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Characterization of Microtubule Organizing Centers in the genus Protostelium, Including Evolutionary Implications

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Characterization of Microtubule Organizing Centers in the genus *Protostelium*, Including Evolutionary Implications

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Abstract

Microtubule organizing centers (MTOCs) are cellular regions of microtubule nucleation. The best known MTOCs are those associated with the centrosome, but several non-centrosomal MTOCs are known in eukaryotes, especially in land plants. MTOCs are poorly characterized across the breadth of amoebozoan diversity, but are well-known in certain amoebozoan lineages, including the genus of protosteloid slime molds *Protostelium*. The structure of the MTOC is known for two non-ciliated species, *P. nocturnum* and *P. mycophaga*, as well as *P. aurantium*, which can reversibly become ciliated under appropriate conditions. *P. nocturnum* and *P. mycophaga* have acentriolar centrosomal MTOCs while *P. aurantium* has a centriole-bearing pro-kinetid that differentiates into a kinetid when the cell becomes ciliated. It was previously thought that the MTOCs of *P. mycophaga* and *P. nocturnum* were homologous to each other, and were derived from a structure reminiscent of the kinetid of *P. aurantium*, but recent changes in our understanding of the group's phylogeny, as well as the realization that most isolates of *P. aurantium* cannot become ciliated, have called this hypothesis into question. In this thesis, a new strain of *P. aurantium* was isolated. This strain, which was unable to produce cilia when isolated, was characterized ultrastructurally and found to have an MTOC typical of non-ciliated *Protostelium* spp. After ultrastructural work was complete, ciliated cells were unexpectedly found in one culture of the new isolate. The significance of these findings, and their implications for the evolutionary history of *Protostelium*, are discussed.

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Chapter 1: Introduction

Introductory biology courses need to lay the foundation for more advanced courses, and often must be accessible to students not entering a biology-related field. Possibly in an attempt to satisfy these requirements, such courses focus on examples that will be familiar to students: animals, especially humans, and to a lesser extent, flowering plants. Although this approach helps ease students into what can be a difficult subject, it leaves them with the notion that these organisms are "typical" and represent a standard for what living things are supposed to be like. They often lack an appreciation for how unusual multicellularity is, and tend to see unicellular organisms as "primitive" or transition states to "higher organisms". There tends to be an assumption that evolution always progresses toward complexity, and that simpler organisms are primitive while more complex organisms are more evolved. This type of thinking is further reinforced by the fact that vertebrates are among the most structurally complex organisms known, which allows us as humans to think of ourselves as being highly evolved.

Many people don't consider the fact that any two extant organisms are separated from their last common ancestor by the same length of time. As such, no extant organism is any more or less evolved than any other extant organism. Multicellularity is simply a characteristic of certain lineages, metazoans and embryophytes among them; it is not some end goal that all organisms are striving for. Evolution does not always lead toward complexity over time. Parasites for instance, are well known to often be highly reduced. The model organism *Saccharomyces cerevisiae* is almost certainly descended from a more complex filamentous fungus with a mycelium in its life cycle (Dee et al. 2015).

Amoebozoa is major clade of eukaryotes composed almost entirely of organisms that are amoeboid during at least part of their life cycle (Adl et al. 2019; Kang et al. 2017). The group

includes “naked” amoebae, testate (shelled) amoebae, ciliated forms, and several so-called slime molds that produce spore-bearing fruiting bodies.

Protosteloid amoebae or protosteloid slime molds are amoeboid organisms in which a single cell can develop into a fruiting body with one to a few spores atop an acellular stalk. Although all known protosteloid slime molds are amoebozoans, they are widely spread in the group and do not form a monphyletic group or a paraphyletic group within a monophyletic Eumycetozoa sensu Olive, along with the myxogastrid and dictyostelid slime molds (Olive, 1975; Spiegel, 1990; Spiegel et al. 1995). This view was refuted by Shadwick et al. (2009). These organisms were formerly referred to as protostelids when they were thought to be closely related, but that term has fallen into disuse to emphasize this fact (Shadwick et al. 2009; Spiegel et al. 2017; Tice et al. 2016).

Simplification and trait loss are major evolutionary trends in Amoebozoa (Adl et al. 2019; Kang et al. 2017; Spiegel et al. 2017). The group’s last common ancestor was a sexual, ciliated organism that may have possessed other traits as well, including the ability for single cells to develop into spore-bearing fruiting bodies, a behavior known as sporocarpy (Adl et al. 2012; Spiegel et al. 2017). Sexuality may have involved an alternation of haploid and diploid generations (Adl et al. 2019; Kang et al. 2017). Today, only a few lineages in Amoebozoa contain organisms in which ciliated cells have been observed or in which a sexual cycle has been fully characterized (Adl et al. 2012; in press; Kang et al. 2017; Spiegel et al. 2017), although there is molecular evidence for sex in nearly all amoebozoan lineages (Hofstatter et al. 2018). Intriguingly, cilia and sporocarpy are erratically distributed throughout Amoebozoa, though there is a tendency for them to co-occur in life histories consistent with sex (Adl et al. 2012, in press; Kang et al. 2017; Spiegel, 1990; Spiegel et al. 1995, 2017). Cilia and sex are both very complex

traits that are synapomorphies of eukaryotes as a whole, and are each believed to have a single origin. As such, the only reasonable explanations are that both have been lost independently numerous times throughout the group's history, or have simply not been observed. The history of sporocarpy is less clear. Though it is unique to amoebozoa (Adl et al. 2019; Shadwick et al. 2009; Kang et al. 2017), it is currently impossible to rule out multiple origins. Still, it is possible that sporocarpy is a synapomorphy of Amoebozoa, and if so, it too appears to have been lost multiple times.

The amoebozoan genus *Protostelium* was first protosteloid slime mold recognized, and was described by Olive and Stoianovitch (1960) with *P. mycophaga* as the type species. It is an amoeboid organism found on decaying plant parts that can develop into a simple fruiting body consisting of a single spore atop an acellular stalk. Two years later, Olive (1962) published description of two new members of the genus, *P. fimicola* and *P. arachisporum*. At this point, Olive also proposed the family Protosteliaceae, distinguishing these organisms from other slime molds, i.e. the myxogastrid and cellular slime molds. A few other organisms were added to the genus over the years, namely *P. zonatum, P. pyriformis, P. irregularis* (Olive and Stoianovitch, 1969), *P. expulsum* (Olive and Stoianovitch, 1981), *P. nocturnum* (Spiegel, 1984) and *P. okumukumu* (Spiegel et al. 2006). L. Shadwick et al (2009) obtained preliminary results suggesting that *Planoprotostelium aurantium*, which had been thought to be sister to *Protostelium*, actually branched within *Protostelium*. This was later confirmed by J. Shadwick et al (2017), who also described a new member of the genus, *P. apiculatum*. Most of these organisms have since been moved out the genus.

Protostelium fimicola was moved to the genus *Protosteliopsis* on the basis of sporocarp morphology and the fact that its spores are non-deciduous (Olive and Stoianovitch, 1966). Later,

it was found to branch within the genus *Vannella* based on molecular data, though the authors of the study note that its amoeba morphology is highly typical of vannellids (Shadwick et al. 2009). It is now treated as a species of *Vanella* (Kang et al. 2017). *Endostelium zonatum* was moved from *Protostelium* based on sporocarp ultrastructure and development and amoebal morphology (Olive et al. 1984). *Protostelium irregularis* and *P. expulsum* were found to be unrelated to other *Protostelium* spp. on the basis of sporocarp development, and significant differences in amoeba morphology, including microtubule organization, lack of a detectable MTOC, and the presence of multiple nucleoli per nucleus, among other differences. (Spiegel, 1990; Spiegel et al. 1994). Both were moved to a new genus, *Soliformovum*. *Protostelium pyriformis* and *P. arachisporum* had their affinities to *Protostelium* called into question due to their ultrastructure (Bennett, 1986; Spiegel, 1990; Spiegel et al. 1994). Bennett (1986) noticed similarities between the centrosome of *P. pyriformis* and those of *Acanthamoeba spp.*, and suspected that they were related. Molecular data later showed both *P. pyriformis* and *P. arachisporum* to be acanthamoebids (Shadwick et al. 2009). They have been renamed *Acanthamoeba pyriformis* and *Luapeleamoeba arachispora*, respectively (Tice et al. 2016).

Olive defined *Protostelium* spp. as having uninucleate amoebae and sporocarps with a single, deciduous spore (i.e. the spore is readily shed from the stalk)(Spiegel et al. 1994). The genus was emended by Spiegel to only include organisms with non-ciliated amoebae containing orange lipid drops, microtubular cytoskeletons focused on one or two MTOCs, and in which the prespore cell passes through an elipsoid stage prior to sporocarp development. After the discovery that *P. aurantium* falls within the genus, it was further emended by Shadwick et al (2017). The current description is very similar the description from 1994, but it is now understood that ciliated cells may be present in the group, and that prespore cells undergo a

counter-clockwise rotation during fruiting body formation. As it stands, there are currently five recognized species of *Protostelium*: *P. mycophaga, P. aurantium, P. apiculatum, P. okumukumu,* and *P. nocturnum*.

