Received: 29 September 2022 | Revised: 31 January 2023 | Accepted: 17 February 2023

DOI: 10.1111/jeu.12971

ORIGINAL ARTICLE

Phylogenetic placement of the protosteloid amoeba *Microglomus paxillus* identifies another case of sporocarpic fruiting in Discosea (Amoebozoa)

Alexander K. Tice[1] | **Frederick W. Spiegel**[2] | **Matthew W. Brown**[1]

[1]Department of Biological Sciences, Mississippi State University, Mississippi State, Mississippi, USA

[2]Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas, USA

Correspondence
Alexander K. Tice and Matthew W. Brown, Department of Biological Sciences, Mississippi State University, Mississippi State, MS, 39762 USA.
Email: atl422@msstate.edu; matthew.brown@msstate.edu

Funding information
Texas Ecological Laboratory; United States National Science Foundation Division of Environmental Biology, Grant/Award Number: 1456054 and 2100888

Abstract

Protosteloid amoebae are a paraphyletic assemblage of amoeboid protists found exclusively in the eukaryotic assemblage Amoebozoa. These amoebae can facultatively form a dispersal structure known as a fruiting body, or more specifically, a sporocarp, from a single amoeboid cell. Sporocarps consist of one to a few spores atop a noncellular stalk. Protosteloid amoebae are known in two out of three well-established major assemblages of Amoebozoa. Amoebae with a protosteloid life cycle are known in the major Amoebozoa lineages Discosea and Evosea but not in Tubulinea. To date, only one genus, which is monotypic, lacks sequence data and, therefore, remains phylogenetically homeless. To further clarify the evolutionary milieu of sporocarpic fruiting we used single-cell transcriptomics to obtain data from individual sporocarps of isolates of the protosteloid amoeba *Microglomus paxillus*. Our phylogenomic analyses using 229 protein coding markers suggest that *M. paxillus* is a member of the Discosea lineage of Amoebozoa most closely related to *Mycamoeba gemmipara*. Due to the hypervariable nature of the SSU rRNA sequence we were unable to further resolve the phylogenetic position of *M. paxillus* in taxon rich datasets using only this marker. Regardless, our results widen the known distribution of sporocarpy in Discosea and stimulate the debate between a single or multiple origins of sporocarpic fruiting in Amoebozoa.

KEYWORDS
evolution, fruiting body, phylogenomics, protist, slime mold

INTRODUCTION

PROTOSTELOID amoebae (previously called protostelids) were thought to be a monophyletic group of amoeboid organisms. They were united primarily by their shared ability to produce a sporocarp facultatively during their lifecycle (Olive, 1975; Spiegel et al., 1995). Early molecular phylogenies based on the SSU rRNA gene showed all protosteloid amoebae sampled at that time were members the eukaryotic supergroup Amoebozoa. However, they were a paraphyletic group with a nearly Amoebozoa-wide distribution (Shadwick et al., 2009).

Interestingly, these studies demonstrated certain protosteloid taxa were members of "well-studied" and established groups of amoebozoans, such as the vannellids and acanthamoebids where protosteloid life cycles were not previously known (Shadwick et al., 2009). The ability of these amoebae to produce a sporocarp was emphasized because protosteloid amoebae were discovered and mostly studied by mycologists who placed little emphasis on amoeba morphology as a systematic character. Upon further inspection of the trophic cells, these results were congruent with morphological expectations (Shadwick et al., 2009). More recent studies on additional taxa using the SSU rRNA gene as well as multiprotein phylogenomic data have continued to corroborate this (Tice et al., 2016).

While 29 out of the ca. 38 species that have been considered protosteloid amoebae have been sequenced and placed in the tree of Amoebozoa using single and/or multigene phylogenetic analyses, no molecular data exist for only one remaining genus, the unusual

 © 2023 International Society of Protistologists.

monotypic, *Microglomus* (Olive et al., 1983; Olive & Stoianovitch, 1977). Due to its low rate of occurrence on sampled substrates (occurring in 1%–5% of collections; Ndiritu et al., 2009; Shadwick et al., 2009), cryptic morphology, and resistance to laboratory cultivation, the protosteloid amoeba *Microglomus paxillus* has yet to be included in any molecular phylogenetic study.

In the original description Olive noted similarities between *Microglomus paxillus* and species in the protosteloid genus *Protosporangium* (Amoebozoa, Eumycetozoa, Protosporangiida). This was due to the production of fruiting bodies with multiple apical cells by members of both genera (Olive et al., 1983; Olive & Stoianovitch, 1977). Additionally, in the original description of *Semimorula liquescens*, an amoeboid taxon with a complex life cycle that includes a stalkless fruiting body, the authors noted the amoebae of both *S. liquescens* and *M. paxillus* reabsorb their spore walls after germination. This trait was uncharacteristic of other protosteloids and myxomycetes known at the time and, therefore, perhaps a character that unites the two (Haskins et al., 1983). However, further observations on *M. paxillus* later revealed that spore wall absorption did not actually occur in this taxon (Olive et al., 1983).

