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A New Species of *Flamella* (Amoebozoa, Variosea, Gracilipodida) Isolated from a Freshwater Pool in Southern Mississippi, USA

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Abstract. We isolated and identified a freshwater amoebozoan species that belongs to the genus *Flamella* Schaeffer, 1926 by single cell isolation and light microscopy. Our specific strain was isolated from a water sample obtained on the cover of a swimming pool in Petal, Mississippi, USA collected during the winter of 2015. Morphologically, our isolate is a fan-shaped amoeba with a large, frontal hyaloplasm and distinctive granuloplasm. It is capable of encystment and trophozoites occasionally have two nuclei. The isolate (GFP151sc) is phylogenetically sister to but unique from the freshwater environmental flamellid clone from Borok, Yaroslavl region, Russia originally published in 2006. Here we describe and place this isolate into a new species, *Flamella piscinae* n. sp.

Key words: Amoeboid, amoeba, SSU rDNA, taxonomy, protist, phylogenetics, *Flamella*, Gracilipoida

INTRODUCTION

Gracilipodida is an order of fan shaped amoebae, which group within Variosea of the supergroup Amoebozoa (Lahr *et al.* 2011). The order contains the genera *Filamoeba*, *Flamella*, and *Telaepolella* (Lahr *et al.* 2012), previously referred to as '*Arachnula*' in Tekle *et al.* 2008. This group is further subdivided into the families Flamellidae, which consists of *Flamella* and *Telaepolella* (Lahr *et al.* 2011) and Filamoebidae (Cavalier-Smith *et al.* 2004). Variosea contains a morphologically diverse set of amoebae with and without flagella, from one (i.e., *Phalansterium, Planoprotostelium*, and *Cavostelium*) to many flagella (*Multicilia*) (Adl *et al.* 2012). Amoebae in the group are quite diverse and taxa may have pointed to reticulate pseudopods (Cavalier-Smith *et al.* 2004, Smirnov *et al.* 2011, Berney *et al.* 2015). In phylogenetic analyses, Variosea has some affinity to grouping with Macromycetozoa, which contains the dictyostelid social amoebae and the plasmodial myxogastrid slime molds. Additionally, in nuclear encoded small subunit (SSU) rDNA phylogenies (e.g., Berney *et al.* 2015) Variosea and Macromycetozoa have a phylogenetic affinity to the anaerobic Archamoebae (i.e., *Entamoeba* and *Mastigamoeba*). This larger group is referred to as Conosa in some analyses (see Cavalier-Smith *et al.* 2004, Smirnov *et al.* 2011). In a recent work by Berney *et al.* (2015), Variosea was greatly expanded by through sampling of freshwater and soil

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reticulate plasmodia which group throughout the clade, representing 6 new genera.

Flamella (Schaffer 1926) is a genus of fan-shaped amoebae with most of the cell body comprised of hyaloplasm and a smaller amount comprised of a conspicuous granuloplasm. The hyaloplasma frequently has subpseudopodia at the leading edge of locomotive amoebae (Kudryavtsev *et al*. 2009). Until recently *Flamella* consisted of only a few known species, but over the course of the last 8 years the genus has received substantial attention and several new species have been described (Kudryavtsev *et al.* 2009, Berney *et al.* 2015, Shmakova *et al.* 2016). The genus currently consists of ten species (Shmakova *et al.* 2016). Only six species have been examined molecularly using SSU rDNA sequences, the rest of which have only been examined microscopically. Additionally, there are SSU rDNA sequences from several unnamed *Flamella* isolates as well as uncultured environmental clones (i.e., Nikolaev *et al.* 2006, Stock *et al.* 2009, Berney *et al.* 2015). Here, we identify a new species of *Flamella* collected from a freshwater ephemeral pool in southern Mississippi, USA. This isolate (GFP151sc) was determined to belong to the genus *Flamella* through light microscopy and nuclear encoded SSU rDNA sequence. This new species is morphologically very similar *to Flamella fluviatilis*, but groups away from *F. fluviatilis* in phylogenetic analyses of SSU rDNA as sister to an uncultured environmental clone from Borok, Russia.