In 1969, Olive and Stoianovitch published a paper describing a new morphotype of *P. mycophaga* which was characterized by the presence of a swelling in the stalk, usually at the base, which they named *P. mycophaga* var. *crassipes*. Two years later, they described a new species, which they named *Planoprotostelium aurantium*. *Planoprotostelium aurantium* is identical to *P. mycophaga,* except that it sheds spores less readily, and its trophic cells become ciliated in liquid medium. In describing it, Olive and Stoianovitch (1971), noted that its sporocarps occasionally had a bulbous swelling at the stalk base. They alluded to the similarity of these swellings to those seen in *P. mycophaga* var. *crassipes,* and even proposed a common mechanism by which those swellings might appear, but apparently did not consider their presence in both organisms to be particularly significant. *Planoprotostelium aurantium* was thought to be sister to the genus *Protostelium,* as this explanation is the most parsimonious in regards to cilia; it assumes a single loss in an ancestor of *Protostelium* spp. However, molecular data show that *Pl. aurantium* branches within the genus *Protostelium*, not sister to it. As such, it was renamed as *Protostelium aurantium* (Shadwick et al. 2017). Additionally, *P. mycophaga var. crassipes* interbranched with *P. aurantium* in a monophyletic clade, indicating that it represents non-ciliated isolates of *P. aurantium*.

The term "microtubule organizing center" (MTOC) was first coined by Pickett-Heaps (1968) in reference to structures or regions responsible for coordinating spindle fibers during mitosis, including the structure in animal centrosomes now referred to as the pericentriolar material (PCM). It is now used to refer to any structure that nucleates and organizes microtubules

(Lüders & Stearns, 2007). By far the best-known type of MTOC is that associated with the animal centrosome. This structure consists of a pair centrioles oriented at a 90° angle to each other, and surrounded by an electron dense layer known as the pericentriolar material (PCM) (Azimazadeh, 2014, Lawo et al. 2012). It is thought to coordinate microtubules both during interphase and mitosis, nucleating them through the action of γ-tubulin-containing complexes, and also functions as a kinetid or flagellar apparatus. The centriole was once thought to be required for mitotic spindle assembly, but several organisms routinely assemble mitotic spindles that lack centrioles (see Table 1). Several eukaryotes possess centriole-less structures that are otherwise reminiscent of animal centrosomes, and these structures are sometimes referred to as acentriolar centrosomes (Gräf et al. 2015); perhaps the best-known such structure is the spindle pole body (SPB) in *Saccharomyces* and other fungi. Acentriolar centrosomes seem to be functionally equivalent to animal centrosomes for the most part, and at least some components of the yeast SPB are homologous to components of the animal PCM (Jasperson & Winey, 2004; Lawo et al. 2012). However, it is not known if centrosomes are homologous across the eukaryotes.

One difference between centriolar and acentriolar centrosomes is that only centriolar centrosomes ever act as kinetids, also known as flagellar apparatuses. While centrioles do not appear to be required for spindle formation or organization of cytoplasmic microtubules, they seem to be necessary for producing cilia. All known kinetids contain centrioles in the form of basal bodies (Yabuki and Leander, 2013), even in organisms that do not otherwise use centrioles (see Table 1). In fact, centrioles seem to be absent in organisms that have lost cilia.

There are also MTOCs that do not include a centrosomal structure (Lüders and Sterns, 2007). These are best known in land plants, in which no structures reminiscent of a centrosome

are seen, except in liverworts (see Table 1). The preprophase band and phragmoplast are involved in coordinating microtubules during land plant mitosis (Buschmann and Zachgo, 2016; Pickett-Heaps and Northcote, 1966), and γ-tubulin-containing complexes have been shown to be recruited to existing microtubules to nucleate new microtubular branches (Murata et al. 2005).

In Amorphea (Amoebozoa + Obazoa, the latter including animals and fungi), the centrosome acts as a kinetid in ciliated cells (see Table1), at least in amoebozoans and opisthokonts. Opisthokonts, the subgroup of Obazoa that includes animals and Fungi that produce ciliated cells seem to have centrioles at all times (Karpov and Mylnikov, 1993; Powell, 1980). There are a few possible exceptions to this, such as metazoan myotubes, in which the nuclear envelope functions as an MTOC, instead of centrosomes (Tassin et al. 1985). Other non-centrosomal MTOCs have been observed in this group, such as mitotic chromosomes (Maiato et al. 2004) and spindle microtubules (Mahoney et al. 2006) in animals. Anucleate cells of fission yeast, *Schizosaccharomyces pombe*, that lack an SPB, are able organize interphase microtubular arrays, probably at least partially from existing microtubules (Daga et al. 2006). Experiments in animals have shown that mitosis proceeds normally even when centrioles or entire centrosomes are absent, but these cells cannot form cilia. Cultured mammalian cells with their centrosomes surgically removed during S-phase will complete mitosis, but stall at G1 phase after mitosis (Hinchcliffe et al. 2001). When genes needed for centriole synthesis in *Drosophila* are absent, centrosomes fail to form. These flies are able to reach adulthood, but are uncoordinated due to a lack of neuronal cilia, and soon die as a result (Basto et al. 2006).

Amoebozoa is currently divided into three major groups: Discosea, Evosea, and Tubulinea (Kang et al. 2017). MTOCs are poorly understood across the diversity of Amoebozoa, but there are a few specific species and lineages with very well-characterized MTOCs. No

ultrastructural data on MTOCS seem to exist for Tubulinea. Centrosomal structures are known in several members of Discosea, mostly in the centramoebids (Table 1). Most information on MTOCs in Amoebozoa comes from Evosea, largely from spore-forming organisms and the anaerobic Archamoebae.

In contrast to opisthokonts, amoebozoans only contain centrioles when cilia are present, or can become present without transitioning through another life stage (Table 1). Some of these organisms have dimorphic life cycles in which a stage that cannot become ciliated—the obligate amoeba, alternates with a stage that can become ciliated under the right conditions—the amoebomastigote (Adl et al. 2019; Spiegel and Feldman, 1985; Spiegel et al. 2017). Obligate amoebae have a variety of MTOCs (Spiegel and Feldman, 1988; Spiegel et al. 1985) none of which contain centrioles. Amoebomastigotes contain a structure known as a pro-kinetid (originally described as the pro-flagellar apparatus; Wright et al. 1980), that develops into the kinetid when the cell produces cilia (Spiegel et al. 1986; Spiegel and Feldman, 1991; Wright et al. 1980). The pro-kinetid undergoes structural changes during mitosis, losing its microtubular arrays, but retaining centrioles (Aldrich, 1969; Spiegel, 1982b).

The genus *Protostelium* is particularly interesting in regards to cilia, as, unless they are undiscovered in some species, they have been lost no fewer than four times (Shadwick at al, 2017). Cilia have been observed in a few strains of *P. aurantium* (Spiegel, 1981, 1982a), but never in any other nominal species in the genus (Shadwick et al. 2017). The structure of the centrosome is known in both *P. mycophaga*, which is sister to *P. aurantium*, and *P. nocturnum*, which is sister to the rest of the genus. Interestingly, it is nearly identical in both (Spigel et al. 1994). It has been proposed (Spiegel, 1982; Spiegel et al. 1994) that the centrosomes of *P. mycophaga* and *P. nocturnum* may be homologous to and derived from a degenerate kinetid like

that of ciliated *P. aurantium*. This hypothesis was presented prior to the realization that *P. aurantium* branches within rather than sister to the rest of *Protostelium*. Considering recent developments of the phylogeny of the genus, the centrosomes of *P. mycophaga* and *P. nocturnum* could be independently derived from a kinetid similar to that of *P. aurantium*, they could be direct homologues of each other, or they could have an entirely separate origin.

The fact that not all strains of *P. aurantium* produce cilia, and neither ciliated nor non-ciliated strains form a monophyletic group (Shadwick et al. 2017) suggests that there may be even more independent losses of cilia in *Protostelium*. There are currently three non-ciliated members of the genus for which the structure of the centrosome is unknown: *P. apiculatum, P. okumukumu,* and "crassipes"-type *P. aurantium*. Knowing the structure of these centrosomes may help shed light on the question of homology and possibly the evolutionary history of cilia within the group. Are the centrosomes of *P. mycophaga* and *P. nocturnum* homologous? In this thesis, the structure of the MTOC of a non-ciliated isolate of *P. aurantium* was determined, and found to be highly reminiscent of the centrosomes of *P. mycophaga* and *P. nocturnum*, suggesting that all three structures are probably direct homologues. Late developments strongly suggest that this homology is more related to the ancestral life history of the genus than to reduction of the kinetid.