A close relationship to either *Protosporangium* spp. Or *S. liquescens* were both suggested cautiously due to the lack of flagellated cells in the lifecycle of *M. paxillus* unlike the former taxa (Haskins et al., 1983; Olive et al., 1983; Olive & Stoianovitch, 1977). Subsequent molecular phylogenetic studies have shown *S. liquescens* to be a derived myxomycete (Fiore-Donno et al., 2009) and the protosporangiids to be the closest relatives to the myxomycetes (Kang et al., 2017). While all protosporangiids have a flagellated stage in their life cycle at least one instance of loss of this cell type has occurred in the myxomycetes (Fiore-Donno et al., 2019). Additionally, ultrastructure of the sporocarps of *M. paxillis* demonstrated that spores appear to be the site of meiosis, a character associated with protosporangiids (Olive et al., 1983; Spiegel et al., 2017).

We have recently been able to collect and germinate *M. paxillus* and confirm its identity through examination of its amoebae. To clarify the phylogenetic position of *M. paxillus* and to further investigate the evolution of sporocarpic fruiting, we obtained transcriptomic data from geographically distinct isolates of *M. paxillus* using the single-cell transcriptomics. We use our resulting transcriptomes to confidently place *M. paxillus* in a highly resolved tree of Amoebozoa. Additionally, we bioinformatically extracted the SSU rRNA gene from our transcriptomes for use in more taxon rich phylogenetic analyses with the hope of more accurately deciphering the closest relatives of *M. paxillus*. We discuss our results in the context of the origin of sporocarpy and the nature of the last common ancestor of Amoebozoa.

MATERIALS AND METHODS

Isolation

Bark from *Juniperus* sp. was collected from two locations in Texas, USA. Just outside of Kerville, TX N:30.065927 E:−99.135255 (K-2016-24-1C) and in Cross Mountain, TX N:29.648483, E:−98.672592 (BX4L). The bark was placed onto the surface of weak malt yeast agar (wMY) plates (0.002 g yeast extract, 0.002 g malt extract, 0.75 g K_2HPO_4, 15 g agar, and 1 L distilled H_2O), moistened with sterile deionized water, and incubated at room temperature (~20°C). After 2 days and continuing for 10 days the bark was examined using a Zeiss AxioSkop2 Plus equipped with a 10× objective. A single instance of spore germination was achieved by placing a sporangium on a thin sheet of wMY agar melted onto a microscope slide then a drop of liquid wMY was added to create a wet mount slide (see https://www.yumpu.com/en/document/read/11344400/a-beginners-guide-to-isolating-and-culturing-eumycetozoans for illustration). Micrographs of intact sporocarps were taken using a Canon EOS 650D digital camera on a AxioSkop2 Plus under 10×s or 20× objective using brightfield microscopy. Micrographs of spores and amoebae were taken with a Canon EOS 5Ds on an AxioSkop2 Plus under 40× objective using Differential Contrast Interference microscopy. All images were taken using the Canon EOS Utilities software.

RNA extraction, cDNA library preparation and Illumina sequencing

RNA was extracted from four individual sporocarps (referred to as isolates K-2016-24-1C-1, K-2016-24-1C-2, K-2016-24-1C-3, and K-2016-24-1C-4) from a single fructification on substrate collection K-2016-24-1C (Figure S1). RNA was also extracted from two individual sporocarps (referred to as isolates BX4L-10 and BX4L-11) from a single fructification on substrate collection BX4L (Figure S1). For RNA isolation and cDNA construction single sporocarps were picked with a flame and ethanol sterilized platinum needle and successively washed with deionized water to remove contaminating organisms before being processed using a modified version of Smart-Seq2 (Picelli et al., 2014) that includes six freeze–thaw cycles to aid in cell lysis as in Onsbring et al. (2020). The resulting cDNA libraries were prepared for sequencing on the Illumina platform using a NexteraXT DNA Library Preparation Kit (Illumina) following the manufacturer's recommended protocol. The libraries were then pooled along with libraries of unrelated studies and sequenced on either an Illumina HiSeq 4000 or Illumina HiSeq X sequenced by Psomagen.

15507408, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jeu.12971 by Matthew Brown - Mississippi State University , Wiley Online Library on [27/03/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Transcriptome assembly and Bioinformatic retrieval of SSU sequences

Adaptors, primer sequences, and low-quality bases were removed from the raw sequencing reads using Trimmomatic v. 0.36 with the options ILLUMINACLIP 2:30:10 SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25 (Bolger et al., 2014). The remaining reads for each library were assembled using Trinity v. 2.12.0 (Haas et al., 2013). Proteins were predicted from the assembled transcripts using Transdecoder v. 5.5.0 (https://github.com/TransDecoder/TransDecoder/). Assembly metrics such as number of contigs and predicted proteins for each individual transcriptome can be found in Table S1.

Ribosomal rRNA gene sequences from representative amoebozoans were used as queries in blastn (Camacho et al., 2009) searches against each *M. paxillus* transcriptome to retrieve the contig that represented the SSU rRNA gene sequence from each of our six individual transcriptomes.

