independently in the Eukaryotic Supergroup Rhizaria, *Current Biology* 22, 1123-1127. https://doi.org/10.1016/j.cub.2012.04.021

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics*, *10*, 1–9. https://doi.org/10.1186/1471-2105-10-421

Capella-Gutiérrez, S., Silla-Martínez, J. M., & Gabaldón, T. (2009). trimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics*, 25(15), 1972–1973. https://doi.org/10.1093/bioinformatics/btp348

Cavalier-Smith, T., Chao, E.-Y., Oates, B. 2004. Molecular phylogeny of Amoebozoa and the evolutionary significance of the unikont Phalansterium. *European Journal of Protistology*, **40,** 21–48.

Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2016). GenBank. *Nucleic Acids Research*, *44*(D1), D67–D72. https://doi.org/10.1093/nar/gkv1276

Criscuolo, A., & Gribaldo, S. (2010). BMGE (Block Mapping and Gathering with Entropy): A new software for selection of phylogenetic informative regions from multiple sequence alignments. *BMC Evolutionary Biology*, *10*(1). https://doi.org/10.1186/1471-2148-10-210

Dyková, I., Pecková, H., & Kostka, M (2008). Introduction of *Mayorella gemmifera* Schaeffer 1926 into phylogenetic studies of Amoebozoa. *Acta Protozoologica*, 47: 205-210.

Eme, L., Sharpe, S. C., Brown, M. W., & Roger, A. J. (2014). On the Age of Eukaryotes: Evaluating Evidence from Fossils and Molecular Clocks. *Cold Spring Harbor Perspectives in Biology*, *6*(8), a016139. https://doi.org/10.1101/CSHPERSPECT.A016139

Fry, N., Schuler, G.A., Jones, R.E., Kooienga, P.G., Jira, V., Shepherd, M., Tice, A.K., Brown, M.W. (2024). Living in the cracks: Two novel genera of Variosea (Amoebozoa) discovered on an urban sidewalk. *Journal of Eukaryotic Microbiology*, 00, e13020. https:// doi.org/10.1111/jeu.13020

Haas, B., Papanicolaou, A., Yassour, M. *et al.* (2013). *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols,* **8**, 1494–1512. https://doi.org/10.1038/nprot.2013.084

Jamy M, Foster R, Barbera P, Czech L, Kozlov A, Stamatakis A, Bass D, Burki F. (2020). Long metabarcoding of the eukaryotic rDNA operon to phylogenetically and taxonomically resolve environmental diversity. *Mol. Ecol. Resourc*. 20(2):429-443. https://doi.org/10.1111/1755-0998.13117

Jones, R. E., Tice, A. K., Eliáš, M., Eme, L., Kolísko, M., Nenarokov, S., Pánek, T., Rokas, A., Salomaki, E., Strassert, J. F. H., Shen, X.-X., Žihala, D., & Brown, M. W. (2024). Create, Analyze, and Visualize Phylogenomic Datasets Using PhyloFisher. *Current Protocols*, *4*(1), e969. https://doi.org/10.1002/cpz1.969

Kang, S., Tice, A. K., Spiegel, F. W., Silberman, J. D., Pánek, T., Cepicka, I., Kostka, M., Kosakyan, A., Alcântara, D. M. C., Roger, A. J., Shadwick, L. L., Smirnov, A., Kudryavtsev, A., Lahr, D. J. G., & Brown, M. W. (2017). Between a Pod and a Hard Test: The Deep Evolution of Amoebae. *Molecular Biology and Evolution*, *34*(9), 2258–2270. https://doi.org/10.1093/molbev/msx162

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Kudryavtsev, A., Voytinsky, F., & Volkova, E. (2022). *Coronamoeba villafranca* gen. Nov. sp. Nov. (Amoebozoa, Dermamoebida) challenges the correlation of morphology and phylogeny in Amoebozoa. *Scientific Reports*, *12*(1), 12541. https://doi.org/10.1038/s41598-022-16721-2

Laurin, V., Labbé, N., Parent, S., Juteau, P., & Villemur, R. (2008). Microeukaryote Diversity in a Marine Methanol-Fed Fluidized Denitrification System. *Microbial Ecology*, *56*(4), 637–648. DOI: https://doi.org/10.1007/s00248-008-9383-x

Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., Haeseler, A. V., Lanfear, R., & Teeling, E. (2020). IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Molecular Biology and Evolution*, *37*(5), 1530–1534. https://doi.org/10.1093/molbev/msaa015

Olive, L., Bennett, W. & Stoianovitch, C. (1983) Redescription of the protostelid genus *Microglomus*, its type species and a new variety. *Transactions of the British Mycological Society*, 81, 449–454.

