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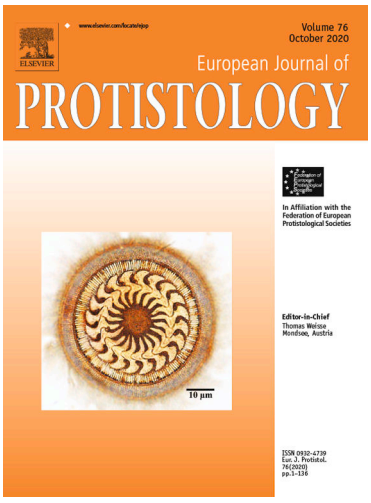

High above the rest: Standing behaviors in amoebae of *Sappinia* and *Thecamoeba*

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TITLE

High above the rest: Standing behaviors in amoebae of *Sappinia* and *Thecamoeba*

SHORT TITLE

Standing Behavior in Terrestrial Amoebae

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Abstract

Many terrestrial microbes have evolved cell behaviors that help them rise above their substrate, often to facilitate dispersal. One example of these behaviors is found in the amoebae of *Sappinia pedata*, which actively lift most of their cell mass above the substrate, known as standing. This standing behavior was first described in *S. pedata* in the 1890s from horse dung isolates but never molecularly characterized from herbivore dung. Our study expands this understanding, revealing the first molecularly confirmed *S. pedata* from herbivore dung in Mississippi, USA, and describing a new species, *Sappinia dangeardi* n. sp., with larger trophozoite cells. Additionally, we isolated another standing amoeba, *Thecamoeba homeri* n. sp., from soil, exhibiting a previously unreported "doughnut shape" transient behavior. In *S. dangeardi* n. sp., we discovered that standing is likely triggered by substrate drying, and that actin filaments actively localize in the “stalk” to support the standing cells, as observed through confocal microscopy. While the purpose of standing behaviors has not been investigated, we hypothesize it is energetically expensive and therefore a significant evolutionary strategy in these organisms. Overall, this study emphasizes behavioral adaptations to terrestrial environments within Amoebozoa, stressing the importance of diverse laboratory conditions that replicate natural habitats.

Keywords

Thecamoebida; Amoebozoa; Discosea; Amoeba; Slime Mold

1. Introduction

The study of non-model protists, particularly those inhabiting terrestrial environments, presents an exciting frontier in biology. With the expansion of molecular

methods, great biological diversity has been exposed and better resolved (Burki et al., 2019, 2021). As we discover novel diversity, it becomes evident that there are more biological mysteries to uncover through direct observation in the lab. We set out to explore terrestrial microhabitats, such as dung and soil, to isolate, culture, and observe eukaryotic microbes focused primarily on amoeboid taxa. From this, we have found novel diversity within the genera *Sappinia* and *Thecamoeba* (Amoebozoa, Discosea, Thecamoebida), and recorded remarkable standing behaviors that have not been previously observed in some members of these genera.

In the late 1890s, Pierre Clement Augustin Dangeard, a pioneering mycologist, discovered *Sappinia pedata* forming macroscopic white patches on old horse dung cultures that had undergone several cycles of desiccation and high humidity (Dangeard 1896). These patches revealed numerous individuals heaped upon each other, forming what he compared to the "pseudoplasmodium" aggregate of cells to the cellular, sorocarpic, slime molds. Dangeard's work paid tribute to the mycologist Mister Sappin-Trouffy, establishing the genus *Sappinia* and naming this dung-dwelling species *Sappinia pedata*. *S. pedata* has a characteristic ability to stand, where the cell attaches to the substrate and pushes most of its cell mass into the open air, resembling a club or baseball bat shape. Dangeard shed light on the standing behavior of *S. pedata* and its remarkable nuclear division where nuclei would divide as closely apposed pairs or quadruples. E. W. Olive (1902) and Kenneth B. Raper (1960) further expanded on Dangeard's work, focusing on *Sappinia*'s aggregative cellular interactions, standing behavior (mistaken as formation of a walled-cell spore), and adaptation to varying environmental factors. Looking at herbivore dung in the United States, E. W. Olive hypothesized that there are two species of standing *Sappinia* differing in size, also noted in Cook (1939) from *Bos taurus* dung in Bristol, England. At this time, *S. pedata* was still held as a slime mold in the Acrasiaceae much like other amoebae from herbivore dung and dead plant material (Dictyostelids, *Copromyxa*, *Guttulinopsis*, *Fonticula*, *Sorodiplophrys*, *Pocheina*, and *Acrasis*) now known to be polyphyletic across Amoebozoa, Rhizaria, Obazoa, Stramenopiles, and Excavata (Brown and Silberman, 2013; Tice and Brown, 2022).
Dangeard, Olive, Cook, and Raper all studied *S. pedata* exclusively from dung samples, but *S. pedata* may also be frequently found on dead plant materials (Brown et al., 2007). Brown et al. (2007) neotypified a leaf isolate of S*appinia pedata* and examined it with molecular phylogenetics along with three additional globally distributed, plant-isolated strains of *S. pedata*. Until now, a dung-dwelling strain of *Sappinia pedata* has yet to be molecularly confirmed calling into question if *S. pedata* from dung is conspecific with *S. pedata* from leaves as proposed by Brown et al. (2007).

Other members of *Sappinia* have much in common with the morphology of *S. pedata* described by Dangeard. They are also primarily terrestrial amoebae, distinguished by paired nuclei (most often a single pair) and locomotive forms are monopodial lingulate ovate amoebae with ridges (wrinkles) along the sides of their cell body (Goodfellow et al., 1974; Page, 1976; Page, 1988; Brown et al., 2007; Walochnik et al., 2010). *Sappinia diploidea* was first described as "*Amoeba diploidea*" by Max Hartmann and Kurt Nägler in 1908. They isolated it from the intestinal material of a lizard's colon and were intrigued by the genus *Sappinia* due to shared morphology with *S. pedata*. In 2014, *Sappinia platani* was described and showed standing in older cultures although it appeared to be much less frequent than in *S. pedata* (Wylezich et al., 2014). This behavior was only mentioned

in the text and no photographic evidence was published of the standing cells. *Sappinia* species have been isolated from various herbivore dung (Dangeard, 1896; Olive, 1902), dead plant material, barks, mosses, soils, and freshwater ponds (Brown et al., 2007, Michel et al., 2006). In 2001, *Sappinia* was reported to be a neurological pathogen causing amoebic encephalitis (Gelman et al., 2001; Gelman et al., 2003), molecularly confirmed to be *S. pedata* sensu Brown et al. 2007 (Qvarnstrom et al., 2009).

Sappinia was placed into the Thecamoebidae (Schaeffer, 1926) by Page (1987) using traditional morphological characterizations, and later placed molecularly into the clade by Michel et al. (2006). Although the cells of *Sappinia* visually resemble their sister genus, *Thecamoeba*, they lack the characteristic dorsal longitudinal folds indicative of *Thecamoeba* cells (Page, 1976). *Thecamoeba* is recognized by its single, centrally located ovoid nucleus and prominent dorsal longitudinal folds. Found mainly in moist soil and leaf litter (Page, 1977) but also in freshwater habitats (Page, 1976; Page, 1983), *Thecamoeba* cells exhibit a flattened locomotive form with a broad, fan-shaped appearance, and a rugose or striate morphotype (Smirnov and Brown, 2004). Together, *Sappinia* and *Thecamoeba* are relatively common in terrestrial environments and morphologically understood but are not well described in their cellular behaviors.

Here we expand our knowledge of the Thecamoebida group, reporting two new isolates of both *Sappinia* and *Thecamoeba*, representing two new species. Our study contributes to the rich history of research on *Sappinia* and *Thecamoeba*, offering new insights into the interesting cellular behaviors of these commonly found yet morphologically diverse and behaviorally complex amoeboid taxa.

2. Materials and methods

2.1. Sample Processing and Culturing

Locality information for each strain is presented in Table 1. *Sappinia* strains BF22-2A and MSU2206 were isolated from *Bos taurus* dung, incubated at room temperature for 2–3 days, and scanned at 5x magnification on a Leica M205C stereoscope. Standing cells were carefully picked from dung samples using a sterile Minutien Insect Pin (Carolina Biological, Burlington, NC, USA) (Suppl. Video 1). These cells were then transferred to sterilized cow dung that had been soaked in a dense suspension of *Escherichia coli* (K-12 strain MG1655) in commercially available spring water. To prepare sterilized dung, approximately 0.5 kg of fresh cow dung was collected from Mississippi State University cattle farms, placed into a 1 L beaker, and covered with aluminum foil. This setup was autoclaved for 45 minutes to ensure sterilization. For culturing, about 2 g of sterilized dung was placed on the surface of a non-nutrient spring water agar Petri dish (1 L Deer-Park spring water and 15 g agar) (Deer Park Spring Water, BlueTriton Brands, Stamford, CT, USA). The suspension of food bacteria (*E. coli*), as above, was made by scrapping four culture plates of *E. coli* (on LB medium) and vortexing it with 5 mL of spring water. This suspension had an estimated optical density (OD) of eight, calculated by measuring the OD of a 1/16 dilution of the suspension. Clonal cultures were maintained at room temperature, with passages onto fresh media every three weeks. To sub-culture, a small piece of culture dung with standing cells was transferred to the same *E. coli* soaked sterilized dung medium mentioned above.