Phylogenetic analyses

Phylogenomic dataset construction and analyses

The phylogenomic dataset was constructed using tools in the software package PhyloFisher (Tice et al., 2021). Candidate orthologs were collected for up to 240 protein coding genes from the *M. paxillus* predicted proteomes using *fisher.py* via the "phylogenetically aware route" using previously identified orthologs from the amoebozoans *Dictyostelium discoideum*, *Acanthamoeba castellanii*, *Protostelium aurantium* var. *fungivorum* as BLAST queries. Candidate orthologs were added to their corresponding alignments via *prep_working_dataset.py*. Each alignment contained previously identified orthologs and closely related paralogs from a set of eukaryotic wide taxa. Homolog trees were constructed using *sgt_constructor.py* using the default settings. Homolog trees were inspected by eye to ensure correct ortholog selection and elimination of any contaminating sequences. To mitigate the effect of missing data on our final analysis, predicted proteomes for all isolates from the same sample location were concatenated together using *taxon_collapser.py* to produce a chimeric proteome with as many of the 240 phylogenetic markers present as possible. The number of PhyloFisher marker genes found in each individual transcriptome as well as in the two chimeric transcriptomes can be found in Table S1. Orthologs that contained at least 33% of our final set of 84 amoebozoan taxa and 11 obazoan outgroup taxa were collected via *prep_final_dataset.py*. Single ortholog datasets were aligned, trimmed, and concatenated via *matrix_constructor.py* using default settings resulting in a matrix of 72,498 amino acid positions from 229 genes. Maximum likelihood phylogenetic reconstruction was performed using the LG+G4+F+C60-PMSF (Wang et al., 2017) model of evolution, with an LG+G4+F+C20 maximum likelihood tree as a PMSF guide input tree in IQ-TREE2 v 2.0-rc1 (Minh et al., 2020). Support values for each node were inferred using 100 real bootstrap replicates under the LG+G4+F+C60-PMSF model of evolution in IQ-TREE2.

SSU rRNA phylogenetic analyses

The SSU sequences of all *M. paxillus* isolates were added to an SSU dataset consisting of described discosean taxa, related environmental sequences (identified through blastn to NCBI nucleotide database), and outgroup sequences from Variosea taxa. Sequences were aligned using mafft v. 7.427 (Katoh & Standley, 2013) with the –auto flag. Sites with low coverage and low confidence sites were removed using BMEG v. 1.12 (Criscuolo & Gribaldo, 2010) with the maximum gap rate allowed per character (−g) set to 0.6, leaving 1452 sites for phylogenetic analysis. The maximum likelihood phylogenetic tree was constructed with RaxML v. 8.2.4 (Stamatakis, 2014) under a GTR+G+I model of nucleotide substitution. Bayesian phylogenetic analysis was performed using Mr. Bayes v. 3.2.6 (Ronquist et al., 2012) also under a GTR+G+I model of nucleotide substitution. Two runs consisting of four chains each were run for 10,000,000 generations sampling trees every 1000 generations. Convergence of all parameters was reached as assessed by a split-deviation value <0.01. The first 25% of trees were discarded as burnin.

RESULTS

Sporocarps matching those originally described for *M. paxillus* appeared on the agar surface ~5 days after plating (Figure 1A). Spores isolated for RNA extraction ranged from 9.4 to 13.4 μm in diameter (average = 11.62 μm, std = 1.52 μm, $n = 6$). Spore germination was achieved once from a two-celled spore isolated from the fructification that appeared on the same substrate as collection K-2016-24-1C (Figure 1B). Two binucleate amoebae emerged from the spore leaving the spore wall behind wall as described by Olive and Stoianovitch (1977; Figure 1C,D). The amoebae were longer than they were wide in their locomotive form with length to breath ratios of 1.08 and 1.12. The average size of the nuclei of the amoebae were 2.75 and 3.45 μm while the nucleoli had an average size of 1.2 and 1.75 μm. Both amoebae observed were isodiametric and displayed broad lamellopodia with broad-based acutely pointed subpseudopodia. Both were

15507408, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jeu.12971 by Matthew Brown - Mississippi State University , Wiley Online Library on [27/03/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

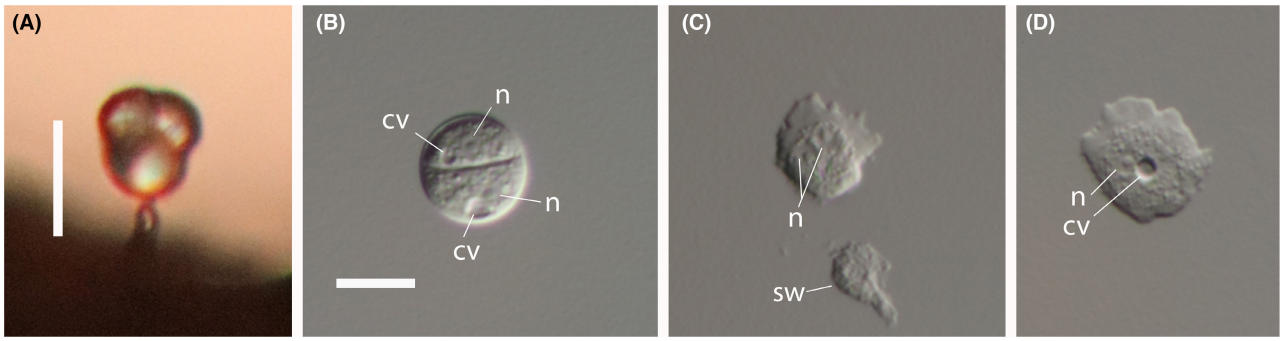

FIGURE 1 Photomicrographs of *M. paxillus*. (A) Sporocarp from the fruitification isolate K-2016-24-1C was collected from. Scale bar = 15 μm. (B) Two-celled spore of a sporocarp from the fructification isolates K-2016-24-1C 1–4 were collected from. Scale bar = 10 μm. (C) Binucleated amoeba that germinated from the sporangium shown in (B). (D) The second binucleated amoeba that germinated from the sporangium shown in (B). Only one nucleus visible. Figures (B–D) are to scale. *n* = nucleus, cv = contractile vacuole, sw = spore wall.

binucleate with contractile vacuoles located near the nucleus (Video S1). No uroid was observed on either of the two amoebae.