Olive, L.S. & Stoianovitch, C. (1977) A New Microsporangial Protostelid, *Microglomus paxillus* gen. and sp. nov.. *The Journal of Protozoology,* 24, 485-489. DOI: https://doi.org/10.1111/j.1550-7408.1977.tb00997.x

Onsbring, H., Tice, A. K., Barton, B. T., Brown, M. W., Ettema, T.J.G., 2020. An efficient single-cell transcriptomics workflow for microbial eukaryotes benchmarked on *Giardia intestinalis* cells. *BMC Genomics* 21, 448.

Page F. C. (1967). Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. *The Journal of Protozoology*, *14*(3), 499–521. https://doi.org/10.1111/j.1550-7408.1967.tb02036.x

Page, F. C. (1975) A new family of amoebae with fine pseudopodia. *Zoological Journal of the Linnean Society*. 56(1): 73–89. https://doi.org/10.1111/j.1096-3642.1975.tb00811.x

Picelli, S., Faridani, O. R., Björklund, Å. K., Winberg, G., Sagasser, S., Sandberg, R., 2014. Full-length RNA-seq from single cells using Smart-seq2. *Nature Protocol* 9, 171–181. https://doi.org/10.1038/nprot.2014.006

Porfirio-Sousa, A. L., Tice, A. K., Morais, L., Ribeiro, G. M., Blandenier, Q., Dumack, K., Eglit, Y., Fry, N. W., Souza, M. B. G. E., Henderson, T., Kleitz-Singleton, F., Singer, D., Brown, M. W., Lahr, D. J. G. (2024). Amoebozoan testate amoebae illuminate the diversity of heterotrophs and the complexity of ecosystems throughout geological time. *Proceedings of the National Academy of Sciences, USA*. 121 (30) e2319628121. https://doi.org/10.1073/pnas.2319628121

Ptáčková E., Kostygov A. Yu., Chistyakova L. V., Falteisek L., Frolov A. O., Patterson D. J., Walker G. & Cepicka I. (2013). Evolution of Archamoebae: Morphological and Molecular Evidence for

Pelobionts Including *Rhizomastix*, *Entamoeba*, *Iodamoeba*, and *Endolimax*. *Protist*, **164**:380–410. https://doi.org/10.1016/j.protis.2012.11.005

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi: https://doi.org/10.7717/peerj.2584

Shadwick, L. L., Spiegel, F. W., Shadwick, J. D. L., Brown, M. W., & Silberman, J. D. (2009). Eumycetozoa = Amoebozoa?: SSUrDNA Phylogeny of Protosteloid Slime Molds and Its Significance for the Amoebozoan Supergroup. *PLoS ONE*, *4*(8), e6754. https://doi.org/10.1371/journal.pone.0006754

Smirnov, A., Nassonova, E., Berney, C., Fahrni, J., Bolivar, I., Pawlowski, J., 2005. Molecular Phylogeny and Classification of the Lobose Amoebae. *Protist* 156, 129–142. https://doi.org/10.1016/j.protis.2005.06.002

Smirnov, A. V., Bedjagina, O. M., & Goodkov, A. V. (2011). Dermamoeba algensis n. Sp. (Amoebozoa, Dermamoebidae) – An algivorous lobose amoeba with complex cell coat and unusual feeding mode. *European Journal of Protistology*, *47*(2), 67–78. https://doi.org/10.1016/j.ejop.2010.12.002

Spiegel, F. W., Shadwick, L. L., Ndiritu, G. G., Brown, M. W., Aguilar, M. & Shadwick, J. D. (2017) Protosteloid amoebae (Protosteliida, Protosporangiida, Cavosteliida, Schizoplasmodiida, Fractoviteliida, and Sporocarpic members of Vannellida, Centramoebida, and Pellitida). In: Handbook of the Protists, Vol. 38. Cham: Springer, pp. 10–1007.