MATERIALS AND METHODS

Isolation and culturing

GFP151sc was isolated from a water sample collected on a pool cover from Petal, Mississippi **(**31.339423°N, –89.194171°W**)** on 18 January 2015. The water sample was about 4-cm deep with a surplus of algae growing on the pool cover. The water was plated onto the surface of a sterile wMY agar (0.002 g yeast extract, 0.002 g malt extract, 0.75 g K_2HPO_4, 15.0 g agar, and 1 liter ddH_2O) Petri dish. The plate was then allowed to incubate at room temperature for five days. Following the incubation period, the plate was observed using a 10 × objective under a compound microscope. The amoebae were located towards the edge of the dried water droplet. A single amoeba cell was isolated using a 30-gauge platinum wire sterilized over an open ethanol-lamp flame. The wire was shaped into a micro loop and was used to drag a single cell across the agar surface to isolate it away from the cells surrounding it. The single cell was then moved to a sterile Petri dish containing wMY agar with a streak of *Escherichia coli* strain MG1655 as a food source. The clonal culture was incubated at room temperature. The culture was maintained in the laboratory by passage onto fresh media with *E. coli* as a food source every 3 weeks. The strain GFP151sc has been deposited to Culture Collection of Algae and Protozoa (CCAP) under accession 1525/5.

Light microscopy

Light microscopy was used to observe morphological features of GFP151sc. The images were taken through a Zeiss AxioSkop 2 (Carl Zeiss Microimaging, Thornwood, NY) attached to a Canon 5DS 50.6MP Full-Frame CMOS color digital camera. The images were taken at 40 × magnification using differential interference contrast. To obtain amoebae, a culture plate was doused in wMY liquid media (with no agar added). The liquid media was then collected in a transfer pipette and placed onto a glass slide. The images were taken using EOS Utility: Canon. Measurements were recorded using ImageJ (http://imagej.nih.gov/ij/) using the Scale Bar tools for Microscopes utility (http://image.bio.methods.free.fr/ImageJ/?Scale-Bar-Tools-for-Microscopes.html).

DNA extraction

For preparation for DNA extraction, the cells were collected using a flamed loop and placed into a PCR strip tube containing 100 µL of QuickExtract (Epicentre, Madison, WI, USA). Two tubes were used: one positive control containing live cells and one negative control containing just the Epicentre QuickExtract. The tubes were placed into a thermal cycler and incubated at 65°C for six minutes followed by 98°C for two minutes and then immediately placed on to ice as per the manufacturer's protocols. This DNA was subsequently used for SSU rDNA polymerase chain reaction (PCR) experiments.

SSU rDNA PCR

The SSU rDNA extracted using the steps above, was amplified using the eukaryotic primers S1 (5'-AACCTGGTTGATCCTGCC-3') and RibBR (5'-GATCCTTCTGCAGGTTCACC-3') (Fiore-Donno *et al*. 2007) and GoTaq Green Master Mix (Promega, Madison, WI). The sample was ran in a thermal cycler using the following parameters: initial denaturation at 94°C for 45 seconds, then 33 cycles of denaturation at 94°C for 25 seconds, annealing at 42°C for 60 seconds, and polymerization at 72°C for 3 minutes 30 seconds (Watson *et al.* 2014). Following amplification, the amplicon was visualized and cleaned through gel electrophoresis. The amplicon was placed onto a 1% agarose gel made with Tris-Acetate buffer (Brown *et al.* 2012) with the use of ethidium bromide as a fluorescing agent. The gel was ran at 113 V for 45 minutes and then observed under an ultraviolet light using a transilluminator. The bands containing the SSU rDNA amplicons were cut and placed into a barrier pipette tip. The tip was then placed into a 1.5 mL tube and centrifuged at 14000 rcf for 10 minutes (Brown *et al.* 2012). The liquid that was left in the tip of the pipette was sterilely transferred into the 1.5 mL tube containing the SSU rDNA. The purified SSU rDNA amplicon was then Sanger sequenced completely in both orientations using the forward and reverse PCR primers as well as internal primers. The sequence data was assembled in Sequencher 5.2.4 (GeneCodes, Ann Arbor, USA).