In our phylogenomic analyses using 229 genes (72,498 amino acid positions) we recover the three well-established major assemblages of Amoebozoa (Tubulinea, Evosea, and Discosea) all with full support (100 MLBS; Figure 2). Both of our representative *M. paxillus* isolates branch within Discosea sister to *Mycamoeba gemmapera* with full bootstrap support (Figure 2). *Microglomus paxillus* and *M. gemmapera* together are sister to the Dermamoebida (*Dermamoeba*, *Paradermamoeba*, and *Mayorella*) also with full MLBS support (Figure 2).

We were able to retrieve partial SSU rRNA sequences from three of our six individual transcriptomes. They were 1762 (K-2016-24-1C-4), 1767 (Bx4L-11), and 1570 (Bx4L-10) bp in length. All were highly divergent resulting in an extremely long branch and inconclusive phylogenetic placement. In our SSU phylogenetic analyses using a dataset enriched in discosean taxa, and related environmental sequences, but absent of other long-branching members of the group both maximum likelihood and Bayesian analyses show *M. paxillus* branching sister to *D. algensis* with low support (29/0.63; Figure 3).

DISCUSSION

The first question that must be addressed, since the original cultures no longer exist, is if the material shown here is *Microglomus paxillus*. The spores/sporangia are completely consistent with those shown in the original publications (Olive et al., 1983; Olive & Stoianovitch, 1977). The short stalk (shorter than the diameter of the spore/sporangium) with a sudden apical narrowing to an acute point is the same and is unique among sporocarpic Amoebozoa. Because we were able to germinate material, we were able to show that the amoebae are morphologically similar to those shown in Olive and Stoianovitch (1977) and Olive et al. (1983). Though the terminology of the original publications for amoebal morphology are somewhat idiosyncratic because at the time in the Olive lab accepted morphological terminology for amoebae was not used as the lab was a mycological lab. However, the broad lamellopodia with broad-based acutely pointed subpsuedopodia are identical with the original publications. The amoebae are relatively isodiametric, binucleate upon germination, and have a contractile vacuole close to the nuclei. Additionally, the second author of this manuscript was in the Olive lab in the late 1970s when the species was discovered and had many chances to observe the cultures of the type of material and was also involved in laboratory discussions about it. There is no doubt that the present material is consistent with the original material. Given these observations, we feel confident we have identified an organism that fits the morphospecies concept of *M. paxillus*. However, we cannot rule out the possibility we have isolated a sibling species morphologically indistinguishable from the original isolate since our knowledge of cryptic speciation in this genus is lacking. Molecular data will need to be gathered on several more isolates fitting this morphospecies concept to understand the true diversity within the genus *Microglomus*.

Olive et al. (1983) pointed out that the life cycle of *M. paxillus* seems to be sexual due to the presence of synaptonemal complexes in nuclei of the developing sporocarp. However, the complete details of the sexual cycle are not known. While it is well-agreed upon that the last common ancestor of Amoebozoa was sexual (Hofstatter et al., 2018; Lahr et al., 2011; Tekle et al., 2017) examples of organisms with traditionally sexual life cycles are sparse in Amoebozoa, especially within Discosea (Kang et al., 2017). Our phylogenetic placement of *M. paxillus* adds a second instance of an organism that likely exhibits a traditionally sexual life cycle, meiosis followed by karyogamy sometime in the future, to Discosea though the exact details need to be examined further once cultures can be reestablished. Our phylogenetic results also further expand the number of known sporocarpic

15507408, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jeu.12971 by Matthew Brown - Mississippi State University , Wiley Online Library on [27/03/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Journal of Eukaryotic Microbiology ISOP Published by the International Society of Protistologists