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*(9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Tekle, Y. I., Grant, J., Anderson, O. R., Nerad, T. A., Cole, J. C., Patterson, D. J., & Katz, L. A. (2008). Phylogenetic placement of diverse amoebae inferred from multigene analyses and assessment of clade stability within 'Amoebozoa' upon removal of varying rate classes of SSU-rDNA. *Molecular Phylogenetics and Evolution*, *47*(1), 339–352. https://doi.org/10.1016/j.ympev.2007.11.015

Tekle, Y. I., Wang, F., Wood, F. C., Anderson, O. R., & Smirnov, A. (2022). New insights on the evolutionary relationships between the major lineages of Amoebozoa. *Scientific Reports*, *12*(1), 11173. https://doi.org/10.1038/s41598-022-15372-7

Tice, A. K., Shadwick, L. L., Fiore-Donno, A. M., Geisen, S., Kang, S., Schuler, G. A., Spiegel, F. W., Wilkinson, K. A., Bonkowski, M., Dumack, K., Lahr, D. J. G., Voelcker, E., Clauß, S., Zhang, J., & Brown, M. W. (2016). Expansion of the molecular and morphological diversity of Acanthamoebidae (Centramoebida, Amoebozoa) and identification of a novel life cycle type within the group. *Biology Direct*, *11*(69). https://doi.org/10.1186/s13062-016-0171-0

Tice, A. K., Spiegel, F. W., & Brown, M. W. (2023). Phylogenetic placement of the protosteloid amoeba *Microglomus paxillus* identifies another case of sporocarpic fruiting in Discosea (Amoebozoa). *Journal of Eukaryotic Microbiology*, *70*(4), e12971. https://doi.org/10.1111/jeu.12971

Tice, A. K., Žihala, D., Pánek, T., Jones, R. E., Salomaki, E. D., Nenarokov, S., Burki, F., Eliáš, M., Eme, L., Roger, A. J., Rokas, A., Shen, X. X., Strassert, J. F. H., Kolísko, M., & Brown, M. W. (2021). PhyloFisher: A phylogenomic package for resolving eukaryotic relationships. *PLoS Biology*, *19*(8). https://doi.org/10.1371/journal.pbio.3001365

Volkova E. & Kudryavtsev A. 2021. A morphological and molecular reinvestigation of *Janickina pigmentifera* (Grassi, 1881) Chatton 1953 – an amoebozoan parasite of arrow-worms (Chaetognatha). *International Journal of Systematic and Evolutionary Microbiology*, **71**. https://doi.org/10.1099/ijsem.0.005094

Wichelen J. V., D'Hondt S., Claeys M., Vyerman W., Berney C., Bass D. & Vanormelingen P. 2016. A Hotspot of Amoebae Diversity: 8 New Naked Amoebae Associated with the Planktonic Bloom-forming Cyanobacterium *Microcystis*. *Acta Protozoologica*, **55**:61–87. https://doi.org/10.4467/16890027AP.16.007.4942

