EVOLUTION OF ZYGOMYCETOUS SPINDLE POLE BODIES: EVIDENCE FROM *COEMANSIA REVERSA* MITOSIS[1]

DAVID J. MCLAUGHLIN[2,6], ROSANNE A. HEALY[2,7], GAIL J. CELIO[3], ROBERT W. ROBERSON[4], AND T. K. ARUN KUMAR[5]

[2]Department of Plant Biology, University of Minnesota, St. Paul, Minnesota 55108-1095 USA; [3]University Imaging Centers, University of Minnesota, St. Paul, Minnesota 55108-1095 USA; [4]School of Life Sciences, Arizona State University, Tempe, Arizona 85287 USA; and [5]Department of Botany, The Zamorin's Guruvayurappan College, Calicut, Kerala 673014 India

- *Premise of the study:* The earliest eukaryotes were likely flagellates with a centriole that nucleates the centrosome, the microtubule-organizing center (MTOC) for nuclear division. The MTOC in higher fungi, which lack flagella, is the spindle pole body (SPB). Can we detect stages in centrosome evolution leading to the diversity of SPB forms observed in terrestrial fungi? Zygomycetous fungi, which consist of saprobes, symbionts, and parasites of animals and plants, are critical in answering the question, but nuclear division has been studied in only two of six clades.
- *Methods:* Ultrastructure of mitosis was studied in *Coemansia reversa* (Kickxellomycotina) germlings using cryofixation or chemical fixation. Character evolution was assessed by parsimony analysis, using a phylogenetic tree assembled from multigene analyses.
- *Key results:* At interphase the SPB consisted of two components: a cytoplasmic, electron-dense sphere containing a cylindrical structure with microtubules oriented nearly perpendicular to the nucleus and an intranuclear component appressed to the nuclear envelope. Markham's rotation was used to reinforce the image of the cylindrical structure and determine the probable number of microtubules as nine. The SPB duplicated early in mitosis and separated on the intact nuclear envelope. Nuclear division appears to be intranuclear with spindle and kinetochore microtubules interspersed with condensed chromatin.
- *Conclusions:* This is the sixth type of zygomycetous SPB, and the third type that suggests a modified centriolar component. *Coemansia reversa* retains SPB character states from an ancestral centriole intermediate between those of fungi with motile cells and other zygomycetous fungi and Dikarya.

Key words: centriole; centrosome; fungi; nuclear division; spindle pole body; ultrastructure; zygomycetes.

The earliest-evolved eukaryotes have been inferred as flagellates, with centrioles that nucleate centrosomes, the microtubule organizing center (MTOC) for nuclear division (Carvalho-Santos et al., 2011). The MTOC in higher fungi, which lack flagella, is the spindle pole body (SPB). One of the hypotheses being tested in the second Assembling the Fungal Tree of Life (AFTOL 2) project is that flagellum loss is associated with differentiation of the SPB and a change to filamentous body form or hyphae in later-diverging fungi (James et al., 2006a; Stajich et al., 2009). The early-diverging fungi typically produce motile spores, and sometimes motile gametes, and their nuclei have a MTOC that consists of a centrosome containing paired centrioles as in animal, algal, and some plant cells (Roberson et al., 2010; Carvalho-Santos et al., 2011). Higher fungi, i.e., zygomycetous fungi and Dikarya, lack motile cells as well as centrioles and the MTOC for nuclear division is a SPB, a derivative of the centrosome. SPBs have distinctive morphologies in those phyla and subphyla studied to date and have proved phylogenetically informative (Celio et al., 2006; McLaughlin et al., 2009, 2015). Spindle pole bodies may be globular or plaque- or disc-shaped and form a middle piece that connects the duplicating SPBs (Roberson et al., 2010). Zygomycetous fungi are critical in testing the SPB differentiation hypothesis, because some taxa have a SPB structure that appears to be intermediate between MTOCs with centrosome and centrioles and a SPB, but nuclear division and SPB morphologies are incompletely known in these fungi.