Thecamoeba strain SK13-4B and SK13-4H were isolated from soil collected in Northern Mississippi, USA (Table 1, 33.455812° N, -88.788126° E). About 1g of the upper soil crust was sterilely collected into a sterile Petri dish, immediately brought into the lab, and suspended in 10mL of sterile spring water. Four drops (ca. 40µL each) of this suspension were put onto a sterile non-nutrient spring water agar Petri dish separated by roughly 2 cm of space between each drop. Once the drops soaked into the agar surface the plate was put upside-down. Observation of the plate using a 10x objective of a compound microscope occurred daily, focusing primarily on the edges of the dried drop of soil suspension. After five days post-plating single amoeba cells were isolated using a 30-gauge platinum wire loop, sterilized by an ethanol flame. To do this, cells were dragged using the Pt loop in the meniscus created by touching the loop to the agar surface from one area of the plate to an area and transferred onto spring water agar with a streak of *E. coli* (MG1655) as a food source. A clonal culture of each was established using this technique. Unfortunately strain SK13-4H was lost and no further cultures remain of this strain.

2.2. Microscopy and Morphometrics

For detailed observations, agar culture slides were made using a small block (ca. 4mm^3 cube) of spring water agar melted onto glass slides with a coverslip on top of the agar block. This was achieved by gently heating the bottom of a glass slide over a Bunsen burner flame to heat the slide surface causing the agar to melt and be sandwiched between the slide and coverslip. After 10 minutes of cooling at room temperature the coverslip was removed by sliding off the thin agar surface, inoculated with amoebae, and covered with a fresh cover slip and sterile spring water. 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 for *Sappinia* spp. and Canon EOS 650D, 18.0MP 4:3 APS-C DSLR for *Thecamoeba* SK13-4B & SK13-4H) controlled by Canon EOS Utility software for Macintosh. Imaging of standing amoebae of *Sappinia* and *Thecamoeba* isolates were imaged under brightfield illumination with a 10x Plan-NeoFluar (NA 0.30) objective directly on the dung substrate or straw by removing the Petri dish lid and imaging from above. Cellular measurements to generate morphometric data for both *Sappinia* strains were obtained by measuring images of active locomotive trophozoites on agar surfaces only taken under a 10x Plan-NeoFluar (NA 0.30) objective in ImageJ software (http://imagej.nih.gov/ij/) with the *Scale Bar tools for Microscopes* utility (http://image.bio.methods.free.fr/ImageJ/?Scale-Bar-Tools-for-Microscopes.html). Length, breadth, and length to breadth ratios were plotted for both strains of *Sappinia* and *Thecamoeba* SK13-4B in R (R Core Team, 2021). Significant differences were tested using two-sample t-tests between both strains of *Sappinia* also in R.

2.3. Video Microscopy and Time-Lapse Microscopy

Our *S. dangeardi* n. sp. isolates from dung habitats regularly stand on sterilized dung (Suppl. Video 2, 3, 4). To capture the process of standing in “natural” habitats, we

employed time-lapse microscopy. For supplemental video 2 and 3, we used sterile forceps to move a piece of dung with numerous standing cells from a dense culture from a dung culture plate, placed it on an agar-coated slide (described above, but without a coverslip overlay), and took an image every five seconds under a 10x objective with transmitted light (same camera setup as above). Alternatively for Suppl. Video 4 we took time-lapse microscopy on dung culture plates under reflected light, using a Canon EOS 650D camera connected to a Leica M205C stereomicroscope. The resulting image sets were compiled together in Adobe Premiere Pro (Mountain View, CA, USA) and exported as an MP4 video at 30 frames per second. To create a timecode in the video, Adobe After Effects and custom JavaScript were used (found on TheBrownLab GitHub: https://github.com/TheBrownLab/time-lapse.tools). The process of doughnut cell morphology formation in *Thecamoeba* SK13-4B was captured with brightfield real-time video microscopy (Suppl. Video 5) as noted above using brightfield microscopy under 10x with the plate inverted upside-down imaging through the agar medium. Canon EOS utility was used for video acquisition.

2.4. Induction of standing in Thecamoeba

The formation of standing bodies was tested for *Thecamoeba* strain SK13-4B by soaking sterilized straw in a slurry of sterile spring water (ca. 500 µL) containing loop of *E. coli* grown on a nutrient agar and placing the soaked straw on an agar Petri dish containing the amoeba culture near a dense patch of amoebal growth according to Brown et al. (2013). Straw was subsequently observed for one week by scanning the edges of the substrate using a 10x objective with a compound microscope. After a few days of observation, standing was observed on the straw edge. The same technique was used for *Thecamoeba* strain SK13-4H, but no standing was observed.

2.5. Cytoskeletal Staining

About 15 standing cells of *Sappinia* (BF22-2A) were picked directly off the agar/sterile dung culture using a flame sterilized Minutien needle. This was achieved by picking one cell then using that cell to pick the next cell, creating a chain of standing cells. The chain was placed onto the center of one well of a sterile 2-well cell culture chamber glass slide (NEST Scientific USA, Woodbridge, NJ, USA). Immediately under a dissecting stereoscope, 1000µL of -80°C methanol was gently put into the well of the slide, while watching the chain of cell being careful to not disrupt or cause cells to detach. Cells were fixed for 10 seconds in the methanol after washing in sterile spring water the cells were stained following the protocol from Porfirio-Sousa et al. (2023). Cells were labeled with ActinGreen 488-nm ready probes (Life Tech | R37110) followed by NucBlue ReadyProbes (Life Tech | R37605). Subsequently after 3 rounds of washing, the cells were mounted in Fluoromount-G (Life Tech | 00-4958-02) with a clean 1.5H coverslip (0.170mm +/- 0.005mm), allowed to dry, and sealed with clear nail polish. Cells were visualized with an inverted confocal microscope (Leica TCS SPE-II, Leica Microsystems, Wetzlar, Germany) 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.

231
232 *2.6. Genomic DNA Extraction*
233
234 Cells of *Sappinia* isolates (BF22-2A and MSU2206) were maintained on sterile
235 dung inoculated with a slurry of *E. coli*. Since these isolates only grew well on dung as a
236 medium, to isolate genomic DNA from these strains about 100 individual cells were picked
237 using a Minutien insect needle and placed directly into QuickExtract solution (LGC
238 Biosearch Technologies-Epicentre, Madison WI, USA) following the manufacturer's
239 recommended protocol. *Thecamoeba* SK13-4B cells were grown on agar surfaces where
240 they ate the *E. coli* streaked onto the plate. Dense cultures of amoebae on agar plates
241 were used for DNA extraction. An area of dense cells were picked using a metal loop and
242 transferred to a 200µL PCR tube with 100 µL of QuickExtract. DNA was processed
243 following the manufacturer's recommended protocol and in accordance with Walthall et
244 al. (2016).
245
246 *2.7. SSU rDNA Amplification*
247
248 The near full-length SSU rDNA was PCR amplified using the universal eukaryotic
249 SSU rDNA primers, 5AmF forward 5'- AACCTGGTTGATCCTGCC (primer S1 in Fiore-
250 Donno et al., 2008) with MedlinB reverse 5'-
251 CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACCTAC (Medlin et al., 1988) and
252 GoTaq Green Master Mix (Promega). The PCR cycling parameters were 3m at 95°C,
253 followed by 34 cycles of 30s at 95°C, 25s at 48°C, and 3.5min at 72°C. PCR products
254 were purified used Mag-Bind TotalPure NGS magnetic beads (Omega Bio-tek, Inc.,
255 Nocross, GA, USA). The purified PCR amplicons were sequenced directly using the PCR
256 primers and internal sequencing primers. All rDNAs were fully sequenced in both
257 orientations.
258
259 *2.8. Molecular Phylogenetic Analyses*
260
261 The 18S rRNA gene sequences of *S. dangeardi* n. sp. (BF22-2A), *S. pedata* from
262 dung (MSU2206), and *T. homeri* n. sp. (SK13-4B), and sequences from GenBank (Clark
263 et al., 2016) from 72 taxa from Thecamoebida, and the closely related genera
264 *Dermamoeba* and *Paradermamoeba* as outgroup taxa were collected. In addition, the
265 locality information of each strain is presented in Suppl. Table 1, which was used to
266 visualize strain locality in the SSU rDNA phylogenetic tree. All these SSU rDNA
267 sequences were aligned using MAFFT with the L-INS-I algorithm (Katoh and Standley,
268 2013). The aligned sequences were then trimmed with BMGE software (Criscuolo &
269 Gribaldo, 2010) with a global entropy (-g) value of 0.8. The final dataset was visually
270 inspected for alignment errors and contained 1732 unambiguously aligned nucleotide
271 characters. The maximum likelihood tree of SSU rRNA was built from RAxML V. 8.2.12
272 (Stamatakis, 2014) with GTRGAMMAI model of nucleotide substitution. Topological
273 support for this phylogeny was assessed with 1,000 rapid bootstrap replicates.
274 Uncorrected pairwise distances of SSU rRNA genes for *Sappinia* spp. and
275 *Thecamoeba* spp. were inferred by individually collecting all taxa within each genus,
276 realigning these generic sequences alone using MAFFT with the AUTO algorithm. The

aligned sequences were then trimmed with BMGE software (Criscuolo & Gribaldo, 2010) with a global entropy (-g) value of 0.9, yielding an alignment per genus without ambiguously aligned sites. From these uncorrected pairwise distances were calculated in PAUP* (Swofford, 2002) using the "showdist" function (Suppl. Table 2). From these trimmed alignments we also inferred phylogenetic trees in RAxML with GTRGAMMA model of nucleotide substitution. Topological support for each phylogeny was assessed with 100 rapid bootstrap replicates.