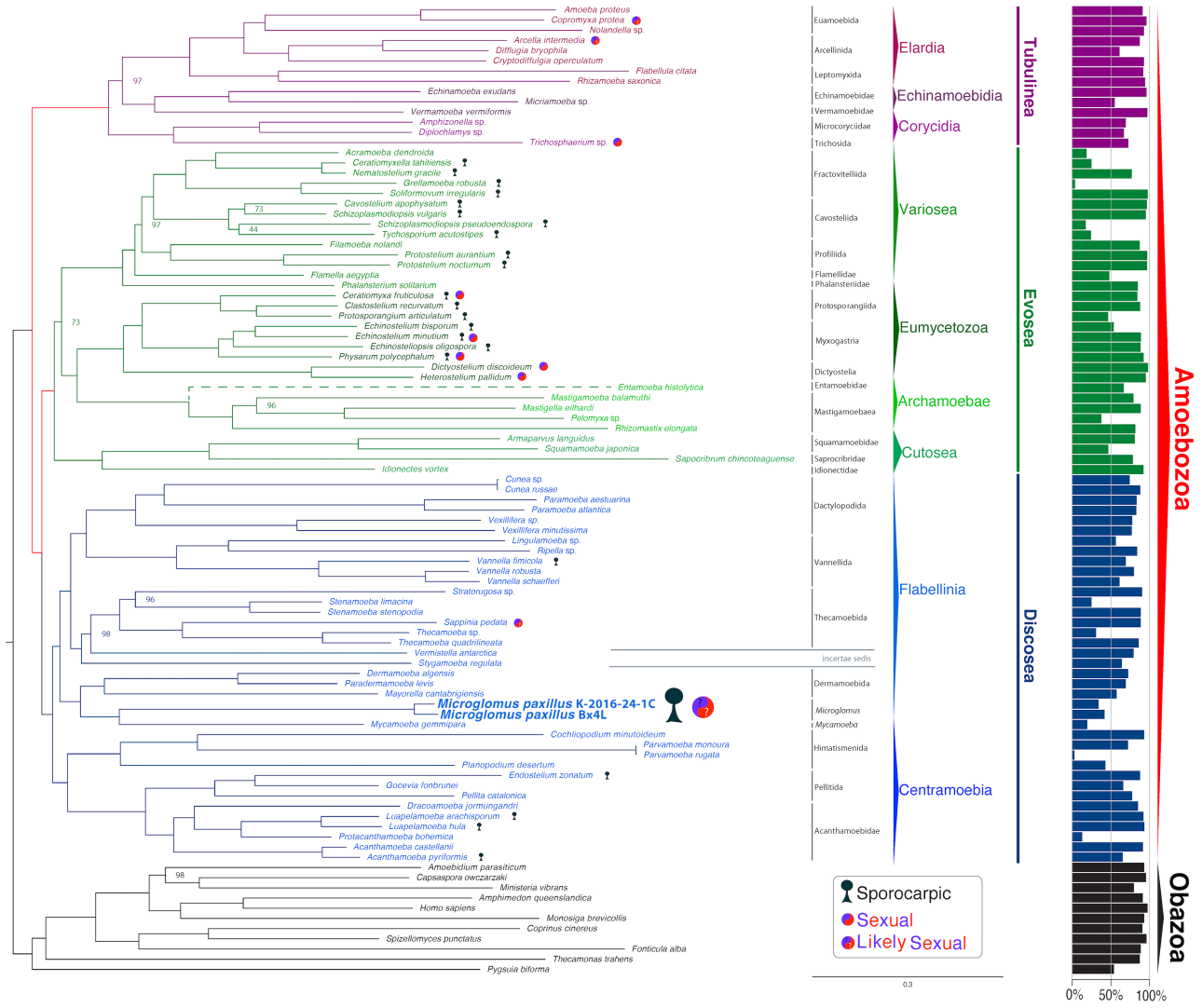

FIGURE 2 Maximum likelihood phylogenomic tree of Amoebozoa with obazoan outgroup taxa. The tree was constructed from a matrix of 72, 498 amino acid positions from 229 genes using the LG+G4+F+C60-PMSF model of evolution, with an LG+G4+F+C20 maximum likelihood tree as a PMSF guide input tree in IQ-TREE2. Support values are derived from 100 nonparametric bootstrap replicates. All nodes are fully supported (100 MLBS) unless otherwise shown. The length of branches displayed as dashed lines has been reduced by 50%. Cartoon fruiting bodies are displayed next to lineages where sporocarpic fruiting is known. Yin yang symbols without internal question marks denote an organism is known to be sexual. Yin yang symbols with internal question marks denote an organism is likely to be sexual but the details of the sexual cycle are incompletely known. The bar chart to the right indicates a taxon's completeness in the phylogenomic matrix. The scale bar represents 0.3 substitutions per site.

lineages within Amoebozoa, specifically the Discosea clade of Amoebozoa. In addition to *M. paxillus*, sporocarpy is found in the Vannellida, Acanthamoebidae, and Pellitida clades of Discosea. Outside of Discosea sporocarpic fruiting is known in the Fractovitelliida, Cavosteliida, and Profiliida lineages of Variosea as well as the Protosporangiida and Myxogastria lineages of Eumycetozoa (Figure 2; Kang et al., 2017). When considering the sculpted spore morphology and meiosis synchronized sporulation of *M. paxillus*, its phylogenetic placement in Discosea near the Dermamoebida rather than in the Eumycetozoa (i.e. myxogastrids and protosporangiids) was somewhat unexpected (Olive et al., 1983; Olive & Stoianovitch, 1977). However, with the recent placement of the limax amoeba *Coronamoeba villafranca* (Kudryavtsev et al., 2022) and the large multinucleate amoeba *Thecochaos fibrillosum* (Mesentsev et al., 2022) in Discosea it is obvious the group is more morphologically diverse than previously recognized.