Molecular phylogenetics

Fourteen publically available flamellid SSU rDNA sequences (from isolates as well as environmental clones) were used for our

phylogenetic analyses along with other amoebozoan and outgroup Opisthokonta taxa. The SSU rDNA sequences were added to the alignment program, Mesquite 2.75 (Maddison and Maddison 2015), and were manually aligned using alignments from Watson *et al.* (2014) as a seed alignment. This alignment was manually masked of uninformative and ambiguously aligned sites, resulting in an inclusion set of 1244 bp. The trimmed and untrimmed alignments are available upon request. Phylogenetic trees were inferred from the dataset. A general-time-reversible + gamma distribution + estimation of the proportion of invariant sites (GTR+Γ+I) was implemented as suggested by the Akaike Information Criterion in ModelTest v3.7 (Posada and Crandall 1998). Maximum Likelihood (ML) trees were inferred using RAxML 8 (Stamatakis 2014). A total of 1000 ML bootstrap (MLBS) psuedoreplicates were inferred with the same model as above in RAxML and mapped on to the best scoring ML tree 300 independent ML inferences. For Bayesian inferences, two independent runs consisting of four Markov Chain Monte Carlo were run in MrBayes v3.12 (Ronquist and Huelsenbeck 2003) for 10,000,000 generations, sampling trees every 100 generations. The 'burnin' was set to 2,500,000 generations, by which time all parameters converged as assessed by the 'sump' function of MrBayes. Bayesian posterior probabilities (BP) were mapped onto the best-scoring ML tree.

RESULTS

Light Microscopy

GFP151sc exhibits typical *Flamella*-like morphology (Page 1988, Dykova and Kostka 2013). The amoebae are fan shaped with a broad hyaloplasm and a very granular granuloplasm (Fig. 1A–E). When in locomotion, the amoebae exhibit occasional trailing filaments (Fig. 1A) and subpseudopods (Fig. 1C). The lengths of the amoebae are 14.3–23.2 μm (average = 18.7 μm, standard deviation = 2.5 μm, n = 42). The breadths of the amoebae are 17.7–39.4 μm (27.7 μm, 5.2 μm, 42). The average length to breadth ratio is 0.7. The amoebae move in a wave-like fashion, starting in the uroid and extending towards the hyaloplasm. The organism shows various nuclear morphologies. Some cells are binucleate (Fig. 1C), whereas most are uninucleate (Fig. 1B). No evidence for more than two nuclei has been observed. The nucleolus is approximately one-half to three-quarters of the diameter of the nucleus. The nuclei sizes are 2.8 μm–8.2 μm (4.3 μm, 0.9 μm, 42). The nucleolus sizes are 1.4–3.7 μm (2.4 μm, 0.6 μm, 42). Throughout growth, cysts regularly form (Fig. 1H). The cysts are 4.213–12.6 μm (7.7 μm, 2.2 μm, 33) in diameter. Cysts appear to be double walled, containing an endo- and ectocyst (Fig. 1H). The cysts form singly (Fig. 1H, I), in groups (Fig. 1J), or in chains (Fig. 1K). A few of the cysts appear to share an ectocyst while maintaining their own endocyst (Fig. 1J), similar to *Flamella fluviatilis* (Kudryavtsev *et al.* 2009). Pore-like structures are also observed in cysts (Fig. 1I, J). When in liquid media, the amoebae morph into a floating derivative (Fig. 1F, G). The floating forms show extensive subpseudopods, as well as numerous contractile vacuoles (Fig. 1F).

Phylogenetic Analysis

Our phylogenetic analysis of SSU rDNA sequences (Fig. 2) supports that GFP151sc is sister to the uncultured flamellid clone amplified from Borok, Yaroslavl region, Russia (Nikolaey *et al.* 2006). This clade also includes another flamellid environmental clone, GoC1_G12, which is from a marine anoxic seep in the Baltic Sea (FJ153641). Our isolate is phylogenetically unique to those sequences publically available and likely represents a novel *Flamella* species based on a phylogenetic species concept, discussed below (Fig. 2).

DISCUSSION

Identification of a new species of *Flamella*

The amoeba identified above shows classic characteristics of the genus *Flamella*. The genus *Flamella* currently is home to ten species (Schaeffer 1926, Bovee 1956, Fishbeck and Bovee 1993, Michel *et al.* 1999, Kudryatsev *et al.* 2009, Shmakova *et al.* 2016) (Suppl. Table 1). The amoebae exhibit a flattened, fan-shaped morphology, with occasional subpseudopods and trailing filaments. The amoebae possess a wide, frontal hyaloplasm with a highly granular granuloplasm. The locomotion of the amoebae is always a smooth fan shape with narrow but conical subpseudopods. Amoebae never display eruptive-like movement or polytactic forms thus eliminating the idea of the species belonging to genera *Flabellula* or *Paraflabellula* (Page 1988). The size ranges and length to breadth ratios of locomotive trophozoites are within the ranges of several described *Flamella* species (i.e., *F. tiara, F. balnearia, F. arnhemensis, F. pleistocenica, F. beringiania*, and *F. aegyptia*) (see Suppl. Table 1). The average cyst sizes were similar to those of *F. beringiania* and *F. balnearia* (Shmakova *et al*. 2016). The cysts exhibited typical flamellid morphology, most of them bearing multiple walls with the presence of pore-like structures (Fig. 1I). These pore-like structures on cyst walls may be