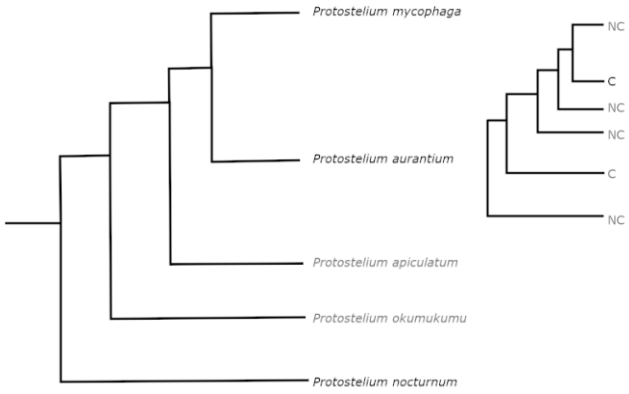

Fig. 1: Topology of the genus *Protostelium*, based on Shadwick et al (2017) from 18S rDNA sequences. Results are based on 22 isolates of *P. mycophaga*, six isolates of *P. aurantium*, two of which were ciliated, one isolate each of *P. apiculatum* and *P. okumukumu*, and three isolates of *P. nocturnum*. Grey lineages (*P. apiculatum* and *P. okumukumu*) represent species for which ultrastructural data on MTOCs is unavailable. Inset: Topology of ciliated (C) and non-ciliated (NC) isolates of *P. aurantium* used in the analysis.

Table 1: Known MTOCs in select groups of eukaryotes.

Organism/group	Typical MTOCs	Changes during Life cycle	Other features	References
Land Plants	Preprophase band (PPB), Phragmoplast, Existing microtubules	Centrioles only in sperm	Acentriolar centrosomes in liverworts	Buschmann and Zachgo, 2016 Murata et al. 2005
Ochrophytes	Animal-like centrosomes in brown algae, Microtubule centers and polar complexes in diatoms	Centrioles in diatom sperm	N/A	Katsaros et al. 2006 Drum and Pankratz, 1963 Tippit and Pickett-Heaps, 1977 Menton et al. 1970
Naegleria	Basal bodies,	Sudden appearance of microtubules during mitosis	No microtubules in amoeboid state, except during mitosis	Walsh, 1984; Walsh, 2012
Trypanosoma	Basal bodies; separate acentriolar structure in mitotic spindle; γ-tubulin in various cellular regions	Reorganization of γ-tubulin during mitosis	N/A	Ogbadoi et al. 2000; Scott et al. 1997
Metazoans	Centriolar centrosomes, Existing microtubules, Mitotic chromosomes, Golgi bodies	Minor changes to centrosome structure during mitosis	Centrosomes act as kinetids	Chabin-Brion et al. 2001; Conduit et al. 2014; Mahoney et al. 2006; Maiato et al. 2004
Fungi	Acentriolar centrosomes, Nuclear membrane	Size varies depending on ploidy	Centriolar centrosomes in zoosporic fungi	Barr, 1981; Jasperson and Winey, 2004
Breviates	Kinetid	Unknown	Has amoeboid state without visible cilia; MTOC unknown	Walker et al. 2006
Apusomonads	Kinetid	Unknown	N/A	Heiss et al. 2013
Discosea	Acentriolar centrosomes	Unknown	N/A	Bennet, 1986; Bowers and Korn, 1968; Geisen et al. 2014; Shadwick et al. 2016
Tubulinea	Unknown	Unknown	Unknown	N/A
Evosea	(Pro)-kinetids, acentriolar centrosomes, possibly existing microtubules	Centrioles present in ciliated cells, absent otherwise, change in pro-kinetid structure during mitosis	Pro-kinetid develops into kinetid when cilia are formed	See footnote[a]
Protostelium	(Pro)-kinetids, acentriolar centrosomes	Pro-kinetid structure changes dramatically during mitosis	Centrioles only known in ciliated *P. aurantium*; pro-kinetid develops into kinetid when cilia form	Spiegel, 1982a,b; Spiegel et al. 1994

[a] Aldrich, 1969; Pánek et al. 2016; Spiegel, 1981; Spiegel et al. 1985; Spiegel et al. 1986; Spiegel and Feldman, 1988; Spiegel and Feldman, 1991; Spiegel et al. 1994; Wright et al. 1980

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Chapter 2: Evidence for a Novel Life History in the Protosteloid Genus *Protostelium*

ABSTRACT

Protostelium aurantium is the only member of the monophyletic genus *Protostelium* in which any isolates have ever been shown to produce ciliated cells. Contrary to what one might expect, *P. aurantium* is not sister to the rest of the clade, but instead emerges from one of the more terminal nodes. Furthermore, cilia have not been observed in all isolates, and ciliated isolates do not form a monophyletic group. Centrosomes have previously been ultrastructurally characterized in two additional members of the group, *P. mycophaga* and *P. nocturnum*. These organisms lost the ability to produce cilia independently of each other, but the structures of their centrosomes are identical. In this study, a new strain of *P. aurantium* was isolated and found to be non-ciliated. The structure of its microtubule organizing center (MTOC) was characterized to help understand the nature of MTOCs in the genus, and found to bear strong resemblance to the centrosomes of *P. mycophaga* and *P. nocturnum*. After ultrastructural work was completed, amoebae of the new isolate unexpectedly became ciliated, suggesting that ciliated and non-ciliated forms of *P. aurantium* represent alternate life history stages. The implications of these findings for the evolutionary history of the genus *Protostelium* are discussed.

INTRODUCTION

Amoebozoa is a large, but poorly studied eukaryotic supergroup. It is sister to the recently described Obazoa, which includes metazoans, fungi, and several groups of protists (Brown et al. 2013). Although nearly all amoebozoans are amoeboid during at least part of their

life cycle, there are other morphologies present in the group, including testate (shelled) forms and ciliated forms. Many amoebozoans can produce cysts, and several are able to develop into spore-bearing fruiting bodies (Adl et al. 2019; Kang et al. 2017). The amoebozoan genus *Protostelium* consists of 5 nominal species, all of which can develop into simple fruiting bodies bearing a single spore atop an acellular stalk (Shadwick et al. 2017). One member of the genus, *P. aurantium*, has been observed to produce ciliated cells with anywhere from one to nine cilia (Olive and Stoianovitch, 1971a; Spiegel, 1982). *Protostelium aurantium* and the type species of the genus, *P. mycophaga*, are nearly identical morphologically, and the two were once distinguished primarily on the basis of *P. aurantium*'s ability to produce cilia when suspended in liquid, and the fact that it sheds spores less frequently than *P. mycophaga* (Olive and Stoianovitch, 1971a). *Protostelium aurantium* was once placed in its own genus, *Planoprotostelium*, until molecular data showed it to branch within the genus *Protostelium* as sister to *P. mycophaga* J Shadwick et al. 2017). The same study also revealed that isolates originally identified as *P. mycophaga var. crassipes* are actually non-ciliated strains of *P. aurantium* (J Shadwick et al. 2017).

The structure of the kinetid of ciliated *P. aurantium* is known (Spiegel, 1982), as well as a functionally similar structure, the centrosome, in *P. mycophaga* and *P. nocturnum* (Spiegel et al. 1994). The centrosomes of *P. mycophaga* and *P. nocturnum* are nearly identical, and consist of a ring with satellite elements surrounding a central plug (Spiegel et al. 1994). Given the relationships among these organisms (Shadwick et al. 2017), and the assumption that cilia have only evolved once in eukaryotes, *P. nocturnum* and *P. mycophaga* must have lost the ability to produce cilia independently of each other. This does raise the question of whether or not this type of centrosome structure is homologous between *P. mycophaga* and *P. nocturnum*, and how

widespread it is throughout the genus. The MTOC (microtubule organizing center) of non-ciliated strains of *P. aurantium* may be particularly informative. These strains probably represent additional losses of the ability to produce cilia. The structure of their MTOC may be reminiscent of the pro-kinetid of ciliated strains, but never develops into a full kinetid, it may resemble the centrosomes of other non-ciliated members of the genus, or it might have an entirely different structure. Knowing the structure of the MTOC of a non-ciliated isolate of *P. aurantium* should prove very insightful as to the evolutionary history of MTOCs within the genus *Protostelium*. A non-ciliated strain of *P. aurantium* was isolated, and the structure of its MTOC determined using transmission electron microscopy. This strain, *P. aurantium* HF-16, has an acentriolar centrosome that is very typical of non-ciliated members of the genus. However, after all ultrastructural work was completed, one subculture of strain HF-16 exhibited cells with cilia. The significance of this finding is discussed.

MATERIALS AND METHODS

Isolation and culturing

Protostelium aurantium strain HF-16 was isolated from dead, unshed apple leaves in Mayes county Oklahoma. Leaves were plated onto weak yeast-malt extract agar (wMY; 0.002 g yeast extract, 0.002 g malt extract, 0.75 g KH_2PO_4, 15 g Bacto agar, 1 L dH_2O; Shadwick et al. 2009). After approximately one week, plates were viewed under a Zeiss Axioskop 2 plus compound light microscope. *P. aurantium* was identified by the presence of a bulbous swelling at the base of some, but not all sporocarps in a colony. This is a distinctive characteristic of *P. aurantium* (Shadwick et al. 2017). This swelling is never seen in any other protosteloid slime mold, and is sufficient to identify a sporocarp as belonging to *P. aurantium*. Spores were picked up using a flame-sterilized insect needle under a Leica Z6 Apo dissecting microscope, dropped

onto wMY plates streaked with the basidiomycete yeast *Rhodotorula mucilaginosa*, and incubated at room temperature. To verify that the isolated strain of *P. aurantium* could not produce cilia, culture plates were flooded with dH_2O and checked for the presence of ciliated cells.