Though tempting, due to their sister relationship in our phylogenomic analysis, we hesitate to discuss the relationship of *M. gemmapera* and *M. paxillus* in depth. While both exhibit complex life cycles that include the production of "fungal like" structures (the sporocarp in the case of *M. paxillus* and the pseudomycelium in *M. gemmipera*), we believe their close phylogenetic proximity to one another in our phylogenomic analysis is more likely due to insufficient taxon sampling rather than an actual close evolutionary relationship between the two. Unfortunately, due to the divergent nature of the SSU

15507408, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jeu.12971 by Matthew Brown - Mississippi State University , Wiley Online Library on [27/03/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

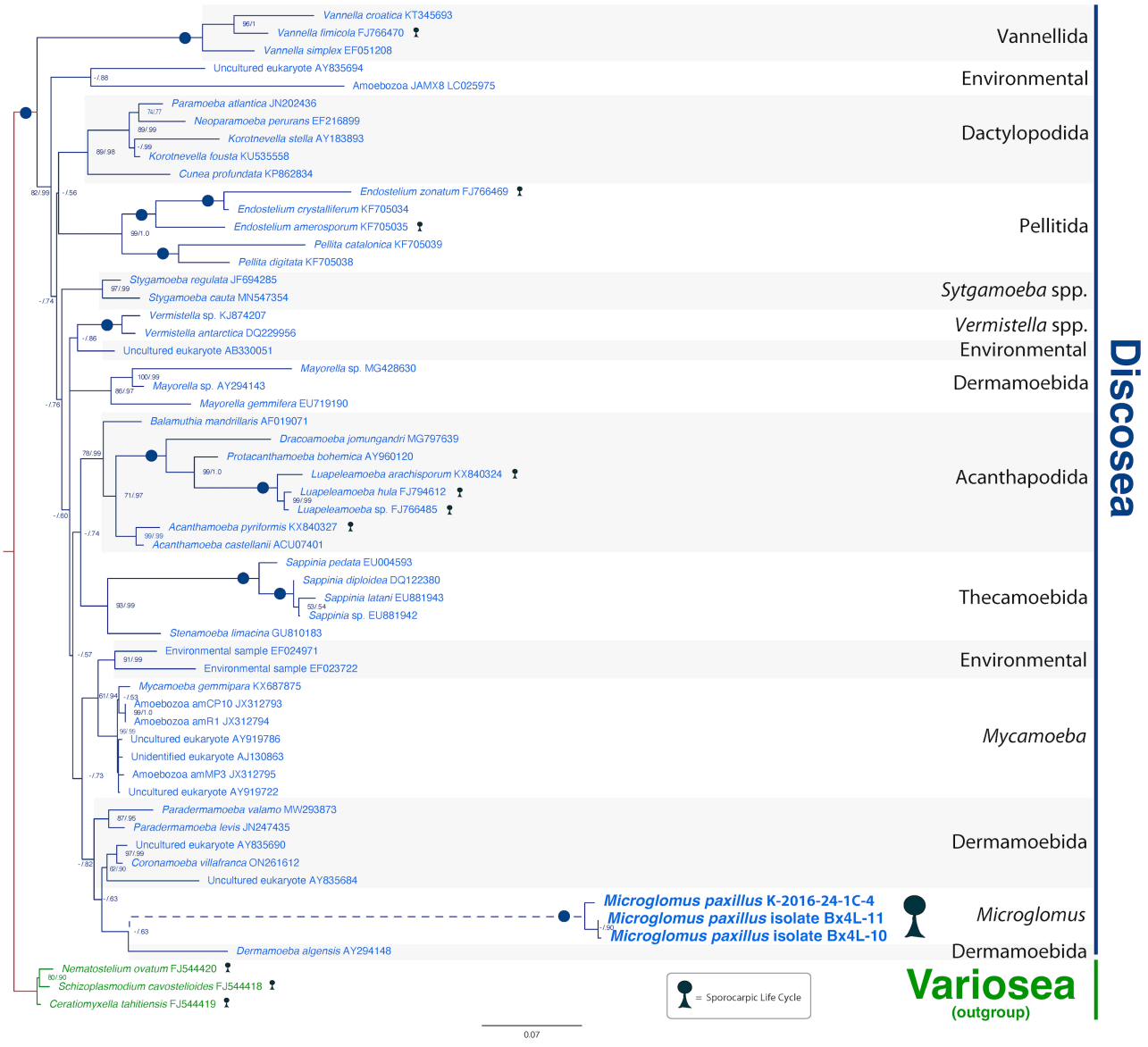

FIGURE 3 Maximum likelihood SSU phylogeny of Discosea with variosean taxa serving as the outgroup. Values at nodes represent Maximum likelihood bootstrap and Bayesian posterior probability values, respectively. Filled in blue circles represent full support (100/1.0) for a node in both analyses. Values less than 50 or 0.50 are not shown or represented with a "–". Cartoon fruiting bodies are displayed next to lineages where sporocarpic fruiting is known. The length of branches displayed as dashed lines has been reduced by 50%. Scale bar represents 0.07 substitutions per site.

sequences of our *M. paxillus* isolates we were unable to confidently resolve the phylogenetic position of *M. paxillus* in more taxon rich datasets that included environmental sequences. As such, taxonomically we chose to leave *Microglomus* as its own lineage within Amoebozoa, Discosea, Flabellinia as has been done with *Mycamoeba* for the time being.