Zygomycetous fungi are highly diverse ecologically and phylogenetically and their deeper relationships are not fully resolved. They are distinguished by the formation of zygospores in sexual reproduction, but zygospores are absent in the arbuscular mycorrhizal fungi (Glomeromycota), which reproduce only asexually (Redecker and Schüssler, 2014). The zygomycetous fungi, which will be referred to hereafter by the common name zygomycetes,

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[6]Author for correspondence (e-mail: davem@umn.edu)

[7]Present address: 4 Farlow, 22 Divinity Avenue, Harvard University, Cambridge, Massachusetts 02138 USA

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are currently treated as six clades, two at the phylum level (Entomophthoromycota, Glomeromycota) and four additional clades at the subphylum level, whose phylum placements are unresolved (Benny et al., 2014; Redecker and Schüssler, 2014). Molecular phylogenetic analyses show that these clades are derived from flagellated fungi, the traditional Chytridiomycota, which now consists of multiple phyla, including Chytridiomycota, Monoblepharidomycota, and Blastocladiomycota (James et al., 2006b; Gryganskyi et al., 2012; Powell and Letcher, 2014), the three clades relevant to this study. Problems in determining relationships within the zygomycete clades have arisen when different rRNA and/or protein-coding genes have been used in phylogenetic reconstruction, and the placement of some genera (*Basidiobolus*, *Olpidium*) has suggested multiple losses of flagella, rather than a single loss, in the transition from ancestral, Chytridiomycota-like fungi with motile cells to the terrestrial zygomycetes and the Dikarya (Ascomycota and Basidiomycota) (James et al., 2006a; White et al., 2006; Sekimoto et al., 2011; Gryganskyi et al., 2012).

Zygomycetes include saprotrophs, such as the common bread mold, *Rhizopus*, whose life cycle is typically included in botany texts, and the dung-inhabiting *Pilobolus* spp., famous for their mode of sporangium discharge (Yafetto et al., 2008). These common fungi with their multispored sporangia are characteristic of the Mucoromycotina clade. The Entomophthoromycota clade contains saprobes and parasites of insects, arthropods, vertebrates, plants, and algae (Benny et al., 2014). They are best known as obligate arthropod parasites. The group includes *Basidiobolus*, which is most consistently isolated from dung of insectivorous reptiles and amphibians; *Pandora*, an important aphid pathogen; and *Ancylistes*, a parasite of desmids (Butt and Beckett, 1984a; Alexopoulos et al., 1996; Stajich et al., 2009; Benny et al., 2014). The Kickxellomycotina clade includes saprobes isolated from soil or dung, such as *Coemansia*, as well as the group commonly referred to as trichomycetes, symbionts in the gut of aquatic insects to which they attach by a holdfast (Stajich et al., 2009; Benny et al., 2014). The arbuscular mycorrhizal fungi (Glomeromycota clade) are important obligate symbionts with land plants with which they have coevolved from the beginning of land plant evolution (Stajich et al., 2009; Redecker and Schüssler, 2014).

Five types of SPB have been reported in zygomycetes, four in the Entomophthoromycota clade (Moorman, 1976; Butt and Beckett, 1984a; Murrin et al., 1984; McKerracher and Heath, 1985; Roberson et al., 2011) and one in the Mucoromycotina clade (Franke and Reau, 1973; McCully and Robinow, 1973; Bland and Lunney, 1975; Heath and Rethoret, 1982). Nuclear division and SPB characters differ greatly between these two clades, most notably the cylindrical SPBs of *Basidiobolus* and *Pandora* (Entomophthoromycota), which are reminiscent of centrioles (Tanaka, 1970; Sun and Bowen, 1972; Gull and Trinci, 1974; Moorman, 1976; Heath and Rethoret, 1982; Butt and Beckett, 1984a, b; Murrin et al., 1984, 1988). The nuclear and SPB data can be viewed online in the AFTOL Structural and Biochemical Database (http://aftol.umn.edu/) (Celio et al., 2006; Kumar et al., 2013; McLaughlin et al., 2015). There are no data on SPB types or nuclear division for the other four clades (Glomeromycota, Kickxellomycotina, Mortierellomycotina, Zoopagomycotina), except for an incomplete report for the trichomycete *Harpella* (Kickxellomycotina) (Reichle and Lichtwardt, 1972).