FIGURE LEGENDS

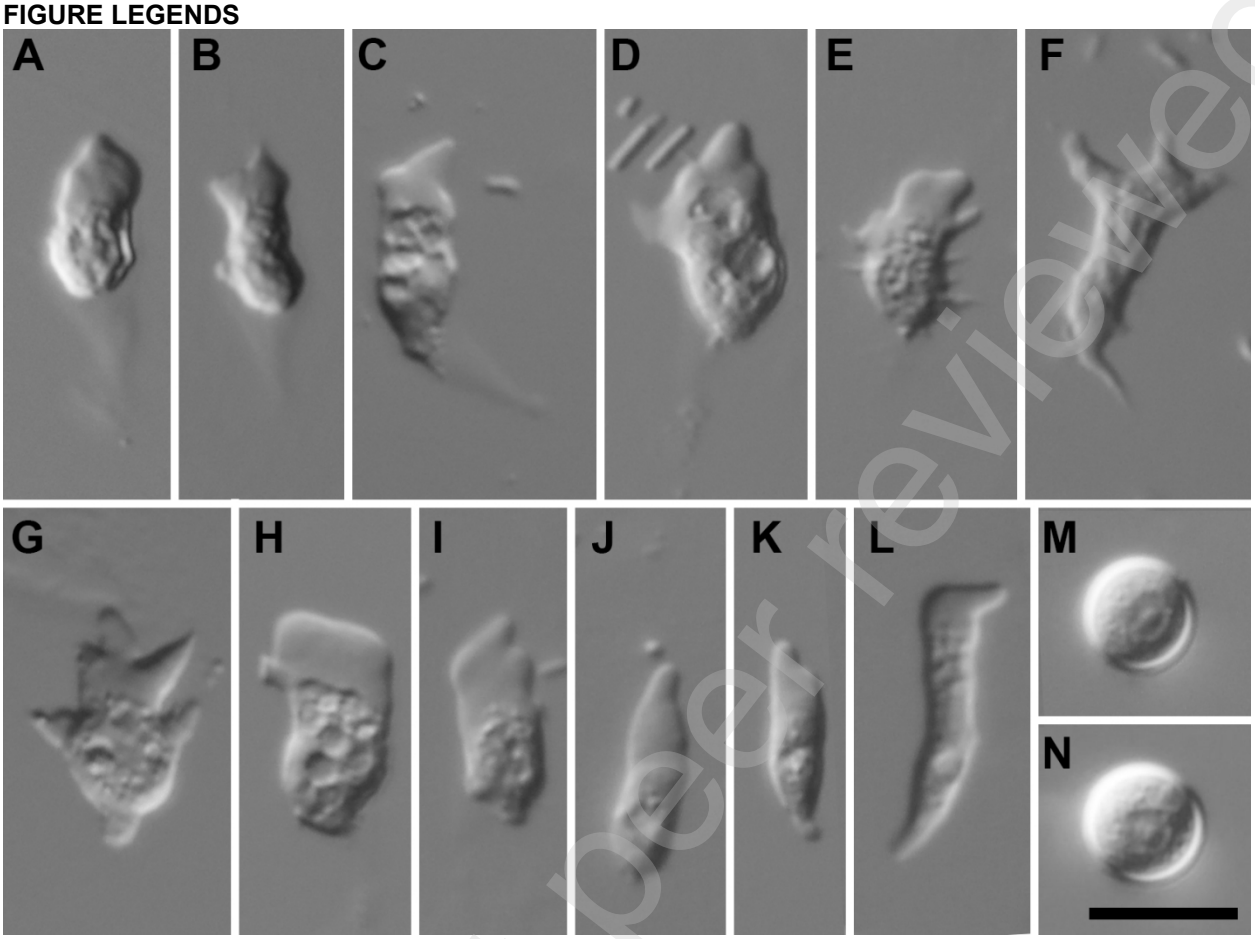


Fig. 1. Light Microscopy. Differential interference contrast (DIC) images of cells of *Janelia veilia* strain HHMI3. A-L) amoeboid cells. M-N) cysts. Scalebar = 10µm, all images are to scale.

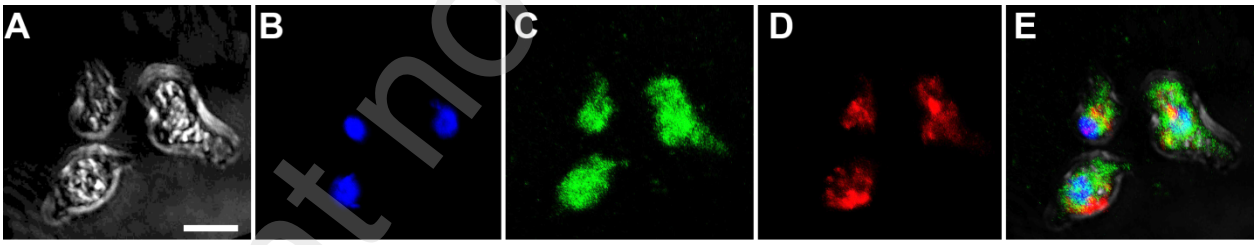


Fig. 2. Confocal microscopy. Confocal micrographs of *Janelia veilia* strain HHMI3. A) Light microscopy, B) DNA NucBlue ReadyProbes, C) ActinGreen 488nm ReadyProbes, D) Microtubule immunocytochemistry antibody stain, E) Overlay. Scalebar = 5µm, all images are to scale.