3. Results and discussion

3.1. Phylogenetic identity and morphological characteristics of strains and new species

This study illustrates two molecularly identified species of standing *Sappinia* inhabiting herbivore dung from cows. One of these species, strain BF22-2A, is new and herein named "*Sappinia dangeardi"* n. sp. in honor of P. A. Dangeard (Fig. 1A–C). The phylogenetic placement of *S. dangeardi* n. sp. is fully supported in its grouping with *S. diploidea* and *S. platani* (Fig. 2). The exact placement of *S. dangeardi* n. sp. in the genus is ambiguous, branching with poor support as sister to *S. diploidea* (Fig. 2) or branching with *Sappinia* sp. LC311582 in our *Sappinia* only phylogeny also with poor support (Suppl. Fig. 1). Based on a phylogenetic species concept, we consider that this strain represents a new species of *Sappinia*. The second strain, MSU2206, was identified as *Sappinia pedata* by its near full support close grouping with other *S. pedata* isolates neotypified by Brown et al. (2007) from dead leaves (bootstrap support of 99% in Fig. 2 in a global Thecamoebida phylogeny, and 100% in Suppl. Fig. 1 in a phylogeny of *Sappinia*). This makes MSU2206 the first molecularly confirmed herbivore dung isolate of *S. pedata*.

Additionally, we examined the morphology of both *Sappinia* dung isolates. The locomotive cells of *S. dangeardi* n. sp. are significantly larger than *S. pedata* both in length (two sample t-test, p-value = 6.51e-16) and breadth (two sample t-test, p-value = 2.159e-08) (Suppl. Fig. 2). From these data, *S. dangeardi* n. sp. cells measure to be about 10 µm longer and 4 µm wider in breadth than *S. pedata* cells. The standing cells of *S. dangeardi* n. sp. and *S. pedata* show remarkable plasticity in their shape and size. We have measured the height of standing cells showing that *S. dangeardi* n. sp. tends to be significantly taller than *S. pedata* (Suppl. Fig. 2). This may support E. W. Olive's hypothesis that there are two distinct dung-inhabiting species of *Sappinia* distinguished by size (Olive, 1902). However, we qualitatively find that the age of the cultures and humidity can affect standing behavior, further complicating reliable measurements. Because of this, we advise that standing form morphology should not be used for diagnosis of species. Interestingly, the *S. dangeardi* n. sp. and *S. pedata* from dung both display hemispherical paired nuclei as well as the circular paired nuclei that are common across *Sappinia* amoebae. We most often observe this morphology in standing cells that were picked onto a glass slide, and it is more common in *S. dangeardi* n. sp. (Suppl. Fig. 3). This nuclear morphology does not appear to have been reported in *Sappinia* before.

Sappinia sp. CCAP 1575/3 was unintentionally obtained from the Culture Collection of Alga and Protozoa (https://www.ccap.ac.uk/) on October 25, 2021. We had originally purchased the strain CCAP 1575/4 (named *Sappinia diploidea* on CCAP's website), which is the type strain of *Sappinia platani* (PL-247). Through a transcriptome

sequencing project, unrelated to this study, we discovered that the isolate labeled as CCAP 1575/4 is actually CCAP 1575/3 based on BlastN (Altschul et al., 1990) of the SSU rDNA contig from the transcriptome generated from this culture to NCBI nucleotide database is 99.96% identical (1 base pair difference across the BlastN alignment) to *Sappinia* sp. CCAP 1575/3 strain Busnog (EU881942), versus 93% identity to that of the *S. platani* CCAP 1575/4 (EU881943) (data not shown). Thus, the isolate we purchased turned out to be the undescribed strain of *Sappinia* sp. CCAP 1575/3 (Busnog). During our cultivation of this organism we observed multiple standing cells on the agar surface. One standing individual was imaged under a 10x objective as below (Fig. 1F).

We also isolated two new strains of *Thecamoeba* (SK13-4B and SK13-4H), the sister to *Sappinia* spp. to further expand our phylogeny of Thecamoebida. We herein name our strain SK13-4B *Thecamoeba homeri* n. sp. named after the cartoon character Homer Simpson, due to the "doughnut" morphology described below. The morphology of both strains are typical of the genus, with lingulate oblong to somewhat round amoebae with longitudinal folds on the dorsum of the cells (Fig. 3A–C, 4A–C). As noted previously by others, because of shared morphological characteristics, morphological identification of *Thecamoeba* isolates to the species level is often not possible under light microscopy and thus requires molecular characterization of the SSU rRNA gene (Mesentsev et al., 2020). These isolates have an uncorrected pair-wise distance of 0.05, i.e., 5% different in 18S rDNA sequence (Suppl. Table 2), in an alignment of strictly *Thecamoeba* isolates which was also used in a phylogenetic analysis (Suppl. Fig. 4). In our SSU rDNA phylogeny, both new strains form a clade fully supported as sister to *T. similis,* which together are sister to *T. foliovenanda* (Fig. 2). These relationships are further supported by similar morphological characteristics between our strains, *T. similis*, and *T. foliovenanda*. Unfortunately, shortly after isolation and molecular characterization of strain SK13-4H, it was lost, and no further characterization is now possible thus we are unable to adequately describe the strain. The locomotive form of cells from SK13-4B measure on average 65 µm in length and 49 µm in breadth falling morphologically between *T. similis* (36–57 µm and 38–44 µm breadth) and *T. foliovenanda* (71 µm length and 55 µm breadth) (Page, 1977; Mesentsev et al., 2020). Like these species, the nuclei of SK13-4B are rounded and elongated with irregular peripheral nucleoli (Fig. 3D, 4). In addition, we have represented the morphological variation of *T. homeri* n. sp. as violin plots in Supplementary Figure 5.

3.2. Investigation into the behaviors of Sappinia dangeardi *n. sp.*

We further investigated the mechanisms of standing behavior of *Sappinia dangeardi* n. sp. For the cytoskeletal architecture of standing cells, we were able to stain DNA and microfilaments. Through confocal fluorescence microscopy, we observed that actin heavily localizes to the "stalk" of standing cells (Fig. 5). Unlike the true stalk created by dormant fruiting forms of other amoebae, such as protosteloid amoebae (see Spiegel et al., 2017), the "stalk" of *Sappinia* is a temporary structure of the cell itself. Standing *Sappinia* are not dormant. They immediately begin moving when transferred to agar. From our observations, we hypothesize the cells must constantly create physical pressure in the stalk, utilizing actin, to maintain standing. This is an intriguing biophysical phenomenon, as cells can maintain standing completely straight up, upside down, and

horizontally for hours (Supplementary Video 2, 3, 4). We also consistently observe nuclei resting at the lower end of the "head" region of the cell.

The initiation of standing in *S. dangeardi* n. sp. appears to be triggered by substrate drying. A qualitative experiment of three parafilm-sealed and three unsealed cultures supported this hypothesis. When parafilm-sealed, the freshly passed culture was kept moist and there was still growth (seen on the clear agar edges), but little to no standing after 10 hours. After removing parafilm, thousands of cells began standing within 10 hours. In the presence of dry conditions, we also predict it is more advantageous to leave the substrate and to disperse to more favorable conditions. Standing behavior may be beneficial to dispersal via abiotic (rain, wind, etc.) or biotic (animals) vectors. Experiments that gauge standing's contribution to dispersal would be beneficial for future work.