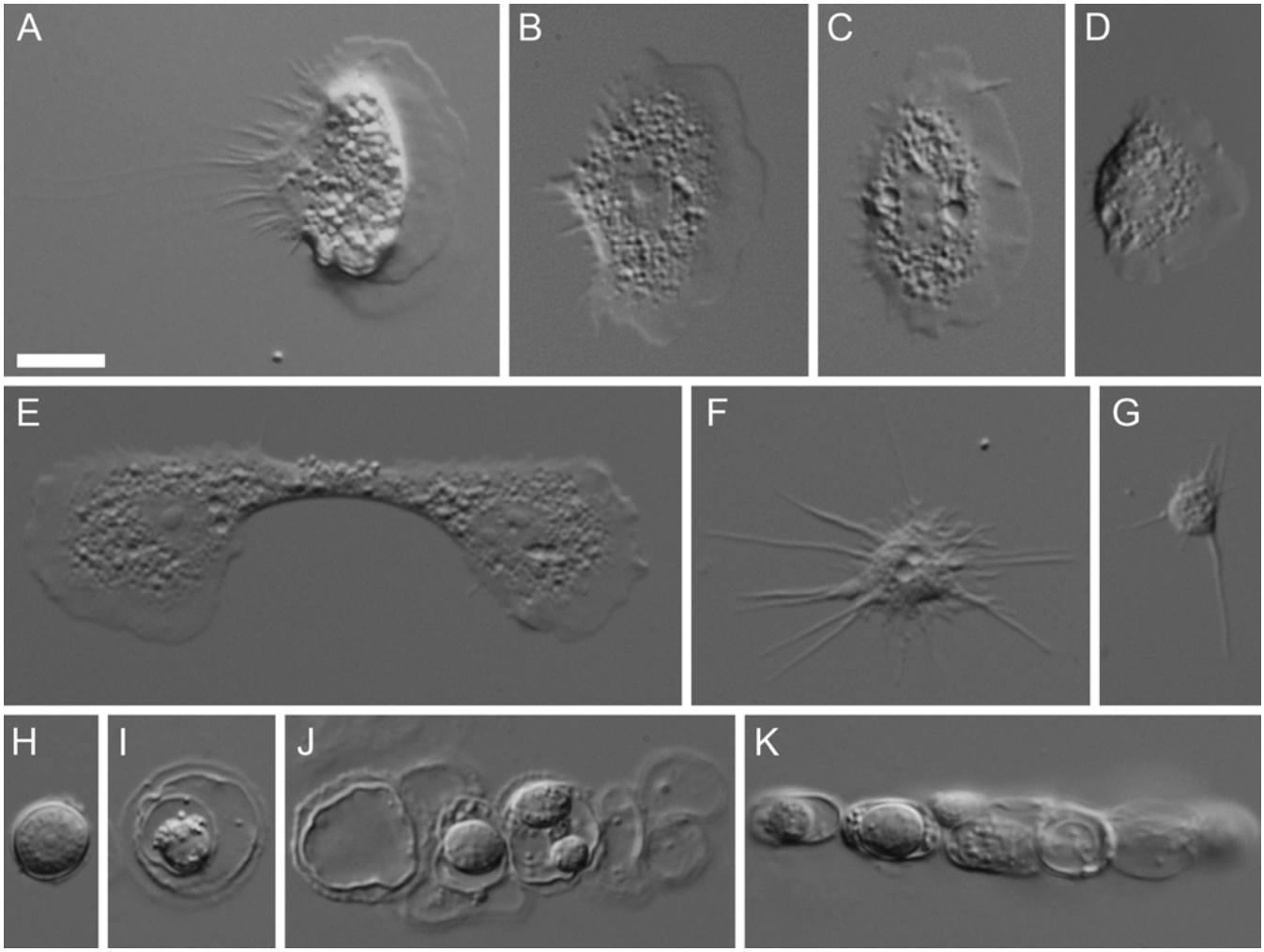


Fig. 1. Photomicrographs of *Flamella piscinae* n. sp. **A** – locomotive form with trailing filaments; **B** – locomotive form; **C** – locomotive form with subpseudopodia; **D** – locomotive form; **E** – cytokinesis; **F** – floating form; **G** – floating form; **H** – single cyst; **I** – cyst enveloped within multiple walls; **J** – cysts sharing walls; **K** – cysts sharing walls in a linear conformation. Scale bar: 10 μm. All images are to scale.