Light microscopy

Trophic cells, cysts, and sporocarps were viewed by placing the whole plate under a Zeiss Axioskop 2 plus with a 50x long working distance objective lens, and photographed using a Canon EOS Mark III digital camera. After collecting TEM data, cells from one plate were transferred to a slide by flooding the plate with wMY broth, suspending the cells with a spear-point needle, and pipetting them onto a slide to obtain images of the floating form amoebae that cannot produce cilia assume when suspended in liquid. Upon doing so, some of the amoebae unexpectedly became ciliated. Other plates were checked for ciliated cells by melting agar on a slide, placing blocks of agar on the surface, letting it sit for approximately 5 min, and adding wMY broth, as this method is less likely to contaminate the plate. This method was confirmed to be able to induce cilium formation in cells from the first plate.

Electron microscopy

In preparation for fixation for electron microscopy, blocks of agar containing trophozoites, cysts, and/or sporocarps of *P. aurantium* were placed on plates containing corn meal+ (CM+; 17 g corn meal agar, 2g dextrose, 2g yeast extract, 5g bacto agar) agar, with *R. mucilaginosa* as a food source. Amoebae were grown under these conditions until the orange color of the amoebae replaced the pink color of the yeast, indicating a high density of amoebae.

Plates were then flooded with wMY broth to act as a buffer. Amoebae were scraped from the agar surface into suspension using a spear-point needle, and a pipet was used to break up aggregates of yeast. Suspended amoebae were transferred to fill either a microfuge tube or Beem capsule about 1/3 of the way full and fixed for 30s to 2 min using enough 8% glutaraldehyde to fill the container about half way. 2% OsO_4 was added to fill the container and cells were left in the dark for 20-30 min. Cells were then centrifuged for 5 min, the supernatant was poured off, and replaced with an equivalent volume of dH_2O; these centrifugation steps were carried out three times, except that after the third centrifugation, 0.5% uranyl acetate was added to fill the container about half full, instead of dH_2O. Cells were then refrigerated overnight.

The supernatant was poured off, replaced with an equal volume of dH_2O, and the cells were centrifuged for 5 min. This was carried out three times. Before pouring off the supernatant for the third time, a small chunk of the pellet was viewed using brightfield microscopy to verify that cells were fixed. Cells were then dehydrated using a series of ethanol washes, with ethanol concentrations of 30%, 50%, 70%, 80%, 95%, and three washes at 100%. Cells were originally left in each wash for 30 min, but 15 minutes was found to be sufficient. It was necessary to centrifuge cells for10 min between each wash to keep the pellet concentrated. After the third wash in 100% ethanol, Spurr's medium was added to create a 50% solution in ethanol, and the mixture centrifuged for 10 minutes and left for one hour. The solution was replaced with 100% Spurr's medium, centrifuged for 10 minutes, and left for an hour. This was repeated once, and cells were left overnight in a desiccator. Cells were then transferred to a 70^{o}C oven for about 14 hrs.

Blocks were sectioned on a Sorvall Porter-Blum ultramicrotome. Blocks were manually trimmed using a razor blade, faced with a glass knife, and cut into ultrathin sections using a

diamond knife. Sections were transferred to either a copper mesh grid or a formvar-coated 1x2mm slotted copper grid. Grids were sometimes stained with 2% uranyl acetate and lead citrate for four min each to increase the visibility of microtubules, but MTOCs were distinct without this staining. Samples were viewed on a JEOL JEM-1011 transmission electron microscope at 100 kV.

RESULTS

General cell morphology

Protostelium aurantium HF-16 was first identified on a primary isolation plate by its sporocarps, some, but not all of which have a bulbous swelling about 3 μm in diameter at the base characteristic of *P. aurantium*. Prone stalks are approximately 50 μm long, and spores have a diameter of about 8-10 μm (Fig. 1a). Fresh cultures display trophic cells, cysts, and sporocarps on wMY agar. As the culture ages, trophic cells and sporocarps become scarcer, leaving mostly cysts. Cysts are round and slightly irregular in outline, with a diameter of approximately 7 μm. (Fig. 1a-c). Trophic cells have broad, lobose pseudopodia with acutely pointed subpseudopodia, one to a few contractile vacuoles, and a single uninucleolate nucleus. Food vacuoles with partially digested yeast cells are visible in some cells. (Fig. 1c). Trophic cells range from approximately 5-28 μm in length and 5-18 μm in width. The smallest cells are rounded, possibly in preparation for encystment (Fig 1b-e). When crawling on a solid substrate, they have numerous pointed subpseudopodia (Fig. 1d-e) characteristic of the genus (Shadwick et al. 2017). They are extremely slow moving on agar medium, and tend to congregate around high concentrations of the yeast used as a food source (Fig 1c). Amoebae contain orange lipid droplets characteristic of *Protostelium* spp. (Fig. 1b). When transferred to a slide, some cells become

detached from the substrate. These cells become much more active, frequently contorting their shape and producing several broad and pointed pseudopodia (Fig. 1f-g).

Amoebae of *P. aurantium* HF-16 may become ciliated

Cells grown on one particular plate that was flooded after obtaining results from electron microscopy unexpectedly became ciliated when transferred to a slide with wMY broth (Fig 2). These cells had recently been transferred as spores and cysts from a plate that was several months old. Each cell typically had approximately two to three cilia (Fig 2a-b), but their three-dimensional shape and constant motion made it difficult to see multiple cilia at the same time (Fig. 3c-d). Orange pigmentation was still present in ciliated cells (Fig 2a, c-d). Cells remained capable of phagocytic feeding while ciliated (Fig. 2c). Kinetids were sometimes visible on ciliated cells (Fig. 2d). These cells were indistinguishable from those characteristic of ciliated isolates of *P. aurantium* (see Spiegel, 1982). The appearance of ciliated cells may be indicative of a transition to another life history stage (see discussion).

Electron Microscopy

General ultrastructure. Cells of *P. aurantium* strain HF-16 contain large numbers of lipid and glycogen bodies (Fig. 3a-b), though these become less abundant if cells are starved (Fig. 3c). This is probably partially due to the food-rich conditions under which they are grown prior to fixation. Glycogen bodies were poorly preserved by fixation. Cells contain mitochondria with tubular cristae as is typical for amoebozoans. Each cell contains a single nucleus, as well as components of the endomembrane system including the endoplasmic reticulum and dictyosomes (Fig. 3a-b). Food vacuoles containing partially digested yeast cells were also present, as were

autophagic vacuoles (Fig. 3a, d). Centrosomes are located among a concentration of endomembrane elements adjacent to the nucleus, in or near an invagination in the nuclear envelope near stacks of dictyosomes (Fig. 3b, Fig. 4). They persist even in walled dormant stages (Fig 3a, c)

Microtubule organizing center structure. The MTOC of *P. aurantium* HF-16 is typical of non-ciliated *Protostelium* spp. It consists of a central plug surrounded by an electron-dense ring (Fig. 4). The central plug protrudes out of the central ring on one end for probably about 50-100 nm (Fig. 4a-c), but does not appear to stretch the entire vertical distance of the ring (Fig. 4e). The plug also does not appear to be a perfect cylinder, as it is narrower inside the ring than outside of it (Fig. 4a-c, f). The plug appears to be about 50-70 nm in diameter at its widest (Fig. 4a-c), and narrows to a diameter of approximately 30 nm (Fig. 4f), possibly smaller. The ring is approximately 180 nm in diameter, and about 40 nm thick. The inside of the ring is about 60 nm around. There is a space between the outer surface of the plug and the inner surface of the ring (Fig. 4f). This inner ring is in turn surrounded by a series of small, electron-dense bumps rich in microtubules that may represent microtubule nucleation sites (Fig. 4c, g). These structures are encircled by pair of irregular ring-like structures (Fig. 4b-f). No single image showed one of the outer rings in its entirety, and it is not clear if they are both the same size. Both seem to have gaps along their length. No pattern to these gaps was discernable. They probably have a diameter of around 200-210 nm. The outer rings may be connected to the inner ring by a series of struts. If the pattern in Fig. 4f holds, there are probably four struts per outer ring. Microtubules originating from the inner components of the centrosome pass through the outer rings (Fig. 4b).

A diagram of the centrosome is shown in Fig. 5. No structures resembling centrioles were ever observed.