Since 2007, one of us (Brown) has attempted and failed many times to isolate and culture *S. pedata* from dung, which is frequently encountered when working with dung amoebae. In these attempts, cells were simply picked from the dung and placed on a non-nutrient agar along with *E. coli* as a food source. While this method was highly successful for *Sappinia pedata* from dead plant materials (mostly leaves) (Brown et al., 2007), this simply has not worked for dung inhabiting *S. pedata*. *S. dangeardi* also shows a reliance on dung to culture. Over the last few years, we have discovered that dung itself and perhaps the prokaryotic community may be required for the successful cultivation of many dung-inhabiting protistan taxa (personal observation). Moreover, we observed aggregates of *Sappinia* cells on wild dung samples. These *Sappinia* aggregates were subcultured by carefully transferring an aggregate with a sterile insect needle onto sterilized dung seeded with *E. coli*. Contrary to our experiences with subculturing both our *Sappinia* isolates (MSU2206 and BF22-2A), these subcultures showed a resurgence of this aggregative behavior using the same culture method. When subculturing by picking standing single cells, aggregative behavior has not been observed.

3.3. Standing and doughnut behaviors of Thecamoeba homeri *n. sp.*

In addition to the well-known standing behavior in *Sappinia* species, we observed standing-like structures of our *Thecamoeba homeri* n. sp. isolate (SK13-4B) (Fig. 3E–F) after adding sterilized straw soaked in an *E. coli* slurry on top of the agar culture medium. This idea came from observations of primary isolation plates with dead plant material, where we have seen presumed *Thecamoeba* cells protrude most of their cell body (standing) from the edges of the plant material. The standing cell observed in this strain was not as obvious or pronounced as the standing cells in *Sappinia* spp. As it is shorter and appears to be much less infrequent, only observed less than five times.

Another interesting behavior found in *T. homeri* n. sp. (SK13-4B), and of a lost strain of *Thecamoeba* (SK13-4H), is the formation of a transient doughnut-shaped cell morphology (Fig. 3GH, Fig. 4 EF, Supplementary Video 5). When viewed from above (or below) the cells have a doughnut ring-like appearance. Currently, the cellular processes to form this transient morphology are completely unknown, but the behavior is worth noting here as it has never been previously documented. First, the cell appears to form a volcano shape with the hole (crater-like) towards the air. However, we cannot make a definitive description of how this hole closes and if there is another opening on the adhered end of the cell. We also have observed this behavior as a separate process from

contractile vacuole formation (Supplementary Video 6). The process of forming the doughnut shape is very rapid (ca. 2–5 minutes) and the cells seem to frequently perform this behavior at random intervals. Due to this, we have been unable to fix cells for imaging under scanning electron, transmission electron, or immunohistochemical microscopy to clarify the underlying mechanism. As far as we know, this doughnut behavior has not been reported in any other *Thecamoeba* isolate, although it has been noted to occur frequently in unidentified *Thecamoeba* species on primary isolation plates while searching for protosteloid amoebae by other researchers (pers. comm. Spiegel & Tice, as well as ourselves). Both standing and doughnut behaviors may indeed be more prevalent across *Thecamoeba* and even Thecamoebida, but often these taxa are cultured in liquid environmental conditions in the laboratory. These behaviors are unlikely to be observed in liquid conditions, as they appear to be terrestrial phenomena. Future studies are needed to examine standing behaviors among *Thecamoeba* and the whole Thecamoebidae family.

Our research has unveiled two novel isolates, *Sappinia dangeardi* n. sp. and *Thecamoeba homeri* n. sp. each exhibiting unique behaviors and morphologies in terrestrial contexts. Our findings emphasize the importance of considering culture conditions when studying amoeboid taxa, as the use of standard liquid media may lead to overlooking behaviors unique to terrestrial contexts. The induction of standing behavior in our *Thecamoeba* isolate SK13-4B by adding sterilized straw to agar media highlights the potential for mimicking environmental conditions to reveal previously undocumented behaviors in these organisms. Furthermore, this study and other studies reinforce the notion that traditional morphological features, such as standing, bicellular cysts, and paired nuclei, are insufficient for species identification within *Sappinia* (Wylezich et al., 2014). Molecular methods, particularly 18S rRNA phylogenetics, provide an accurate and reliable means of identification and classification, particularly when conferred with morphological characters, revealing cryptic species where morphology fails. Notably, our work, along with a recent study on *Thecamoeba terricola* by Mesentsev et al. (2023), suggests that standing behavior is more widespread among *Sappinia* and *Thecamoeba* species than previously understood. Observing *Sappinia* sp. CCAP 1575/3 (Busnog strain) and its standing behavior further corroborate this expanded view. These findings collectively underscore the dynamic nature of amoeboid taxonomy and the ongoing need to refine our understanding of their ecological adaptations and evolutionary relationships.

Taxonomy of novel species

***Sappinia dangeardi* Henderson & Brown n. sp.**

Taxonomic Summary

Eukaryota (Chatton, 1925) Whittaker & Margulis, 1978
Amorphea Adl et al., 2012
Amoebozoa Lühe, 1913 emend. Cavalier-Smith, 1998
Discosea Cavalier-Smith et al., 2004
Flabellinia Smirnov et al., 2005 *sensu* Kang et al., 2017
Thecamoebida Smirnov and Cavalier-Smith, 2011
Thecamoebidae Schaeffer, 1926
Sappinia Dangeard, 1896

Sappinia dangeardi n. sp. Henderson & Brown, 2024

Diagnosis. *Sappinia dangeardi* n. sp. can be diagnosed by its specific SSU rRNA sequences and by its phylogenetic placement. Ligulate monopodial amoeboid trophozoite cells with lateral side folds. Length in locomotion as observed on agar surfaces is 47–117 µm (average 72 µm, SD = 11, n = 161), width in locomotion 26–63 µm (average 42 µm, SD = 7, n = 161), length to breadth ratio 1.09–2.57 (average 1.75). The nuclei of cells most often display hemispherical morphologies. Cells with two closely appressed hemispherical nuclei measure in diameter across both nuclei at widest point is 9–14 µm (average 12 µm, SD = 1.4, n = 21). The length of hemispherical nuclei measure between 8–11 µm (average 9 µm, SD = 0.8, n = 21). The flat surface of the hemispherical nucleus pairs with flat surface of the other nucleus. Each nucleus is most often observed to have a central hemispherical nucleolus measuring in length 6–10 µm (average 8 µm, SD = 1, n = 21) and breadth 3–5 µm (average 4 µm, SD = 0.5, n = 21). Rarely rounded pairs of nuclei with central round nucleoli are observed. Trophozoite cells forms multiple small contractile vacuoles, merging into one larger vacuole before expulsion (termed "polyvacuole") often associated with the nucleus. Standing cells show a variety of forms, often associated with an erect baseball bat-shaped morphology of 53–159 µm (average 85 µm, SD=201, n=103) in height (length). Aggregative forms have been observed in this strain on the tips of small dung pieces. Cysts have not been observed in this strain. Primarily a bacterivore.

Type location. Strain BF22-2A of *Sappinia dangeardi* n. sp. Was obtained from *Bos taurus* dung on private farm that is home to both cattle and horses near the Mississippi State University campus in Starkville, Mississippi, USA Lat. 33.500459° N, Long. -88.752719° E, in March of 2022.

Type material. The type specimen (BF22-2A) is a permanent slide of fixed cells deposited in the Invertebrate Zoology Collection at the Smithsonian National Museum of Natural History. This slide 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 (BF22-2A) is deposited GenBank under accession {awaiting Accession numbers}.

ZooBank ID: urn:lsid:zoobank.org:act:0CD69482-B9B7-46BB-9F08-29545E79CD59

Etymology. For the specific epithet, we chose *dangeardi* n. sp. in recognition of Dangeard who originally described the genus *Sappinia*.

Differential Diagnosis. *S. dangeardi* n. sp. differs from other known species based on SSU rRNA gene sequences.

***Thecamoeba homeri* Henderson & Brown n. sp.**

Taxonomic Summary

Eukaryota (Chatton, 1925) Whittaker & Margulis, 1978
Amorphea Adl et al., 2012
Amoebozoa Lühe, 1913 emend. Cavalier-Smith, 1998
Discosea Cavalier-Smith et al., 2004
Flabellinia Smirnov et al., 2005 *sensu* Kang et al., 2017
Thecamoebida Smirnov and Cavalier-Smith, 2011
Thecamoebidae Schaeffer, 1926
Thecamoeba Fromentel, 1874
Thecamoeba homeri n. sp. Henderson & Brown, 2024

Diagnosis. Cells are typical of the genus. Dorsally wrinkled or parallel folds oblong amoeboid cells with dorsal wrinkles or parallel folds. Length in locomotion 50–79 µm (average 65 µm, SD = 7, n = 102), width in locomotion 37–65 µm (average 49 µm, SD = 6, n = 102), length to breadth ratio 1.03–1.92 (average 1.34). Nucleus commonly ovoid, length 7–21 µm (average 12.4 µm, SD = 3, n = 101) and breadth 4–14 µm (average 7 µm, SD = 3, n = 101). A single nucleus per cell observed. Numerous nucleoli circular to lobed (ca. 0.5–1 µm in diameter) and parietal on the periphery of the internal wall of the nuclear envelope. Cysts have not been observed. Cells often form a transient doughnut-shaped round structure on the surface of agar lasting 2–7 minutes. Primarily a bacterivore. These morphological and behavioral characteristics are similar to other species, thus confident identification of *T. homeri* n. sp. must be done by SSU rDNA sequencing and analysis.