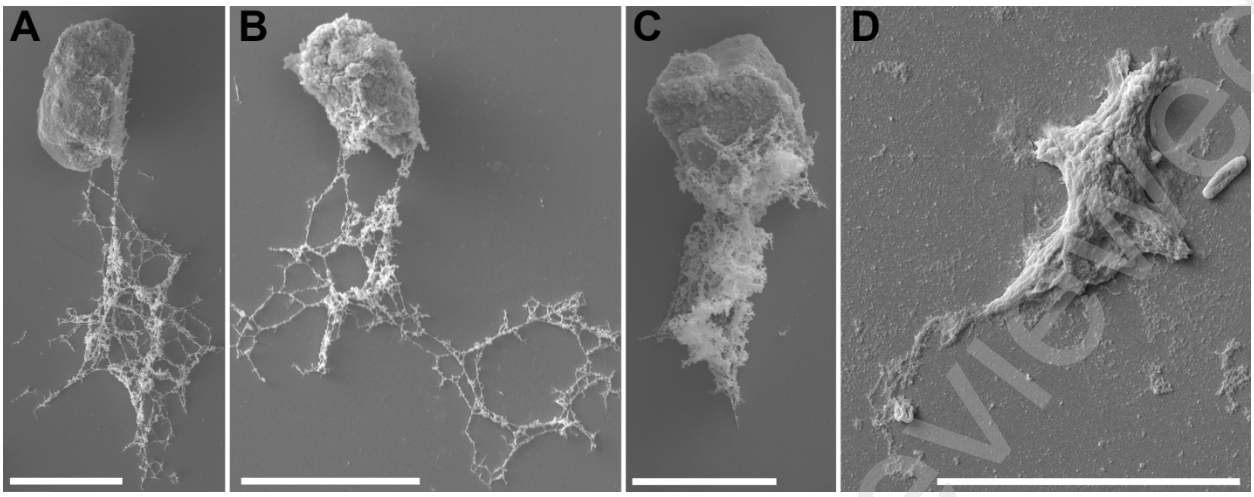


Fig. 3. Scanning electron microscopy images of *Janelia veilia* strain HHMI3. A-D) amoebae with trailing veil. Scalebar in each image = 10μm, images not to scale with one another.

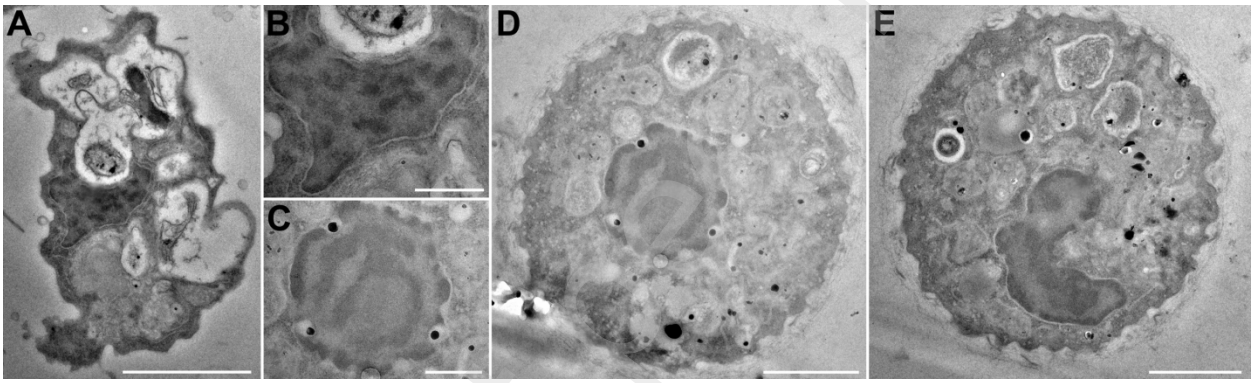


Fig. 4. Transmission electron microscopy images of *Janelia veilia* strain HHMI3. A) Amoeba, Scale bar = 2μm. B) Nucleus of the amoeba in A, Scale bar = 500nm. C) Nucleus of the cyst in D, Scale bar = 500nm. D) Cyst, Scalebar = 1μm. E) Cyst, Scalebar = 1μm.