Continuing to characterize and conduct phylogenetic studies on protosteloid amoebae is important for understanding the nature of the last common ancestor of Amoebozoa. While *M. paxillus* represents the last described protosteloid amoebae genus whose phylogenetic home was previously mysterious, many undescribed species exist. Due to the paucity of researchers focusing on protosteloid amoebae, there remains much to be explored and discovered regarding the biodiversity of these organisms. Whether these distinct lineages represent independent innovations of sporocarpic fruiting or maintenance of sporocarpic fruiting from a shared common ancestor is still a topic of debate. Arguments have been made for both scenarios. Cavalier-Smith et al. (2016) suggested that protosteloid sporocarps arose from nonstalked cysts independently in each protosteloid lineage. Arguing that selection for aerial dispersion is widespread and, therefore, mutations that lead to the development of aerial dispersal structures such as sporocarps must not be particularly complex and, therefore, not particularly rare (Cavalier-Smith

15507408, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jeu.12971 by Matthew Brown - Mississippi State University , Wiley Online Library on [27/03/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

et al., 2016). On the other hand, Kang et al. (2017) favored a single origin in the last common ancestor of Amoebozoa citing the uniqueness of the protosteloid life cycle to Amoebozoa even though amoeboid taxa can be found in most major lineages of eukaryotes, the widespread phylogenetic distribution of sporocarpic fruiting in Amoebozoa, and morphological differences between the cysts and spores of many protosteloid amoebae as evidence for their position (Kang et al., 2017). We would also like to point out that from the little that is known of stalk ultrastructure, stalk morphology is very similar in both the Evosea in Protosteliidae/Profiliida (Spiegel et al., 1979), Cavosteliida (Spiegel & Feldman, 1993), Schizoplasmodiida (Spiegel, unpublished), Protosporangiida (Spiegel, unpublished) and in Discosea in Acanthamoebidae (Spiegel, unpublished) and Pellitida (Olive et al., 1984). If one looks at figure 17 in Olive et al. (1983) There is what appears to be a rather typical protosteloid stalk apex directly below the spore of *M. paxillus*.

Much more clarity on this matter will be provided when further developmental studies have been conducted to examine molecular commonalities and/or differences during sporocarp developmental in a range of taxa. As of now, gene expression throughout sporocarp development has only been examined in a single taxon *Protostelium aurantium* var. *fungivorm* (Hillmann et al., 2018). A well-agreed upon deep structure of the tree of Amoebozoa is also important to understanding the nature of the last common ancestor of Amoebozoa and the evolution of sporocarpic fruiting. Currently two competing hypotheses exist and seem to be dependent on the phylogenetic reconstruction method applied to phylogenomic datasets. In maximum likelihood analyses using both site homogenous and site heterogenous evolutionary models Tubulinea is always sister to the rest of Amoebozoa (Cavalier-Smith et al., 2016; Kang et al., 2017; Tekle et al., 2022). When the site heterogenous infinite mixture model CAT-GTR is applied in Bayesian phylogenetic analyses Discosea is sister to the rest of Amoebozoa (Kang et al., 2017). If the ML topologies are correct the possibility that sporocarpic fruiting arose in the last common ancestor of Discosea plus Evosea rather than the last common ancestor of Amoebozoa cannot be ruled out since there are no protosteloid members of Tubulinea.

In conclusion, the results of our phylogenetic study on the protosteloid amoeba *Microglomus paxillus* demonstrate sporocarpic fruiting is more prevalent in Amoebozoa than previously known. We believe the widespread distribution of this amoebozoan exclusive life cycle within the group is most likely due to its retention from the last common ancestor of Amoebozoa in some taxa and loss in many others. However, future studies across a multitude of biological disciplines will be needed to convincingly support or reject this hypothesis.

ACKNOWLEDGMENTS

We would like to thank Nicholas W. Fry for substrate collection and preparing the Nextera XT libraries for a subset of these samples. This work was supported in part by the United States National Science Foundation (NSF) Division of Environmental Biology (DEB) grants 1456054 and 2100888 (http://www.nsf.gov) awarded to M.W.B, as well as Texas Ecology Laboratory individual grants awarded to A.K.T. and M.W.B.

DATA AVAILABILITY STATEMENT

The *M. paxillus* raw sequence reads have been deposited on NCBI under BioProject PRJNA877218 and the SSU rRNA gene sequences have been deposited under accession numbers OP392029-31. The assembled transcriptome, predicted proteins, trimmed and untrimmed protein and SSU alignments, single ortholog trees, and phylogenomic matrix have been deposited on figshare https://doi.org/10.6084/m9.figshare.21234741.

ORCID

Alexander K. Tice https://orcid.org/0000-0002-3128-1867
Frederick W. Spiegel https://orcid.org/0000-0003-1142-424X
Matthew W. Brown https://orcid.org/0000-0002-1254-0608

REFERENCES

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

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. et al. (2009) BLAST+: architecture and applications. *BMC Bioinformatics*, 10, 421.

Cavalier-Smith, T., Chao, E.E. & Lewis, R. (2016) 187-gene phylogeny of protozoan phylum Amoebozoa reveals a new class (Cutosea) of deep-branching, ultrastructurally unique, enveloped marine Lobosa and clarifies amoeba evolution. *Molecular Phylogenetics and Evolution*, 99, 275–296. Available from: https://doi.org/10.1016/j.ympev.2016.03.023

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, 210.