To answer our question regarding the association between stages in centrosome evolution and the diversity of SPB forms in terrestrial fungi, we examined the SPB structure and details of nuclear division of vegetative cells of *Coemansia reversa* Tiegh. & G. Le Monn. (Kickxellomycotina), a species that grows readily in culture. *Coemansia* species produce dramatic, branched sporangiophores bearing one-spored sporangia arranged in a row along the lower side of the branches, and are saprobic in dung, humus, or other organic matter (Benjamin, 1959; Alexopoulos et al., 1996). For an illustration of the sporangiophore of *C. reversa*, see http://genome.jgi.doe.gov/Coere1/Coere1.home.html. *Coemansia reversa* was selected as the first representative of its clade for genome sequencing in the 1000 Fungal Genomes project to better understand fungal biodiversity (http://genome.jgi.gov/programs/fungi/index.jsf) (Grigoriev et al., 2011). The nuclear stages in *Coemansia* sporangiophores were illustrated by Benjamin (1959), and these structures were initially targeted. However, while the sporangiophores were ideal for cryofixation, the sporangiophore walls interfered with freeze substitution and resulted in inadequately contrasted cytoplasm (Healy et al., 2014). Therefore, we examined the SPBs in nuclei of germlings and young hyphae, where the walls were thinner, less differentiated, and more easily penetrated by staining solutions. Cryofixation followed by freeze substitution produces fewer artifacts than chemical fixation (Hoch, 1986 and references therein); however, the nuclear membrane, among other features, is difficult to distinguish because it does not readily take up heavy metal stains in freeze-substituted cells (Giddings, 2003), and SPBs are clearer after chemical fixation (Heath and Rethoret, 1982). Therefore, both freeze substitution and chemical fixation were used to gain insights into the structure of SPBs and dividing nuclei of *C. reversa*. Here we report on mitosis and the SPB cycle in *Coemansia revers*a using 3- to 4-d-old germinated spores prepared for electron microscopy by chemical fixation (CF) or cryofixation with freeze substitution (FS) and use the data to infer patterns of character evolution, especially in SPBs, during the early evolution of fungi as they transitioned from aquatic to terrestrial habitats.

MATERIALS AND METHODS

Fungal material—*Coemansia reversa* (AFTOL 1401, NRRL 1564, ATCC 12441, originally isolated from rat dung) was grown on malt extract yeast extract (MEYE) agar plates (90 × 15 mm) in a growth chamber at 21°C, on a 12 h light/dark cycle, for 3 wk until sporulation was abundant. An initial study of nuclear condition, using fluorescence microscopy, was carried out, using 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Roche Molecular Biochemicals, Mannheim, Germany), 2 μg/mL in distilled water for 5 min, after fixation for 1 h in 2% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7, followed by three distilled water rinses. Staining on a timed series of spore germination indicated that spores are uninucleate, do not germinate synchronously, and that there are up to eight nuclei in the young hyphae within 3 d of germination. From these initial studies, we decided to use 3- to 4-d-old cultures for ultrastructural analysis because they would include many stages of germinated spores and young hyphae with actively dividing nuclei. Spores were harvested by adding 10 mL of sterile distilled water to the plate and swirling gently for several minutes. Additional spores were loosened with a sterile micropipette tip. The floating spores were harvested by tipping the plate slightly and pipetting up the water. The spore solution was filtered through sterile miracloth to exclude hyphal fragments, placed into sterile 1.5 mL microcentrifuge tubes, and centrifuged for 10 min at 13 500 rpm. The spores tended to clump together, but 10 min of intermittent vortexing effectively separated the majority of clumps. Using a hemocytometer, the spore solution density was adjusted to 400/mL for those intended for fixation of 4-d-old young hyphae, and a density of 725/mL for those intended for fixation of 3-d-old germlings. For fungal material that would be chemically fixed, 360–480 μL of a solution with 400 spores/mL was added to 6-cm-diameter, sterile cellophane membranes on MEYE agar,

TABLE 1. Character states for mitotic and spindle pole body characters used in data matrix for character evolution analysis.

Character	States
1. Centriole	0, absent; 1, present
2. Basic organization of the spindle pole	0, centriolar-associated material; 1, spindle pole body consists of small amount of extranuclear material (Mucoralean zygomycetes); 2, ring-like spindle pole body; 3, spindle pole body a plaque or disc; 4, bar
3. Microtubules in spindle pole body in circle perpendicular to nuclear envelope	0, absent; 1, present
4. Spindle pole body form, interphase–prophase	0, notched ring with middle piece and intranuclear component; 1, ring containing microtubules but lacking centriolar 9-fold symmetry (*Basidiobolus*); 2, centriolar-associated extranuclear and intranuclear components with intact nuclear envelope; 3, centriole-associated material; 4, ring containing microtubules with centriolar 9-fold symmetry; 5, bar
5. Spindle pole body form, metaphase–anaphase	0, unlayered disc with intact nuclear envelope and internal microtubule organizing center; 1, notched ring with persistent half middle piece and clear zone between intranuclear component and nuclear envelope; 2, centriole associated material; 3, ring containing microtubules with centriolar 9-fold symmetry; 4, bar and spindle microtubules often terminating within nucleus some distance from nuclear envelope
6. Spindle development site	0, gap in nuclear envelope; 1, intranuclear (nuclear envelope intact)
7. Spindle pole body migration	0, migration before spindle formation; 1, migration during spindle formation; 2, integration of spindle pole body into invaginated nuclear envelope before spindle formation
8. Metaphase nuclear envelope	0, intact; 1, intact with small polar fenestrae plugged by the spindle pole bodies; 2, loose polar fenestrae, including extensions of nuclear envelope into the cytoplasm at the spindle pole, but mainly intact; 3, partially dispersed
9. Telophase nuclear envelope	0, retention of complete nuclear envelope around the chromatin with median constriction/fragmentation; 1, retention of parts of a disrupted nuclear envelope around the chromatin; 2, new envelope forms within old envelope; 3, dispersed in interzone; 4, retention of the nuclear envelope around the chromatin with constriction/fragmentation near the poles, and interzone is cut off from the daughter nuclei
10. Metaphase plate	0, absent; 1, present
11. Nucleolus behavior	0, nucleolus is dispersed and no longer recognizable during prophase; 1, nucleolus discarded between prophase and metaphase; 2, nucleolus or part of it is more persistent and is discarded after metaphase; 3, nucleolus is persistent throughout division