Type location. Strain SK13-4B of *Thecamoeba homeri* n. sp. Was obtained from the uppermost soil surface on the Mississippi State University campus in Starkville, Mississippi, USA Lat. 33.455812° N, Long. -88.788126° E in May of 2013.

Type material. This type culture (SK13-4B) is deposited in a metabolically inactive state with 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 (SK13-4B) is deposited GenBank under accession {awaiting Accession numbers}.

ZooBank ID: urn:lsid:zoobank.org:act:0D7CD4D5-D76D-4E41-AAAC-8FFECE85AA4E

Etymology. For the specific epithet, we chose "*homeri*" referring to the transient, doughnut-shaped cell morphology observed in this *Thecamoeba* strain. The name is after *The Simpsons* television cartoon character *Homer Simpson*, whose favorite food are doughnut pastries (donut).

Differential Diagnosis. *T. homeri* n. sp. differs from other known species based in SSU rRNA gene sequences.

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.

Acknowledgements

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Data availability

All molecular data and supplemental files (figures and videos) associated with this manuscript are available on FigShare (https://doi.org/10.6084/m9.figshare.24772854. This includes alignments (trimmed and untrimmed) and phylogenetic trees.

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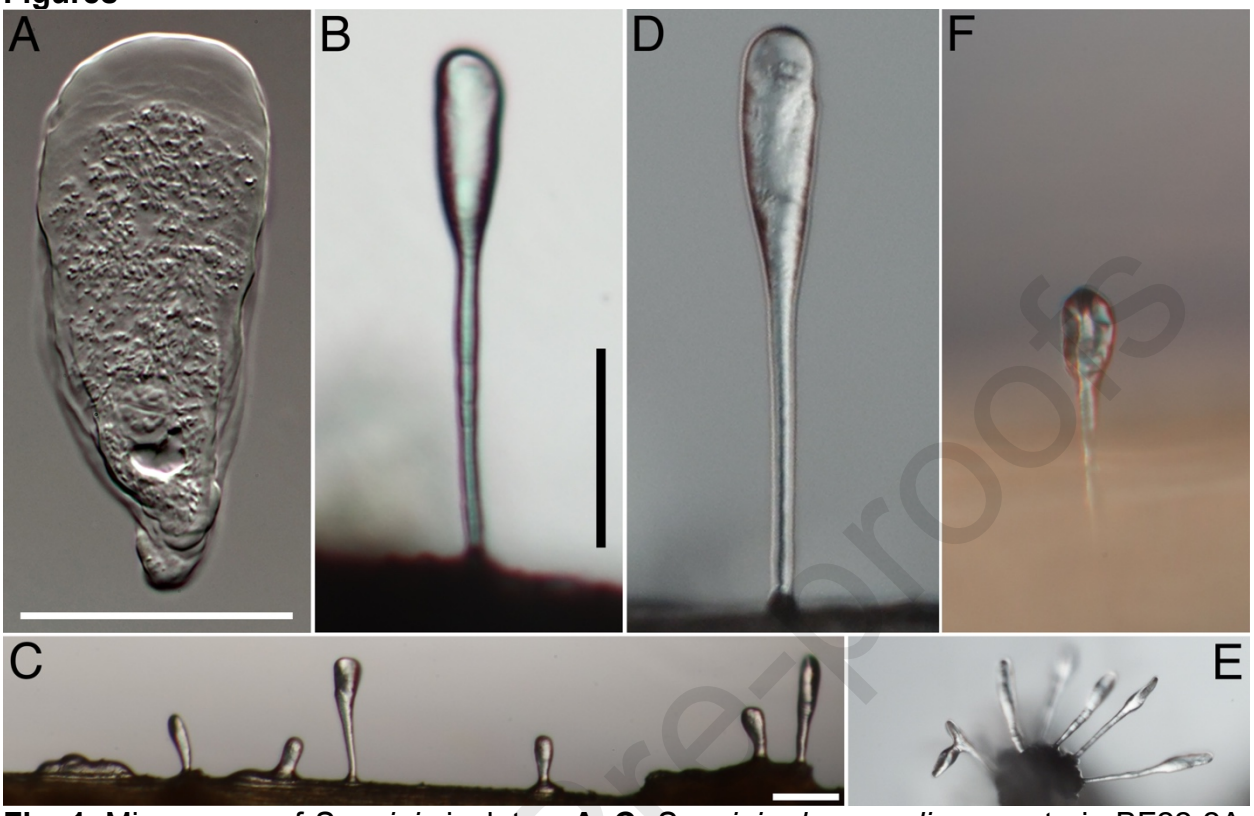
686 **TABLES**

687 **Table 1:** Details of isolates explored in this study.

Isolate ID	Species	Place	Site Name	Substrate	Latitude	Longitude
BF22-2A	"*Sappinia dangeardi*" n. sp.	MS, USA	Byrum Farm	*Bos taurus* dung	33.500459° N	-88.752719° E
MSU2206	*Sappinia pedata*	MS, USA	MSU - Agricultural Research Center	*Bos taurus* dung	33.418104° N	-88.786925° E
SK13-4B	"*Thecamoeba homeri*" n. sp.	MS, USA	MS– - Harned Hall	Soil	33.455812° N	-88.788126° E
SK13-4H	*Thecamoeba* sp.	MS, USA	MS– - Harned Hall	Soil	33.455812° N	-88.788126° E

688

689 **Figures**



690

691 **Fig. 1.** Microscopy of *Sappinia* isolates**. A**–**C.** *Sappinia dangeardi* n. sp. strain BF22-2A.
692 **A.** DIC micrograph of a trophozoite amoeboid cell. **B.** Standing cell on dung. **C.** Numerous
693 standing cells on dung. **D**–**E.** *Sappinia pedata* strain MSU2206 standing cells on dung. **F.**
694 *Sappinia* sp. CCAP 1575/3 (Busnog strain) standing cell in culture. All scale-bars = 50
695 µm. Cells in B, D, F are to scale, as are C and E.