Fig. 2. Maximum likelihood tree of one hundred five amoebozoans and six opisthokonts. The organism of interest, *Flamella piscinae*, is highlighted and bolded. The numbers at the nodes correlate to RAxML ML bootstrap (MLBS) values and Bayesian Interference posterior probabilities (BP), respectively. Values less than 50% MLBS and 0.50 BP were excluded from the tree figure. A black dot refers to a value of 100/1. The '--' refer to either an unrecovered node in Bayesian inferences or values less than 50% MLBS or 0.50 BP.

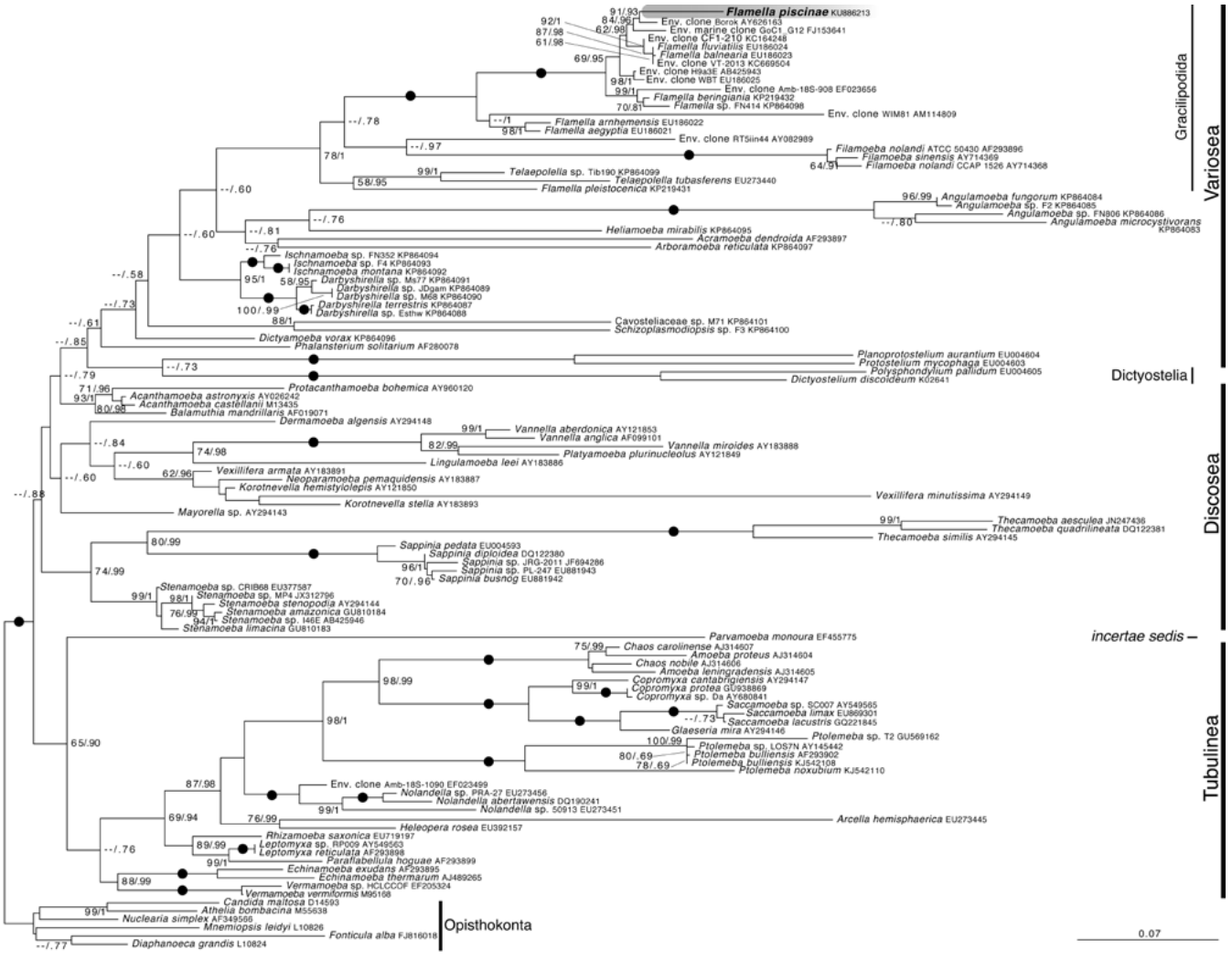
opercula and ostioles; however, careful ultrastructural characterizations are required to confirm these structures.