DISCUSSION

The centrosome of *P. aurantium* HF-16 (Fig. 5) bears striking resemblances to those of *P. mycophaga* and *P. nocturnum* in that all three contain an electron-dense ring surrounding an electron-dense plug (Spiegel et al. 1994). The centrosomes of those organisms do not appear to have the outer ring of *P. aurantium*, but the satellite extensions seen in *P. mycophaga* (Spiegel, 1982), may be equivalent. It was hypothesized that the centrosomes of *P. mycophaga* and *P. nocturnum* may be homologous to one or more components of the kinetid of *P. aurantium* (Spiegel, 1982; Spiegel et al. 1994), particularly the ring-like rootlet element at the base of the centriole. Current understanding of the phylogeny of the genus *Protostelium* would require that both structures are independently derived from a "Planoprotostelium"-like kinetid in order for this to be the case. The other likely possibility is that both structures are direct homologues. The similarity of *P. aurantium* HF-16's centrosome to those of *P. mycophaga* and *P. nocturnum* supports the idea that the ring and plug structure may homologous throughout the genus, though knowing the MTOC structures of the two remaining species, *P. apiculatum* and *P. okumukumu*, would allow us to be more confident in this assumption. The last common ancestor of the genus was clearly capable of producing cilia, as there is no evidence suggesting that cilia in "Planoprotostelium"-type *P. aurantium* are not homologous to those in other eukaryotes (Spiegel, 1982). As such, the kinetid of ciliated *P. aurantium* is an ancestral, not a derived trait. However, acentriolar centrosomes similar to those seen in non-ciliated *Protostelium* spp. have never been reported in ciliated strains of *P. aurantium*. So where did the acentriolar centrosomal MTOC in non-ciliated *Protostelium* spp. come from?

Although strain HF-16 was consistently non-ciliated since its isolation, one plate examined late in the study produced ciliated cells. This plate was several months old, and was flooded to illustrate the so-called floating form amoebae assume when suspended in water (Shadwick et al. 2017). After approximately 5-10 min. however, cells began to develop cilia and assume a morphology typical of "Planoprotostelium"-type strains of the species.

Shadwick et al (2017) suggest that ciliated and non-ciliated forms of *P. aurantium* may represent alternate stages of the same life history, but noted that the data needed to verify this hypothesis are not currently available. In fact, when Olive and Stoianovitch (1971a) described "*Planoprotostelium*" *aurantium*, they noted that some of its sporocarps had a bulbous swelling at the base, and even mentioned the similarity of these sporocarps to those of *"P. mycophaga var. crassipes*", a proposed morphotype of *P. mycophaga* distinguished by the presence of such swellings (Olive and Stoianovitch, 1969) but apparently didn't see it as particularly significant. As mentioned previously, all isolates of *P. mycophga* var. *crassipes* examined thus far have branched with *P. aurantium* in 18S rDNA trees (Shadwick et al. 2017). If these isolates did represent alternate life history stages, it would offer a possible explanation for the conservation of the ring and plug centrosome throughout the genus *Protostelium*. If an alternation of life history stages was an ancestral characteristic of the genus *Protostelium*, the ciliated stage could have been lost or simply undiscovered in all species except *P. aurantium*. Under this assumption, the trophic cells of all other *Protostelium* spp. are homologous to the non-ciliated stage of *P. aurantium*, which has a centrosome that is ultrastucturally distinct from the kinetid of the ciliated stage.

There is precedent for this in Amoebozoa, as several species exhibit dimorphic life cycles that alternate between a reversibly ciliated amoebomastigote like the type strain of *P.*

aurantium and a strictly non-ciliated obligate amoeba (Spiegel and Feldman, 1985; Spiegel, 1990; Spiegel et al. 2017). The myxogastrid slime molds (Stephenson et al. 2011), their sister group, the protosporangiids (Scheetz, 1972; Olive and Stoianovitch, 1972; Olive and Stoianovitch, 1977), *Cavostelium apophysatum* (Spiegel and Feldman, 1985), and *Ceratiomyxella tahitiensis* (Olive and Stoianovitch, 1971b) all exhibit such life cycles. The amoebomastigotes of these organisms possess a pro-kinetid, which functions as an MTOC in the amoeboid state, and develops into a kinetid while the cell is ciliated (Spiegel et al. 1986; Spiegel and Feldman, 1991; Wright et al. 1980), as do ciliated isolates of *P. aurantium* (Spiegel, 1982). The kinetid and pro-kinetid of the amoebomastigote are ultrastructurally distinct from the MTOC of the obligate amoeba (Spiegel, 1981; Spiegel and Feldman, 1985; Spiegel and Feldman, 1988; Salles-Passador et al. 1992). It is worth noting however, that in all the above examples, the amoebomastigote and obligate amoeba stages have very different morphology. Additionally, in all of the above life cycles, only the obligate amoeba stage is capable of fruiting, and amoebomastigotes germinate from the spores in almost all cases, while both ciliated and non-ciliated amoebae of *P. aurantium* can fruit.

The centrosome observed here by electron microscopy (see Fig. 5) is not consistent with the kinetid of ciliated strains of *P. aurantium* (Spiegel, 1982); it lacks several key components of the kinetid, including centrioles, and it is doubtful it could coordinate synthesis of a ciliary axoneme, meaning the cells observed by electron microscopy were almost certainly incapable of producing cilia, at least under current conditions.

The presence of ciliated cells possibly represents a transition to a different life stage, as suggested by Shadwick et al (2017). Trophic cells of non-ciliated *Protostelium* spp., including "crassipes"-type *P. aurantium* have been considered to be homologous to the amoebomastigote

of "planoprotostelium"-type *P. aurantium* (Spiegel, 1990; Spiegel et al. 1994). In other words, they were thought of as amoebomastigotes that have lost the ability to produce cilia. If the events observed here are being interpreted correctly, these cells may be true obligate amoebae. If so, *P. aurantium* is the only known amoebozoan for which the amoebomastigote and obligate amoeba are morphologically indistinguishable, and in which both are capable of fruiting. A proposed life history of *P. aurantium* is shown in Fig. 6.

The transition between the amoebomastigote and obligate amoeba is known to be associated with the sexual cycle in myxomycetes (Aldrich, 1967; Dee, 1962; Stephenson et al. 2011), and this association is suspected in other organisms with similar dimorphic life cycles (Kang et al. 2017; Olive and Stoianovitch, 1972; Sheetz, 1972; Spiegel et al. 1981; Spiegel and Feldman, 1985). Evidence of sex has never been reported in *Protostelium* spp. If the transition observed here indeed represents a life cycle transition, it is unclear if sex is involved, though it is becoming apparent that sex may be much more common in Amoebozoa than previously suspected (Hofstatter et al. 2018; Lahr et al. 2011). The availability of the *P. aurantium* genome opens the possibility of searching it for genes involved in sex. This could also be done with the genome of *P. mycophaga*, and would be possible with other members of the genus, if their genomes are sequenced. This may hint at the possibility of life histories similar to what is proposed here for *P. aurantium*.

Currently, there is no reliable method for inducing life stage transitions in *P. aurantium*. They appear to be a rare occurrence under culture conditions. The fact that electron microscopy consistently revealed acentriolar centrosomes instead of "planoprotostelium"-like kinetids suggests that this is probably not the result of a failure to recognize transitions due to an inability to distinguish the two stages. Currently, the only known distinctions between the obligate

amoeba and amoebomastigote of *P. aurantium* are the nature of the MTOC and ability of the amoebomastigote to become ciliated. It is unclear exactly what other differences, if any, may exist.

Comparative studies of the amoebomastigote and obligate amoeba may help to uncover these differences, and may hint at what factors are responsible for inducing transitions between the two. It is unclear if obligate amoebae are homologous throughout Amoebozoa or have arisen independently multiple times, though it has been proposed that they have independent origins (Spiegel and Feldman, 1985; Spiegel et al. 1995). Comparative work on gene regulation during life stage transitions and between obligate amoebae and amoebomastigotes in the same life history may help resolve this question, which would have significant implications for our understanding of the evolutionary history of Evosea, if not the whole of Amoebozoa.

These findings also hint at the possibility of cryptic life histories in the genus *Protostelium*. Other members of the genus may exhibit similar, undiscovered life histories. Furthermore, Amoebozoa as a whole may have undiscovered complexity. Traits such as sex and cilia could be much more widely distributed in the group than currently suspected (Adl et al. 2019; Kang et al. 2017; Spiegel et al. 2017).