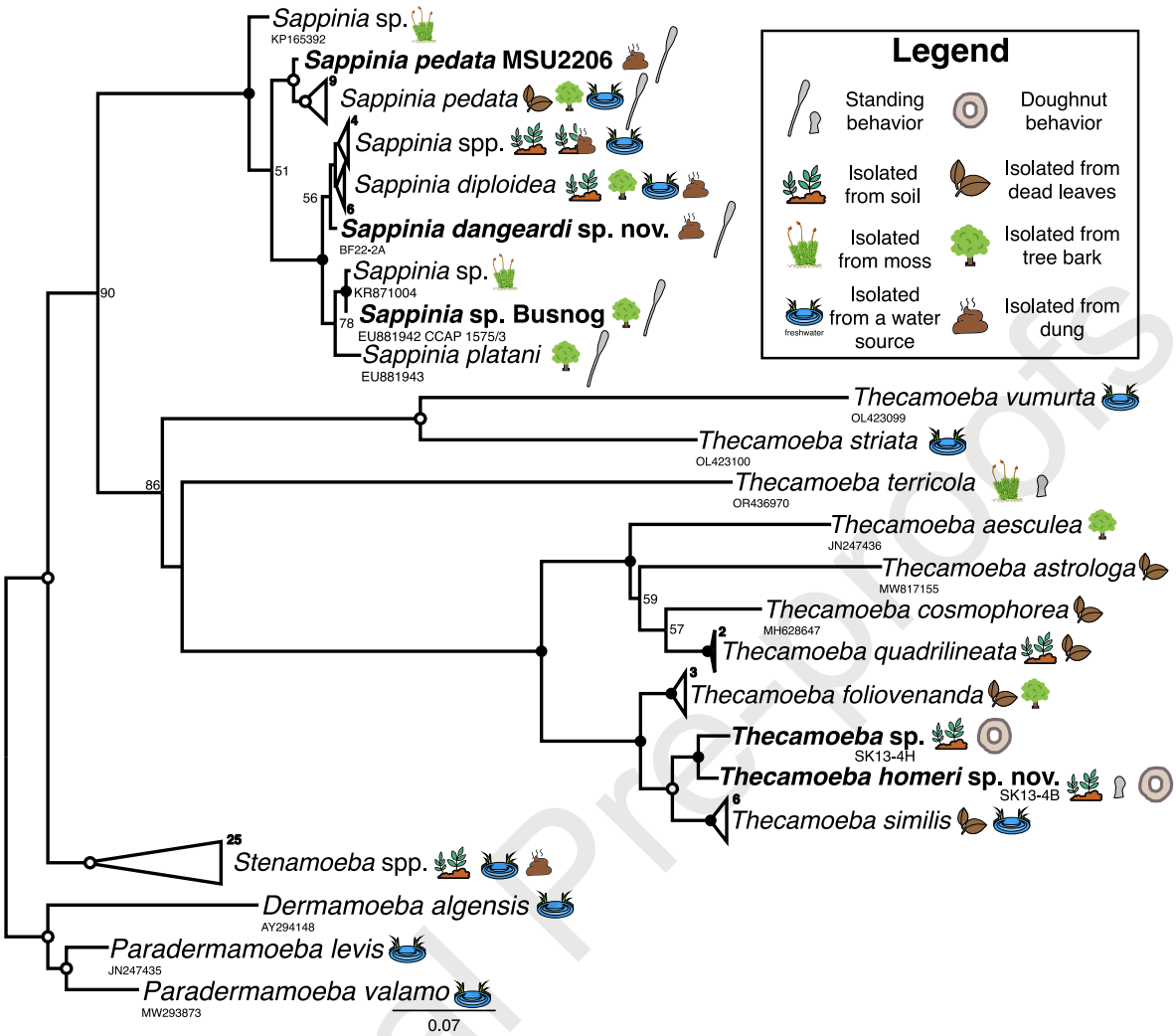


Fig. 2. Maximum likelihood (ML) SSU rRNA gene tree of Thecamoebida, including all available Thecamoebida 18S sequences with *Dermamoeba* and *Paradermamoeba* as outgroup taxa. The tree was constructed with RAxML using the GTRGAMMAI model of evolution with 1772 nucleotides sites, with 1000 ML bootstrap replicates mapped. Bolded taxa are strains observed during the course of this research. Closed dots represent 100 bootstrap support and open dots represent 95–99 bootstrap support. Bootstrap values below 50 are not noted. Additional strain locality information included in Supplementary Table 1.

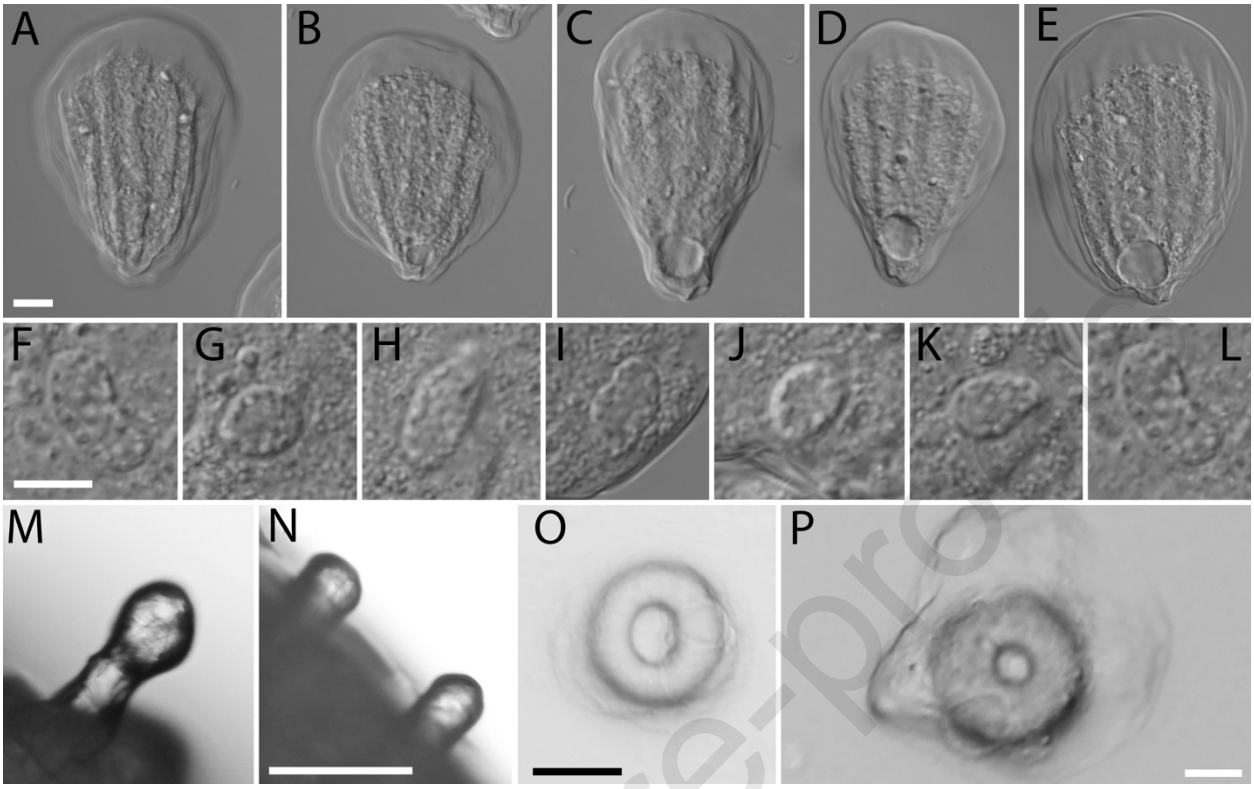


Fig. 3. Microscopy of *Thecamoeba homeri* n. sp. strain SK13-4B. **A**–**C.** DIC micrographs of trophozoite amoeboid cells, showing dorsal ridges. **F**–**L.** nucleus with lobed peripheral nucleoli. **M.** standing cell on a projection of a piece of straw material. **F.** standing cells on straw material. **O**–**P.** Doughnut morphologies on the agar surface. A-E, scale-bar = 10 µm. F–L, scale-bar = 10 µm. M-N, scale-bar = 50 µm. O-P, scale-bar = 25 µm.

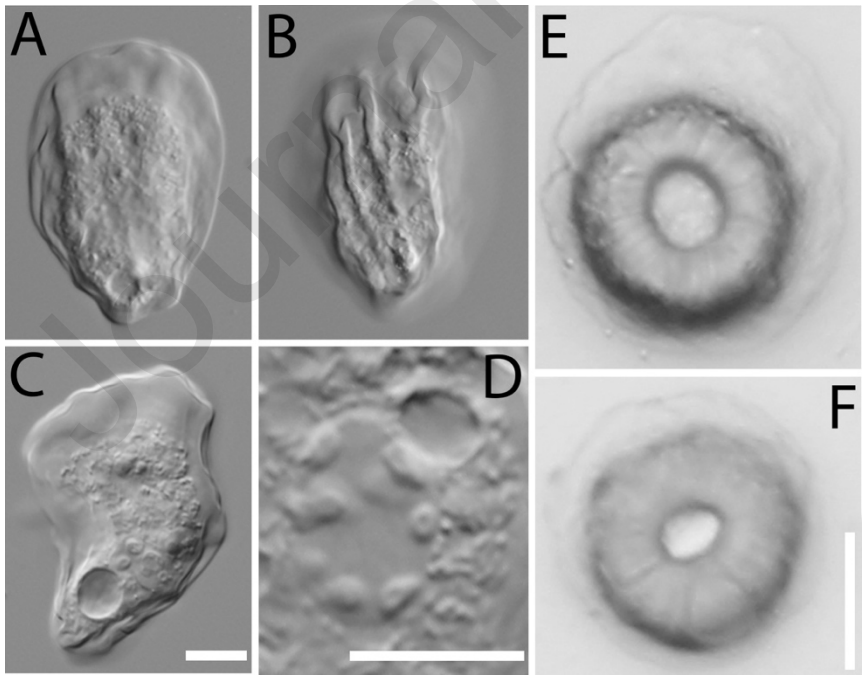


Fig. 4. *Thecamoeba* sp. strain SK13-4H amoebae (A, B, C) and nucleus (D). Doughnut behavior (E & F). Scale-bar A & D = 10 µm (A–C to scale) and F is 25 µm.