Fiore-Donno, A.M., Haskins, E.F., Pawlowski, J. & Cavalier-Smith, T. (2009) Semimorula liquescens is a modified echinostelid myxomycete (Mycetozoa). *Mycologia*, 101, 773–776.

Fiore-Donno, A.M., Tice, A.K. & Brown, M.W. (2019) A non-flagellated member of the Myxogastria and expansion of the Echinosteliida. *Journal of Eukaryotic Microbiology*, 66, 538–544.

Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J. 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.

Haskins, E., McGuinness, M. & Berry, C. (1983) Semimorula: new genus with myxomycete and protostelid affinities. *Mycologia*, 75, 153–158.

Hillmann, F., Forbes, G., Novohradská, S., Ferling, I., Riege, K., Groth, M. et al. (2018) Multiple roots of fruiting body formation in Amoebozoa. *Genome Biology and Evolution*, 10, 591–606.

15507408, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jeu.12971 by Matthew Brown - Mississippi State University , Wiley Online Library on [27/03/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Hofstatter, P.G., Brown, M.W. & Lahr, D.J. (2018) Comparative genomics supports sex and meiosis in diverse Amoebozoa. *Genome Biology and Evolution*, 10, 3118–3128.

Kang, S., Tice, A.K., Spiegel, F.W., Silberman, J.D., Pánek, T., Čepička, I. et al. (2017) Between a pod and a hard test: the deep evolution of amoebae. *Molecular Biology and Evolution*, 34, 2258–2270.

Katoh, K. & Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, 30, 772–780.

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, 1–13.

Lahr, D.J.G., Parfrey, L.W., Mitchell, E.A.D., Katz, L.A. & Lara, E. (2011) The chastity of amoebae: re-evaluating evidence for sex in amoeboid organisms. *Proceedings of the Royal Society B: Biological Sciences*, 278, 2081–2090. Available from: https://doi.org/10.1098/rspb.2011.0289

Mesentsev, Y., Bondarenko, N., Kamyshatskaya, O., Nassonova, E., Glotova, A., Loiko, S. et al. (2022) Thecochaos is not a myth: study of the genus Thecochaos (Amoebozoa, Discosea)—a rediscovered group of lobose amoeba, with short SSU gene. *Organisms Diversity & Evolution*, 23, 7–24.

Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A. et al. (2020) IQ-TREE 2: new models and efficient Methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution*, 37, 1530–1534.

Ndiritu, G., Spiegel, F. & Stephenson, S. (2009) Rapid biodiversity assessment of myxomycetes in two regions of Kenya. *Sydowia*, 61, 287–319.

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., Bennett, W.E. & Deasey, M.C. (1984) The new Protostelid genus Endostelium. *Mycologia*, 76, 884. Available from: https://doi.org/10.2307/3793145

Olive, L.S. & Stoianovitch, C. (1977) A new microsporangial Protostelid, microglomus paxillus gen. And sp. nov. *The Journal of Protozoology*, 24, 485–489.

Olive, L.S. (1975) *The mycetozoans*. New York, NY: New York Academic Press.

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.

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 Protocols*, 9, 171–181.

Ronquist, F., Teslenko, M., Mark, P., Ayres, D.L., Darling, A., Hohna, S. et al. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61, 539–542. Available from: https://doi.org/10.1093/sysbio/sys029

Shadwick, L.L., Spiegel, F.W., Shadwick, J.D., 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, e6754. Available from: https://doi.org/10.1371/journal.pone.0006754

Spiegel, F. & Feldman, J. (1993) Fruiting body ultrastructure in the protostelid Schizoplasmodiopsis vulgare. *Mycologia*, 85, 894–897.

Spiegel, F., Lee, S. & Rusk, S. (1995) Eumycetozoans and molecular systematics. *Canadian Journal of Botany*, 73, 738–746.

Spiegel, F.W., Olive, L. & Brown, R.M., Jr. (1979) Roles of Actin during sporocarp culmination in the simple mycetozoan Planoprotostelium aurantium. *Proceedings of the National Academy of Sciences*, 76, 2335–2339.

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, 1312–1313.

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, 11173.

Tekle, Y.I., Wood, F.C., Katz, L.A., Cerón-Romero, M.A. & Gorfu, L.A. (2017) Amoebozoans are secretly but ancestrally sexual: evidence for sex genes and potential novel crossover pathways in diverse groups of amoebae. *Genome Biology and Evolution*, 9, 375–387.

Tice, A.K., Shadwick, L.L., Fiore-Donno, A.M., Geisen, S., Kang, S., Schuler, G.A. et al. (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.

Tice, A.K., Žihala, D., Pánek, T., Jones, R.E., Salomaki, E.D., Nenarokov, S. et al. (2021) PhyloFisher: a phylogenomic package for resolving eukaryotic relationships. *PLoS Biology*, 19, e3001365.

Wang, H.-C., Minh, B.Q., Susko, E. & Roger, A.J. (2017) Modeling site heterogeneity with posterior mean site frequency profiles accelerates accurate Phylogenomic estimation. *Systematic Biology*, 67, 216–235.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: 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*, 00, e12971. Available from: https://doi.org/10.1111/jeu.12971

15507408, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jeu.12971 by Matthew Brown - Mississippi State University , Wiley Online Library on [27/03/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License