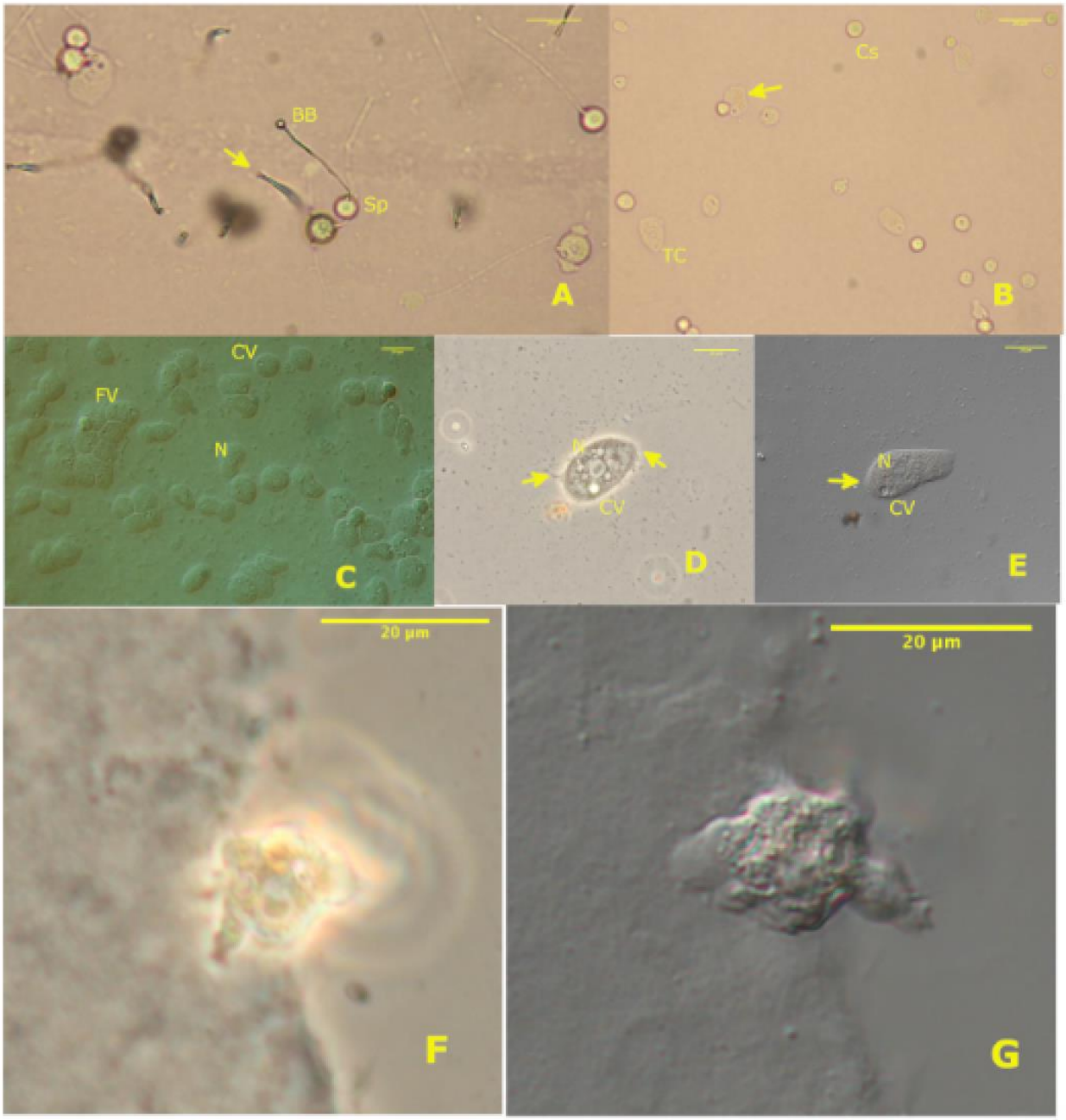

Fig. 1: Light micrographs of non-ciliated *Protostelium aurantium* HF-16. A) Stages of life cycle visible on wMY agar, including sporocarps with and without (arrow) bulbous bases; brightfield. B) Amoebae and cysts. Note orange lipid droplets (arrow) in amoebae; brightfield. C) Dense cluster of trophic cells showing nuclei, contractile vacuoles, and food vacuoles; DIC. D) adherent trophic cell on a slide, showing filose subpseudopodia (arrows); phase contrast. E) The same cell, also showing fine, filose subpseudopodia (arrows); DIC. F) Non-ciliated amoeba suspended in wMY broth with a large, irregular pseudopod; phase contrast. G) The same cell; DIC. Key: BB=bulbous stalk base; Cs=cyst; CV=contractile vacuole; FV=food vacuole; N=nucleus; Sp=spore; TC=trophic cell. Scale bars=20 µm

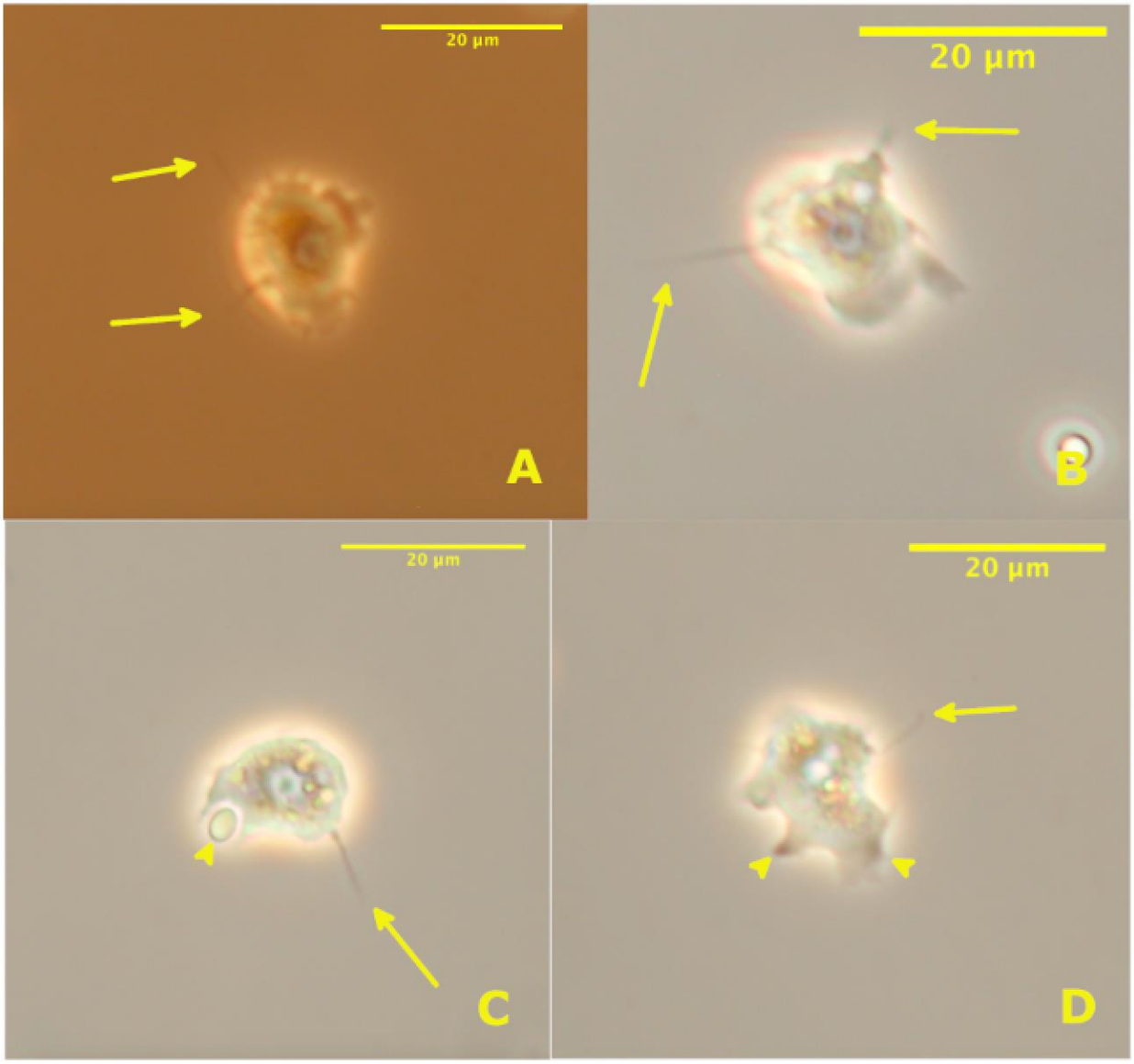

Fig. 2: Light micrographs of a ciliated cell of *P. aurantium* HF-16; phase contrast. A-B) Cell with two cilia visible (arrows). C) Cell phagocytizing a yeast (arrowhead). One cilium is visible (arrow). D) Cell with one cilium (arrow) and two kinetids (arrowheads) visible.