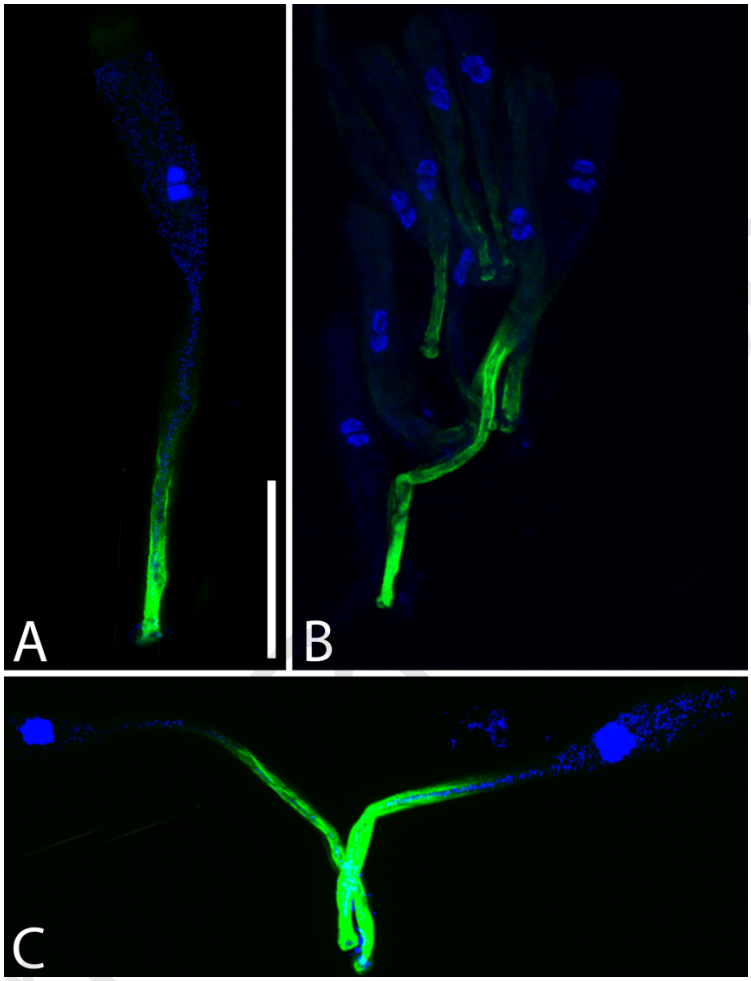
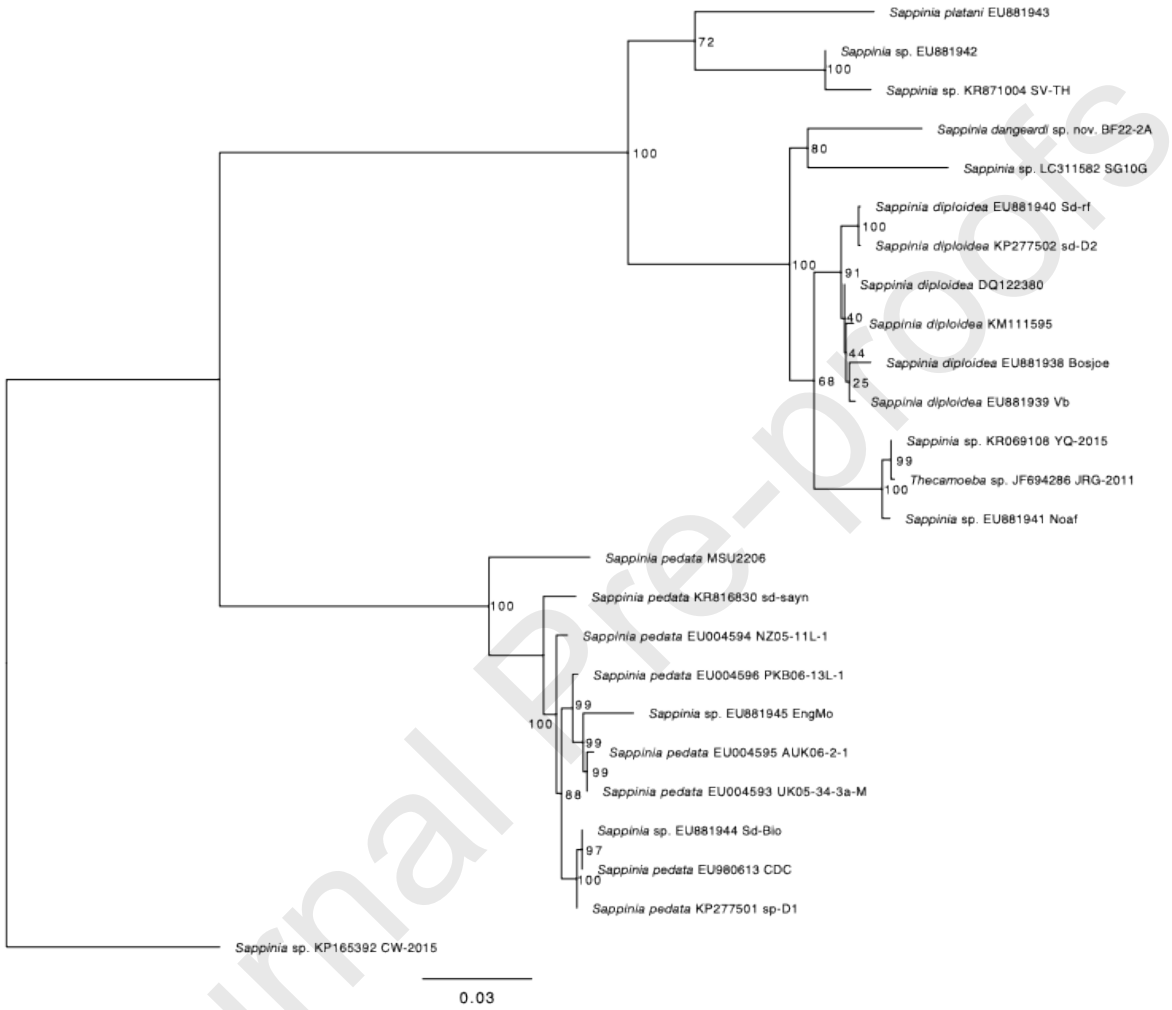


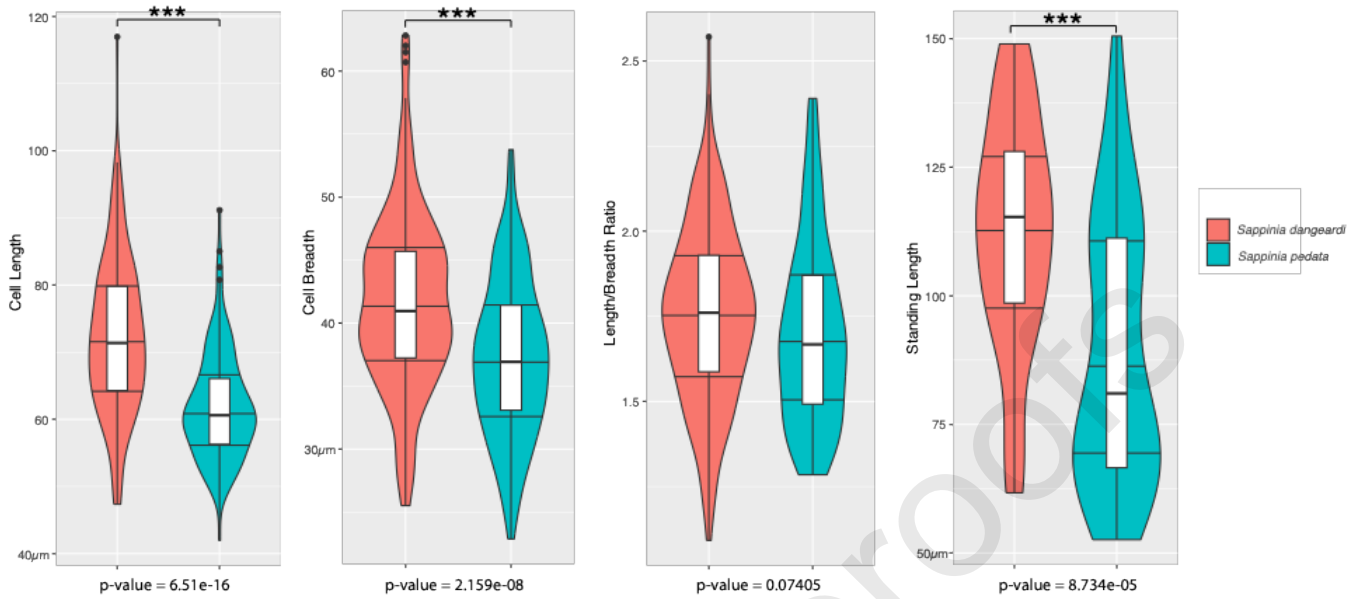
Fig. 5. Histocytochemical fluorescence localization of cellular structures of *Sappinia dangeardi* n. sp. (BF22-2A) standing amoebae using confocal microscopy. **A–C** ActinGreen 488-nm ready probes (Actin, Green) and NucBlue ReadyProbes (DNA, Blue), scale bar = 50 µm (A–C to scale).

SUPPLEMENTAL FILES

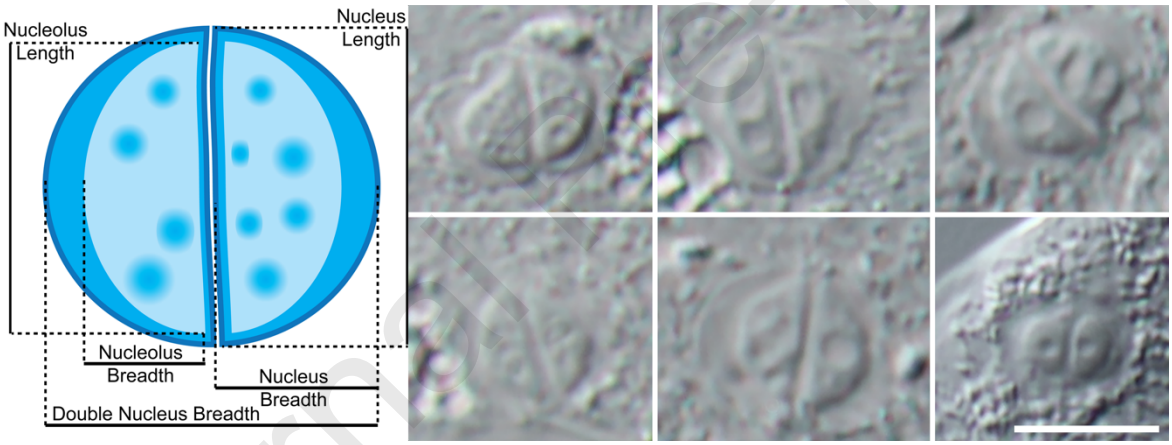
All molecular data and supplemental files (figures and videos) associated with this manuscript are available on FigShare (https://doi.org/10.6084/m9.figshare.24772854).