SSU rDNA sequence data is available from all of the species that morphologically resemble our isolate except for *F. tiara*. However, cysts are not known in *F. tiara* (Fishbeck and Bovee 1993). This suggests that our isolate, which readily forms cysts in culture, is not synonymous with *F. tiara*. Given the similarities in amoeboid, nuclear, and cyst morphologies illustrated in Suppl. Table 1 as well as the molecular variation that is observed in the genus (Fig. 2), we must use a phylogenetic, rather than a morphological, species concept. These data illustrate that for any isolate attributed to *Flamella* spp. an SSU rDNA sequence must be obtained to adequately identify the isolate to species. Molecular

Suppl. Table 1. Morphological details of described species in the genus *Flamella*.*

Species	Length µm	Breath µm	L/B ratio	Diameter nucleus µm	Diameter nucelolus µm	Diameter cysts µm
F. magnifica	30–60	30–60	0.2–1**	–	–	cysts not found
F. citrensis	30–50	35–55	–	5	–	cysts not found
F. tiara	12–25	21–42.5	–	5	–	cysts not found
F. aegyptia	16–36(24.6)	24–80(41.9)	0.44–1.0(0.62)	6–10(6.9)	2.4–7.2(3.8)	19–45
F. lacustris	40–60	45–110	0.6–0.7	8–10	5–8	cysts not found
F. arnhemensis	13–34(20)	23–69(41)	0.29–0.8(0.49)	3–8(5.2)	1.5–4(2.7)	10.3–18(13.3)
F. fluviatilis	13–32(21)	17–56(36)	0.39–0.84(0.59)	4–6(4.7)	1.8–3.2(2.3)	10.7–23.4(15.5)
F. balnearia	11–34(20)	19–51(33)	0.4–1(0.63)	3–6.5(4.6)	1–3(2)	10–17(12.3)
F. beringiania	10–30(17.6)	17.5–42.5(28.6)	0.46–1(0.61)	3–5(3.7)	1–3(1.8)	6–12(8)
F. pleistocenica	9.5–22.2(14)	25–33.9(28.5)	0.2–0.8(0.5)	3–6(4.8)	1.5–4(2.6)	8–13(10.35)
F. piscinae	14.3–23.2(18.7)	17.7–39.4(27.7)	0.47–0.98(0.69)	2.8–8.2(4.3)	1.4–3.7(2.4)	4.2–12.6(7.7)

All sizes in µm and averages are in parentheses. '–' = no reliable data available. *This table is directly adapted from Shmakova *et al.* 2016. **Estimated by Shmakova *et al.* 2016 from Schaeffer 1926.



phylogenetics confirms the phylogenetic position of our amoeba isolate (GFP151sc) belonging to the genus *Flamella*. While sharing the most similar morphologies with *F. fluviatilis*, the base pair composition substantially differs by 140 base pairs (11.3% pairwise difference (pd)). In comparison with the sister environmental sequence clone, Borok, the difference is 56 base pairs (4.6% pd). The difference between the sequences of *F. fluviatilis* and *F. balnearia* was 1.4% pd (Kudryavtsev *et al.* 2009), which is a smaller percent difference than that of our isolate and Borok environmental clone. However, Kudryavtsev *et al.* (2009) were able to identify ultrastructural differences in cyst wall morphologies to morphologically delineate between these two molecularly indistinct species. Given the molecular data from the known diversity of the genus our isolate should be separated as its own species. This evidence suggests our isolate should be included in the genus *Flamella* (Schaeffer 1926). However, this isolate is molecularly unique in the group, which makes the primary basis for the description of it as a new species below.