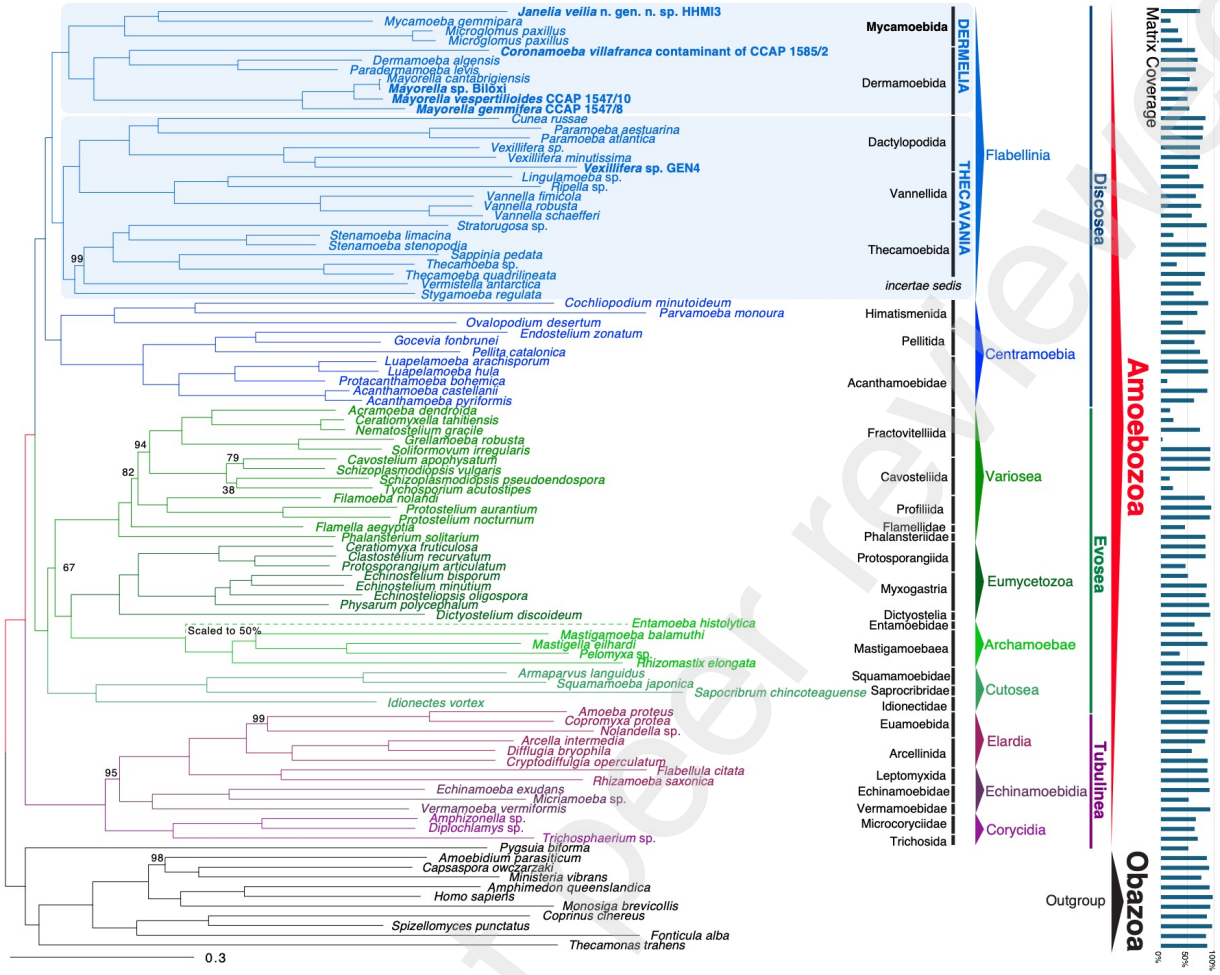


Fig. 5. Phylogenomic tree of Amoebozoa. Maximum Likelihood tree of Amoebozoa including our novel transcriptomes (in bold), using Obazoa as an outgroup. The tree was inferred using our PhyloFisher constructed supermatrix of 240 orthologs and 75,380 amino acid sites in IQTree2 under LG+G4+C60 model. Relevant clades are defined by labeled vertical