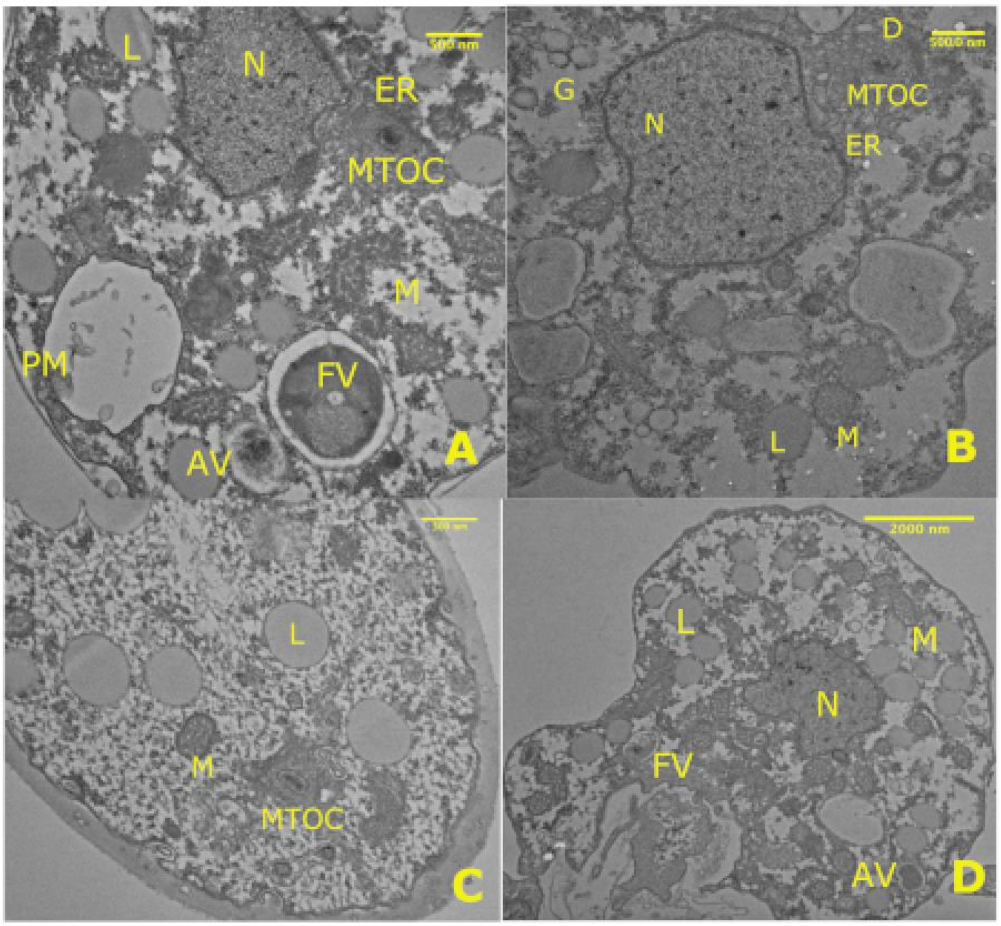

Fig. 3: General ultrastructure of *P. aurantium* HF-16. A) Walled dormant cell. B) trophic cell. C) Walled dormant cell showing signs of starvation. D) General cell morphology. Key: AV=autophagic vacuole; D=dictyosomes; ER=endoplasmic reticulum; FV=food vacuole; G=remnant of glycogen body; L=lipid body; M=mitochondrion; MTOC=microtubule organizing center; N=nucleus; PM=plasma membrane

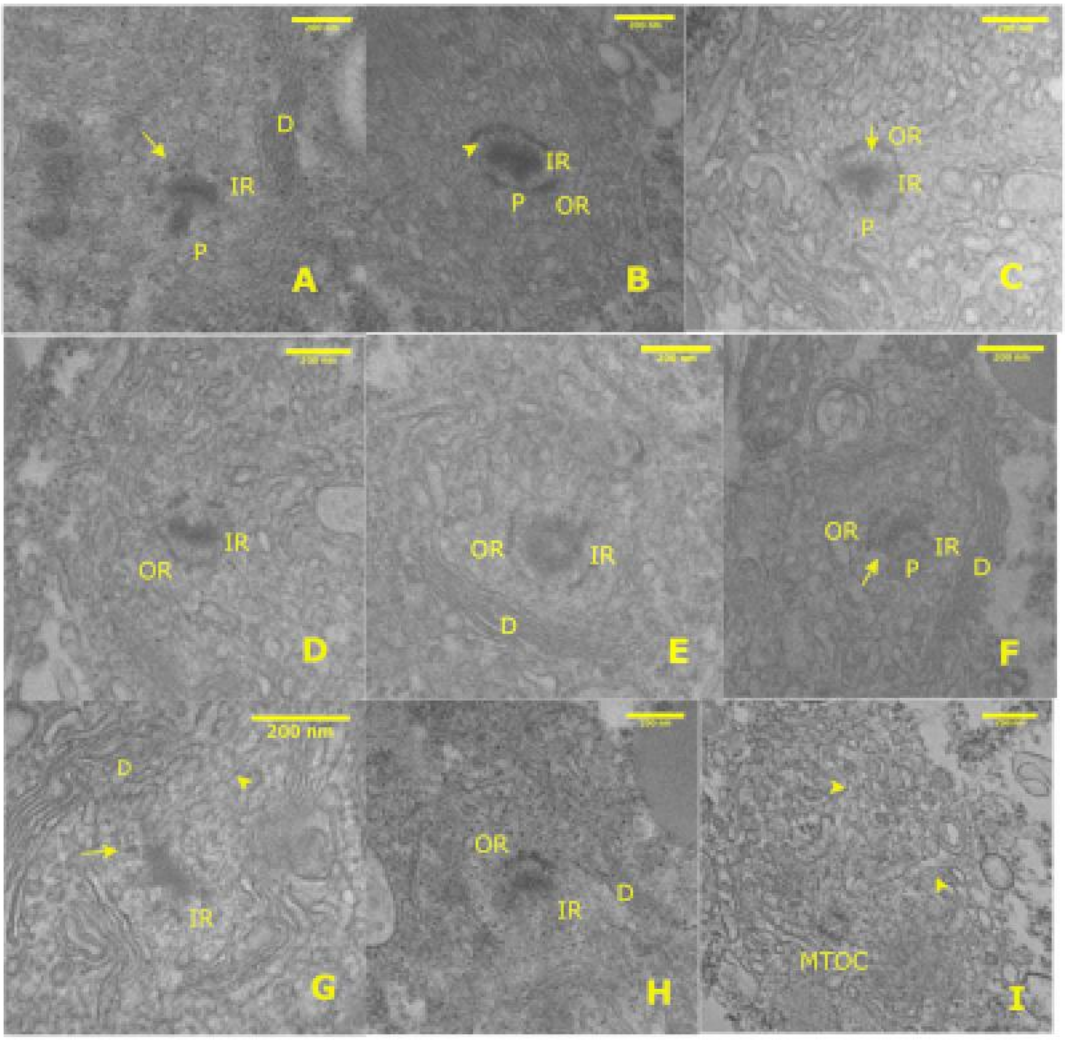

Fig. 4: Ultrastructure of the MTOC of *P. aurantium* HF-16. A) Lateral view of MTOC, with central plug protruding. Note peripheral elements with microtubules (arrowheads). B) Lateral view of MTOC). Plug is slightly visible. Microtubules (arrowheads) can be seen penetrating outer ring structure. C, D) Adjacent sections of inner ring. Plug is visible in C, and spoke-like structures (arrow)are seen between inner and outer rings. E) Cross-section of outer ring. F) Cross-section of outer ring, with plug visible in the center of the ring. Struts (arrow) are seen connecting the inner and outer rings. G) Central ring with surrounding elements (arrows) and attached microtubules (arrowheads). H) Small region of inner and outer rings. I) MTOC with associated microtubules (arrowheads) Key: D=dictyosomes; IR=inner ring; MTOC=microtubule organizing center; OR=outer ring; P=plug

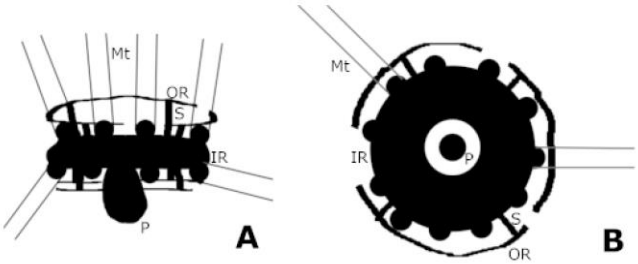

Fig 5: Diagram of centrosome of *P. aurantium* HF-16. A) Lateral view; B) Top-down view. Key: IR=inner ring; Mt=microtubule; OR=outer ring; P=plug

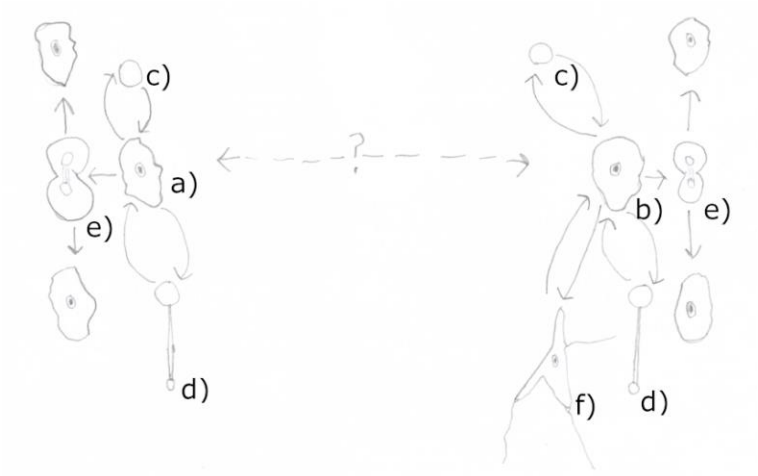

Fig. 6: Proposed life history of *P. aurantium*: a) obligate amoebae and b) amoebomastigotes can c) encyst, d) fruit, or e) divide. Unlike the obligate amoeba, the amoebomastigote can f) become ciliated. The dotted line indicates that the nature of the transition between the obligate amoeba and amoebomastigote is unknown.