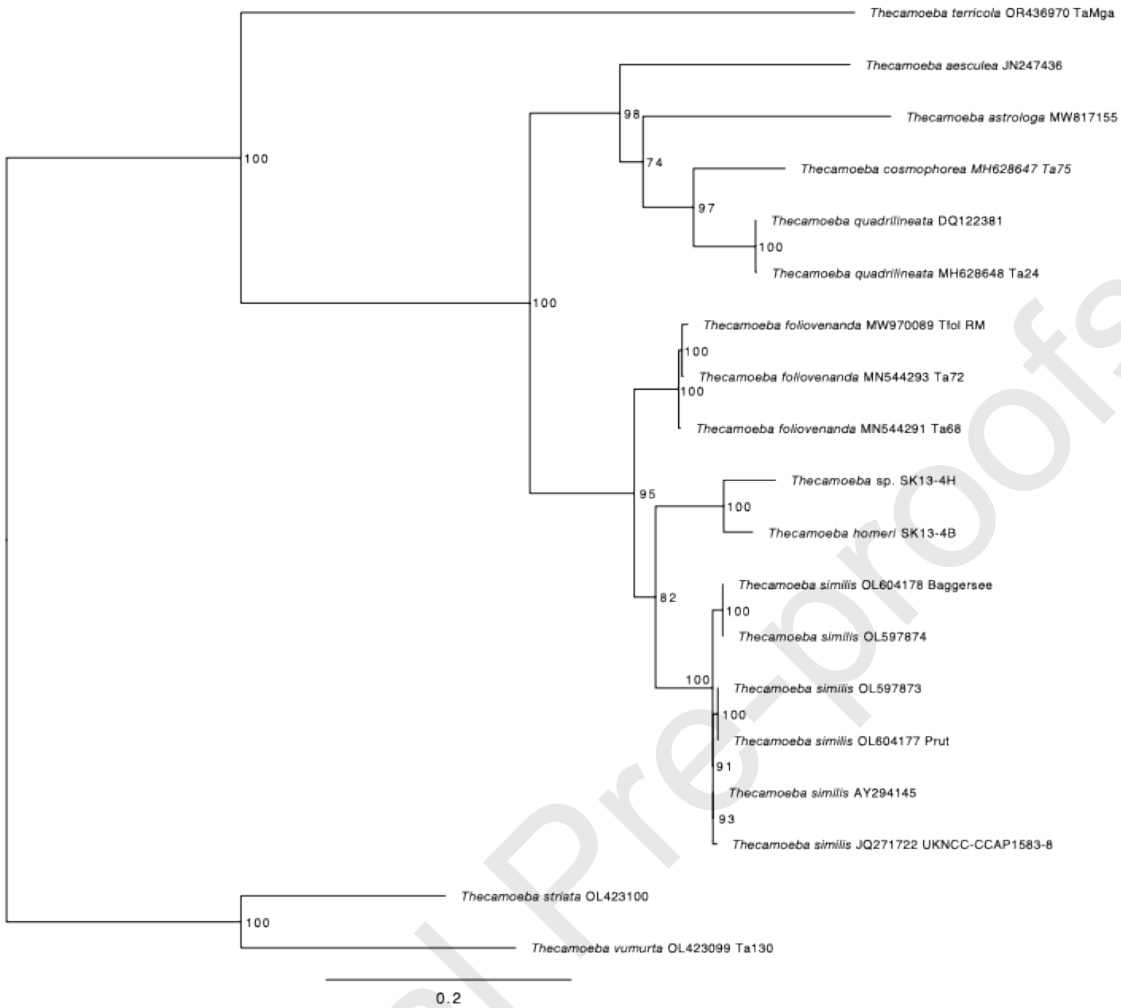
Supplemental Figure 1: Maximum likelihood (ML) SSU rRNA gene tree of *Sappinia.*



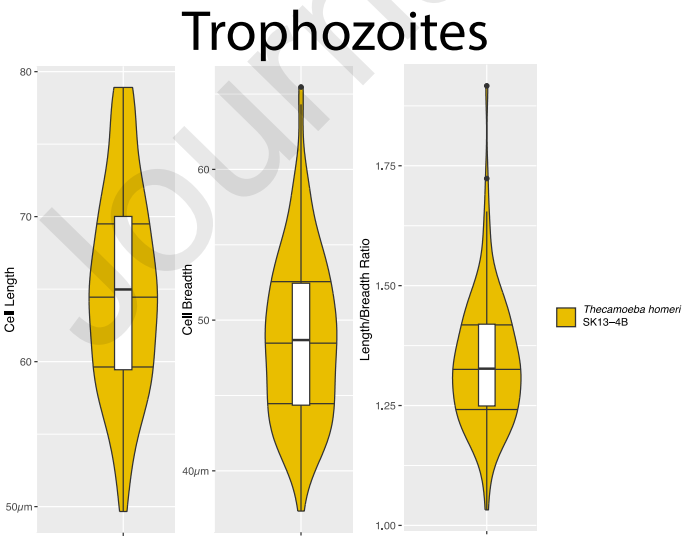
Supplemental Figure 2: Morphological variation of locomotive and standing *Sappinia* cells comparing *S. dangeardi* n. sp. BF22-2A and *S. pedata* MSU2206.



Supplemental Figure 3. Hemispherical nuclei of *S. dangeardi* n. sp.. Scalebar = 10 μm.



743
744 **Supplemental Figure 4:** Maximum likelihood (ML) SSU rRNA gene tree of *Thecamoeba.*
745



746
747 **Supplemental Figure 5:** Morphological variation of locomotive *Thecamoeba homeri* n.
748 sp. SK13-4B cells.

Supplemental Figure 6. Type specimen of *S. dangeardi* n. sp. fixed in methanol and paraformaldehyde, type slide containing this cell has been deposited to the Invertebrate Zoology collection at the Smithsonian Institute. Scale-bar = 50 µm.

Supplemental Video 1.
[SupplementalVideo1.SappiniaPick.mp4] An example of a single cell pick of *Sappinia* sp. using platinum wire.

Supplemental Video 2.
[SupplementalVideo2.20220522 BF22-2A 5spf 30fps standing behavior.mp4] Timelapse video of standing *Sappinia dangeardi* n. sp. BF22-2A. Each frame was taken five seconds apart and compiled at 30 frames per second. Taken under transmitted light.

Supplemental Video 3.
[SupplementalVideo3.Sappinia Timelapse.BF22-2b.20220306.mp4] Timelapse video of standing *Sappinia dangeardi* n. sp. BF22-2A. Each frame was taken five seconds apart and compiled at 30 frames per second. Taken under transmitted light.

Supplemental Video 4.
[SupplementalVideo4.BF22Dense CultureOverview_QT.mp4] Realtime video microscopy of standing *Sappinia dangeardi* n. sp. BF22-2A on dung. Taken under reflected light.

Supplemental Video 5.

774 **[SupplementalVideo5.Thecamoeba.Doughnut.variousmag.realtime.mp4]** Realtime
775 video microscopy of the doughnut formation in *Thecamoeba homeri* n. sp. SK13-4B.
776 Taken under transmitted light. Multiple occurrences compiled into one video.
777
778 **Supplemental Video 6.**
779 **[SupplementalVideo6.Polyvacuole_timeseries.Sappinia_*dangeardi*.40X.mp4]** Time
780 series micrographs of *Sappinia dangeardi* n. sp. polyvacoule formation.
781
782 **Supplemental Video 7.**
783 **[SupplementalVideo7.SK13-4H.Donut.mp4]** Real time video of doughnut formation in
784 *Thecamoeba* sp. SK13-4H.
785
786 **Supplemental Table 1.**
787 **[SupplementalTable1.Tree_Strain_Information.xlsx]** Locality information of strains
788 whose 18S rDNA sequences were used in Fig. 4.
789
790 **Supplemental Table 2.**
791 **[SupplementalTable2.UncorrectedPairwiseDistances.MAFFTAUTO-BMGE0.9.xlsx]**
792 Uncorrected pairwise distance matrices for the genera *Sappinia* and *Thecamoeba*.
793