bars. Values at each node represent bootstrap percentages inferred through real non-parametric maximum likelihood bootstrapping under the LG+G4+C60+PSMF model of evolution. Blank nodes represent full bootstrap support. Histogram to the right represents the percent coverage of the phylogenomic supermatrix per taxon.

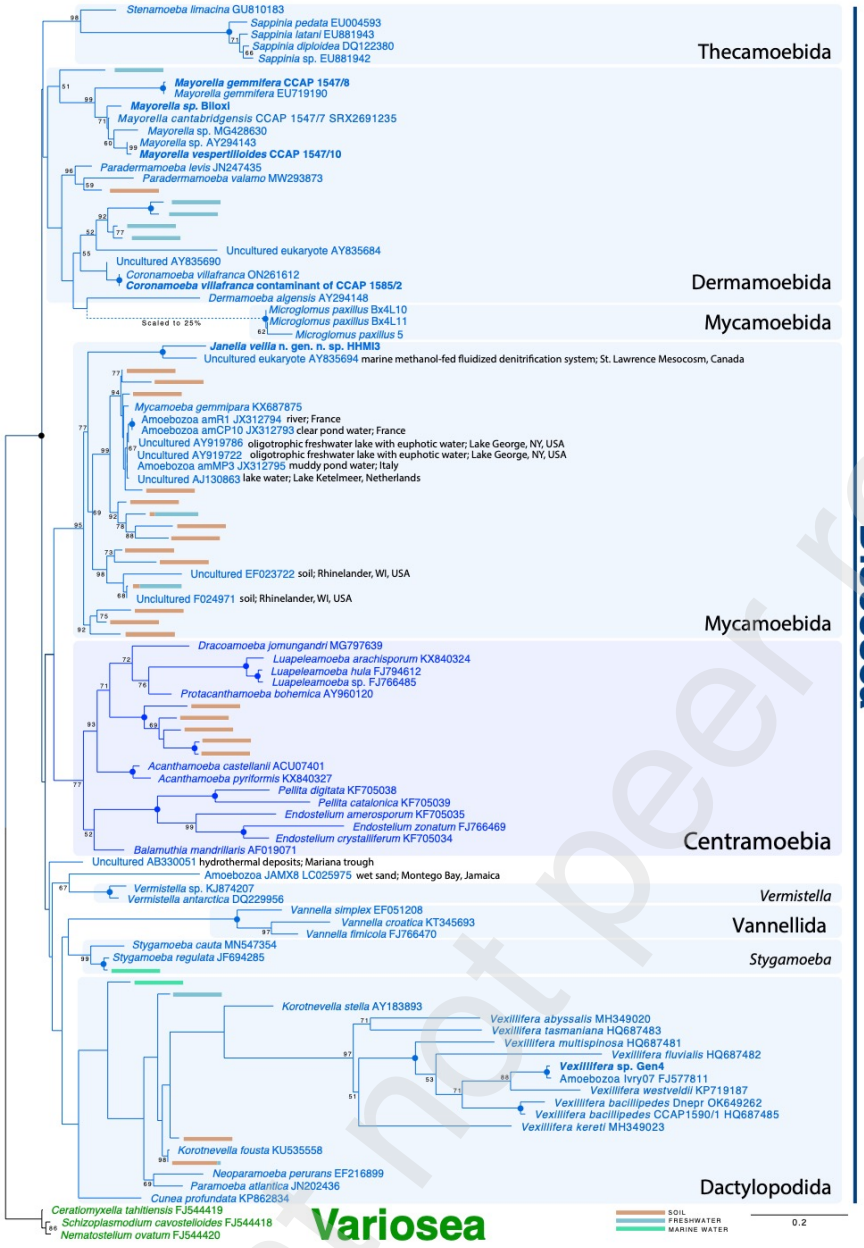
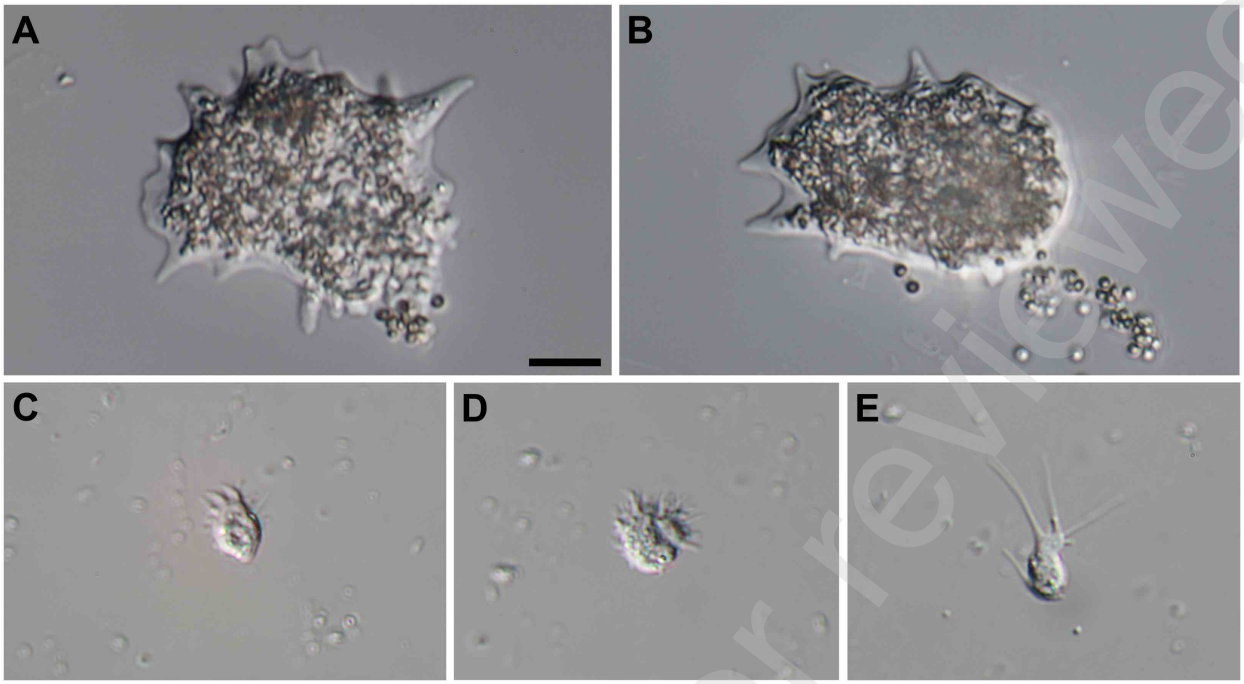