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Chapter 3: Conclusion

Protostelium aurantium is the only species of *Protostelium* in which ciliated cells have ever been reported. While one would expect it to be sister to the rest of the genus such that one clade retained the ability to produce ciliated cells while the other did not, it instead branches within the genus, as sister to *P. mycophaga,* with the grade of *P. apiculatum, P. okumukumu,* and *P. nocturnum* branching basal to it (Fig. 1; Shadwick et al. 2017). The existence of even a single ciliated strain within a lineage is regarded as sufficient evidence to conclude that the last common ancestor of that lineage was ciliated, as all cilia are thought to share a common origin. Therefore, the last common ancestor of the genus *Protostelium* was almost certainly capable of producing cilia. If the current proposed phylogeny of the group is correct, all species of *Protostelium* other than *P. aurantium* have lost the ability to produce cilia independently of one another, though we cannot completely rule out the possibility that ciliated cells have simply never been found in at least some of them. Still, the centrosomes observed in non-ciliated *Protostelium* isolates (Spiegel et al. 1994) are likely not capable of coordinating the synthesis of a ciliary axoneme, as they lack centrioles. Acentriolar centrosomal MTOCs like the structures observed in these organisms and kinetids or pro-kinetids are not known to exist in amoebozoan cells at the same time, and acentriolar MTOCs have not been observed in ciliated isolates of *P. aurantium* (Spiegel, 1982) or any other ciliated amoebozoans that have been examined in any depth (Spiegel, 1981; Spiegel and Feldman, 1985, 1988; Wright et al. 1979).

These structures either evolved independently in these organisms in the wake of losing the ability to produce cilia, or are homologous to each other. The fact that they are also observed in a non-ciliated isolate of *P. aurantium* means that if they are not homologous, the ring and plug centrosome has at least three independent origins, possibly more. centrosomes tend to be highly

conserved structures (Azimzadeh, 2014; Shadwick et al. 2016; Spiegel et al. 1994). While independent origins of ring and plug centrosome cannot be conclusively ruled out, it seems increasingly likely that this type of centrosome is homologous throughout the genus *Protostelium*. However, if the ring and plug centrosome has a single origin, how do we explain its absence in ciliated strains of *P. aurantium*? Since the last common ancestor of the genus was ciliated, and kinetid structure tends be very strongly conserved, it likely had a kinetid as an MTOC similar to what is found in *P. aurantium* today. One possible explanation for this situation is the existence of multiple trophic states as suggested by Shadwick et al (2017); an amoebomastigote with a kinetid, and a nearly identical obligate amoeba with an acentriolar centrosome. The presence of ciliated cells in a culture that could not produce them when it was isolated lends credibility to this hypothesis.

P. aurantium strain HF-16 was isolated over two years prior to the writing of this thesis. Although cells were not checked for cilia by suspending them in liquid during that time, they were examined using electron microscopy, and no signs of kinetids or pro-kinetids were ever observed, indicating that were incapable of producing cilia during that time. This would suggest that it took nearly two years in culture before some cells transitioned to a state in which they could become ciliated by suspending them in liquid. As such, it would appear that this may be a transition that occurs only rarely, at least under laboratory conditions, explaining why it has not previously been observed. It is not known how widespread the transition is in the culture in which it was observed. This could be determined by isolating individual cells and growing them in liquid media on a multi-well plate. Since *P. aurantium* is pigmented, wells with ciliated cells would have orange pigmentation throughout the media, while it would be restricted to the bottom

and sides if ciliated cells were absent. The trigger that induces the transition remains unclear, and finding that trigger is beyond the scope of this thesis.

This also provides a possible explanation for the relationship between *Ceratiomyxella tahitiensis* and *Nematostelium gracile*. *N. gracile* has a plasmodium as its only trophic state, that can cleave into sporocarps. *C. tahitiensis* has a complex life cycle involving an amoebomastigote alternating with a plasmodium identical to that of *N. gracile*, which can either cleave into fruiting bodies or develop directly into amoebomastigotes. Sporocarps produced by both organisms are identical (Olive and Stoianovitch, 1971). In light of the discovery here regarding *P. aurantium*, *N. gracile* may simply represent isolates of *C. tahitiensis* that have lost the amoebomastigote stage or do not enter it under laboratory conditions. This could be tested if phylogenies of the two organisms could be constructed using more isolates of each. If neither forms a monophyletic group, or if *N. gracile* branches from within *C. tahitiensis*, it would provide strong support for this hypothesis.

Because the discovery of ciliated cells was a last-minute occurrence, there was no time to obtain ultrastructural data on them to verify the presence of a kinetid. Additionally, there was no time to use molecular data to verify that both ciliated and non-ciliated forms are indeed the same organism. This could be done easily by comparing the 18S rDNA of both, as they should be identical. This would confirm that the discovery of ciliated cells was due to a life stage transition rather than contamination. Knowing the structure of the MTOCs of *P. apiculatum* and *P. okumukumu* may be helpful in reinforcing the conclusion that the ring and plug centrosome has a common origin among non-ciliated *Protostelium* spp. However, confirming that the ciliated and non-ciliated variants of *P. aurantium* are alternate stages of a life history using 18S rDNA

sequences would provide very compelling support for this idea, and would be much easier to carry out.

In all other amoebozoans in which an amoebomastigote and obligate amoeba stage are known, the two differ morphologically, often significantly (Spiegel and Feldman, 1985, 1991; Spiegel, 1990; Spiegel et al. 2017). Furthermore, only obligate amoebae are known produce spores in these life histories (Spiegel & Feldman, 1985). The obligate amoeba and amoebomastigote of *P. aurantium* however, are both capable of fruiting, and are morphologically identical; the only difference appears to be in the fact that the obligate amoeba has an acentriolar MTOC and cannot produce cilia, while the amoebomastigote has a (pro)-kinetid (Spiegel, 1982) and can produce cilia. This suggests that other amoebozoans may have cryptic life history stages that are virtually identical other than certain behaviors or ultrastructural features. It also suggests that some reductions in Amoebozoa may be due to loss of an entire life stage.

As there is currently no known method for inducing life stage transitions in *P. aurantium*, studying the factors involved in inducing it could be difficult, especially as it seems to occur so rarely in culture. However, it may be possible to determine what differences exist between the amoebomastigote and obligate amoeba. Transcriptomic differences may be the best place to start, as there appear to be no ultrastructural differences aside from the MTOC. This may help to elucidate what factors are involved in life stage transitions as well.

It is thought that obligate amoebae may have multiple origins throughout Evosea, as there do not appear to be any consistent similarities between them (Spiegel & Feldman, 1985; Spiegel, 1990). Obligate amoebae vary widely as to gross morphology, including number of nuclei and the structures of their MTOCs. Amoebomastigotes on the other hand are all

uninucleate amoebae with very similar kinetids (Spiegel, 1981; Spiegel, 1990; Spiegel et al. 2017). Amoebozoans with dimorphic life cycles are known only in Evosea, but sporocarpic fruiting, which is found in nearly all known dimorphic amoebozoan life cycles, is present on both sides of the deepest node in Amoebozoa (Adl et al. 2019; Kang et al. 2017). Interestingly, in dimorphic life cycles with sporocarpic fruiting, it is always the obligate amoeba that fruits (Spiegel, 1985). The only known exception to this is *P. aurantium*. Comparative studies of amoebomastigotes and obligate amoebae may be able to confirm or refute the idea of multiple origins of obligate amoebae. The fact that the obligate amoebae and amoebomastigotes of *P. aurantium* are so similar make it an interesting organism to use in these comparisons. If molecular techniques can reveal homologies that could not be detected through morphology or ultrastructure, it would suggest that a dimorphic life cycle involving amoebomastigotes, obligate amoebae, and sporocarpic fruiting was probably present in the last common ancestor of the subgroups in Evosea that display such life cycles today, Eumycetozoa and Variosea. If significant homologies cannot be found, it would suggest that obligate amoebae do indeed have multiple origins. This would in turn raise the question of why it is nearly always the obligate amoeba the produces fruiting bodies and amoebomastigotes that germinate from spores. An organism like *P. aurantium*, in which both stages appear to be capable of both fruiting and germinating from spores might simply be a novelty, or it could be helpful in explaining this pattern. Determining the origins of obligate amoebae would profoundly affect our understanding of Evosea.

The sister group to *Protostelium*, *Filamoeba*, does not contain any known members with ciliated cells (Kang et al. 2017). It would be interesting to see if *Filamoeba* spp. also contain a ring-and-plug MTOC similar to that seen in non-ciliated *Protostelium* spp. More work should

also be done to determine if centrosomes are homologous across eukaryotes. The composition and organization of the metazoan centrosome is known (Lawo et al. 2012). Finding similar protein sequences in other eukaryotes would help to establish homology between their centrosomes, but it may also be informative to use immunolabeling or fluorescent tagging to demonstrate that the same proteins localize to the centrosome.

These results highlight the potential for hidden complexity among living organisms. Some organisms exhibit changes, features, or behaviors that are very noticeable, leading us to expect all such aspects of living things to stand out and catch our attention. In doing so, we often overlook subtle things, and in many cases, the things we overlook hold the answers to the questions we ask.

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