In our SSU rDNA phylogenetic analyses *Flamella* is a paraphyletic genus (Fig. 2). The species *F. pleistocenica* (Shmakova *et al.* 2016) groups with very poor support (58% MLBS/0.95 BP) outside the genus, instead with *Telaepolella tubasferens* (ATCC 50593) + *Telaepolella* sp. Tib190 (Fig. 2). This clade is sister to a clade of the rest of *Flamella* species + (*Filamoeba nolandi* and an environmental clone AY082989). *Telaepolella* is morphologically unique to *Flamella,* including *F. pleistocenica,* in that it has very large plasmodial stages that can grow up to 500 m (Lahr *et al.* 2012). Interestingly, Shmakova *et al.* (2016) did not recover this relationship. In fact, they show *F. pleistocenica* grouping with very poor support with the rest of *Flamella* in their presented maximum likelihood tree, but it was not recovered in the Bayesian inference (indicated by '--' in Figure 6 of Shmakova *et al.* 2016). Our results may be due to the increased taxon sampling in our analyses of variosean isolates collected from Berney *et al.* 2015. Morphologically, one key difference between *F. pleistocenica* and other *Flamella* species are in the floating forms. Most *Flamella* species have pointed radiating pseudopods whereas *F. pleistocenica* has short conical pseudopodial projections (Shmakova *et al.* 2016). However, this floating form is much different than *T. tubasferens,* with a bulbous floating form (Lahr *et al.* 2012). More molecular data in terms of gene and taxon sampling should be collected from both *Telaepolella* species and *Flamella* species, including *F. pleistocenica,* to clarify the specific relationships within this clade.

Taxonomic Appendix

Amoebozoa Luhe 1913, emend. Cavalier-Smith 1998
Variosea Cavalier-Smith *et al.* 2004
Gracilipodida Lahr *et al.* 2011
Flamellidae Lahr *et al.* 2011
Flamella Schaeffer 1926
Flamella piscinae n. sp. Brown & Walthall

Diagnosis: Species with characteristics of the genus. Freshwater inhabiting fan-shaped amoeba. Breadth is significantly wider than the length of actively moving trophozoites. Front, leading-edge, hyaloplasm devoid of inclusions with an inclusion-rich rear granuloplasm that makes up about 2/3 of the cell length. Frequently with long adhesive uroidal filaments extending from trailing end. Length in locomotion 14–23 μm (average 19 μm), breath 18–39 μm (27.7 μm) (length to breath ratio averages 0.7). Floating form with long acutely pointed pseudopodia radiating from all areas of the cell. Cell with one or two nuclei, more nuclei not observed. Vesicular nuclei, 3–8 μm (4 μm) in diameter, with central nucleolus 1.4–3.7 μm (2.4 μm) in diameter. Cysts readily observed as singular, in groups, or in chains. Cysts with multiple walls common. Cysts 4–13 μm (average 8 μm) in diameter.

Type location: This species' type isolate was obtained from a freshwater pool in Petal, Mississippi, USA (31.339423°N, –89.194171°W). Sample that contained this isolate was collected from the top of an impermeable swimming pool cover, which was covering the pool during the offseason (winter).

Type material: The type culture (GFP151sc) has been deposited with the CCAP under accession 1525/5. This culture is considered the hapantotype (name-bearing type) of the species (see Art. 73.3 of the International Code for Zoological Nomenclature, 4th Edition).

Gene sequence data: The nearly complete SSU rRNA gene of the type isolate (GFP151sc) is deposited in GenBank under accession no. KU886213.

Etymology: The specific epithet, *piscinae*, refers to the type locality, which is the water on top of a swimming pool cover located in southern Mississippi. *Piscinae* is Latin for 'of swimming pool'. The root of the word is *piscis* (fish) because artificial swimming pools often had fishes in ancient Rome.

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REFERENCES

Adl S. M., Simpson A. G., Lane C. E., Lukes J., Bass D., Bowser S. S., Brown M. W., Burki F., Dunthorn M., Hampl V., Heiss A., Hoppenrath M., Lara E., Le Gall L., Lynn D. H., McManus H., Mitchell E. A., Mozley-Stanridge S. E., Parfrey L. W., Pawlowski J., Rueckert S., Shadwick R. S., Schoch C. L., Smirnov A., Spiegel F. W. (2012) The revised classification of eukaryotes. *J. Eukaryot. Microbiol.* **59:** 429–493

Bovee E. C. (1956) Some observations on the morphology and activities of new ameba from citrus wastes, *Flamella citrensis* n. sp. *J. Protozool.* **3:** 151–153

Berney C., Geisen S., Van Wichelen J., Nitsche F., Vanormelingen P., Bonkowski M., Bass D. (2015) Expansion of the 'Reticulosphere': Diversity of Novel Branching and Network-forming Amoebae Helps to Define Variosea (Amoebozoa). *Protist*. **166:** 271–295

Brown M. W., Silberman J. D., Spiegel F. W. (2012) A contemporary evaluation of the acrasids (Acrasidae, Heterolobosea, Excavata). *Eur. J. Protistol.* **48:** 103–123

Cavalier-Smith T., Chao E.-Y., Oates B. (2004) Molecular phylogeny of Amoebozoa and evolutionary significance of the unikont *Phalansterium*. *Eur. J. Protistol.* **40:** 21–48

Dykova I., Kostka M. (2013) Illustrated Guide to Culture Collection of Free-Living Amoebae. Academia, Prague, Czech Republic.