Fig. 6. SSU rRNA gene phylogenetic tree focused on Discosea. Maximum likelihood (ML) SSU rRNA gene tree of 59 Discosea taxa and the 30 discosean clusters identified from environmental data of Jamy et al. 2020, with 3 Variosea taxa as an outgroup. The tree was constructed with RAxML using the GTRCAT model of evolution with 1037 nucleotides sites. Topological support of phylogenetic nodes was assessed with 100 ML bootstrap replicates and are mapped on each node. Nodes without numbers represent bootstrap support values under 50%. Colors of branches and taxa correspond to conventional colors used in Kang et al. (2017). Terminal taxa illustrated as colored bars are clusters of environmental data from Jamey et al. (2020). They are represented as a proportion of reads attributed to each environment sampled in Jamy et al. (2020). For environmental sequences collected from GenBank that are closely related to *Janelia veilia*, environmental information is included next to their names.



Supplemental Fig. 1. Additional discosean isolates

Light microscopy images of various morphologies of additional discosean isolates. A-B) *Mayorella* sp. isolate Biloxi, scale bar 10µm, all images to scale. C) *Vexillifera* sp. isolate Gen4 displaying anterior short, pointed pseudopods. D). *Vexillifera* sp. isolate Gen4 displaying short, pointed pseudopods in all directions. E) *Vexillifera* sp. isolate Gen4 displaying long pseudopods.