Fiore-Donno A. M., Meyer M., Baldauf S. L., Pawlowski J. (2007) Evolution of dark-spored Myxomycetes (slime-molds), molecules versus morphology. *Mol. Phylogenet. Evol*. **46:** 787–889

Fishbeck D. W., Bovee E. C. (1993) Two new amoebae, *Striamoeba sparolata* n. sp. and *Flamella tiara* n. sp., from fresh water. *Ohio J. Sci*. **93:** 134–139

Kudryavtsev A., Wylezich C., Schlegel M., Walochnik J., Michel R. (2009) Ultrastructure, SSU rRNA Gene Sequences and Phylogenetic Relationships of *Flamella* Schaeffer, 1926 (Amoebozoa), with Description of Three New Species. *Protist*. **160:** 21–40

Lahr D. J. G., Grant J., Nguyen T., Lin J. H., Katz L. A. (2011) Comprehensive Phylogenetic Reconstruction of Amoebozoa Based on Concatenated Analyses of SSU-rDNA and Actin Genes. *PLoS One*. **6:** e22780

Lahr D. J. G., Anderson O. R., Kubik G. M., Grant J., Gant A. L., Katz L.A. (2012) Morphological Description of *Telaepolella tubasferens* n. g., n. sp., Isolate ATCC 50593, a Filose Amoeba in the Gracilipodida, Amoebozoa. *Acta Protozool.* **51:** 305–318

Maddison W. P., Maddison D. R. (2015) Mesquite: a modular system for evolutionary analysis. Version 3.04 http://mesquiteproject.org

Michel R., Smirnov A. V. (1999) The Genus *Flamella* Schaeffer, 1926 (Lobosea, Gymnamoebia), with a Description of Two New Species. *Eur. J. Protistol*. **35:** 403–410

Nikolaev S. I., Berney C., Petrov N. B., Mylnikov A. P., Fahrni J. F., Pawlowski J. (2006) Phylogenetic position of *Multicilia marina* and the evolution of Amoebozoa. *Int. J. Syst. Evol. Microbiol.* **56:** 1449–1458

Page F. C. (1988) A New Key to Freshwater and Soil Gymnamoebae. Freshwater Biological Association, Ambleside, Cumbria, UK

Posada D., Crandall K. A. (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818

Ronquist F., Huelsenbeck J. P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574

Schaeffer A. A. (1926) Taxonomy of the Amoebas. *Papers Dept. Mar. Biol. Carnegi. Inst. Washington USA*. **24:** 3–112

Shmakova L., Bondarenko N., Smirnov A. (2016) Viable species of *Flamella* (Amoebozoa: Variosea) isolated from ancient Arctic permafrost sediments. *Protist.* **167:** 13–30

Smirnov A. V., Chao E., Nassonova E. S., Cavalier-Smith T. (2011) A Revised Classification of Naked Lobose Amoebae (Amoebozoa: Lobosa). *Protist*. **162:** 545–570

Stamatakis A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30:** 1312–1313

Stock A., Jürgens K., Bunge J., Stoeck T. (2009) Protistan diversity in suboxic and anoxic waters of the Gotland Deep (Baltic Sea) as revealed by 18S rRNA clone libraries. *Aquat. Microb. Ecol.* **55:** 267–284

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 amoeba inferred from multigene analyses and assessment of clade stability within 'Amoebozoa' upon removal of varying rate classes of SSU-rDNA. *Mol. Phylogent. Evol.* **47:** 339–352

Watson P. M., Sorrell S. C., Brown M. W. (2014) *Ptolemeba* n. gen., a Novel Genus of Hartmanellid Amoebae (Tubulinea, Amoebozoa); with an Emphasis on the Taxonomy of *Saccamoeba*. *J. Eukaryot. Microbiol*. **61:** 611–619

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