and cultures were placed in the growth chamber for 4 d using the conditions outlined above. For fungal material destined for freeze-substitution, 1.5 µL of a solution containing 725 spores/mL was placed on plates of MEYE agar, each of which contained 16, 5 × 4-mm dialysis membranes, which had been boiled for 6 h in distilled water, then separated and autoclaved in distilled water. These membranes had been notched in the upper right corner to mark the inoculated side of the membrane and closely arranged in a square in the center of the plate.

***Electron microscopy*—**For chemical fixation, 4-d-old hyphae on membranes were initially fixed by flooding the plates for 15 min with 2% glutaraldehyde + 2% acrolein in 0.1 M sodium cacodylate buffer, pH 7. The hyphae were scraped off the cellophane membranes with a plastic pipette and liquid plus hyphae decanted into microcentrifuge tubes. Hyphae were pelleted by centrifuging for 10 min at 13 000 rpm, the supernatant discarded, and hyphae resuspended in fresh fixative in foil-wrapped vials and set on a shaker at ca. 50–100 rpm for 12 h at room temperature. Hyphae and fixative were then transferred to microcentrifuge tubes and centrifuged for 10 min and the supernatant discarded. Centrifugation was used prior to all solution changes in this protocol to pellet the hyphae. Fixed hyphae were washed six times in the same buffer, inverting the tube after each change to dislodge hyphae from the bottom of the tube into the fresh solution. After final wash, hyphae were post fixed in 1% w/v OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h on ice in the dark, followed by two buffer and two distilled water washes. Hyphae were stained en bloc in 0.5% w/v aqueous uranyl acetate in the dark over night at room temperature. For the dehydration steps, hyphae were embedded in 1.5% w/v Noble agar (Becton Dickinson and Co., Sparks, Maryland, USA) in a 45°C water bath, pelleted by centrifugation, and solidified at 4°C for 1 h. Dehydration, infiltration, embedding, and polymerization were as in Kumar et al. (2012) except that the initial water wash was repeated six times.

For cryofixation and freeze substitution, membranes with 3-d-old germlings were lifted using forceps from the media plates and immediately plunge frozen in liquid propane at −178 to −185°C (Hoch, 1986). Frozen specimens were transferred to a substitution solution of 1% glutaraldehyde + 1% tannic acid in acetone and held at −80°C for 72 h (Fields et al., 1997). Samples were then washed three times in acetone and transferred to a second substitution fluid of 1% OsO_4 in acetone and held at −80°C for 1 h They were gradually brought to

TABLE 2. Character matrix for character evolution analysis. Clade names (Fig. 11; online Appendix S1) abbreviated to first four letters.

Taxa	Clade	Character no. 1	2	3	4	5	6	7	8	9	10	11	Reference
Allomyces neomoniliformis Indoh	BLAS	1	0	1	2	?	?	?	?	1	?	2	Olson, 1974
Ancylistes sp.	ENTO	0	3	0	?	0	1	?	0	0	?	3	Moorman, 1976
Basidiobolus haptosporus Dreschler	ENTO	0	2	1	1	?	?	?	?	?	?	?	McKerracher and Heath, 1985
Basidiobolus magnus Dreschler	ENTO	0	2	1	1	?	?	?	?	?	?	?	McKerracher and Heath, 1985
Basidiobolus ranarum Eidem	ENTO	?	?	?	?	?	?	0	3	3	1	0	Gull and Trinci, 1974; Sun and Bowen, 1972; Tanaka, 1970
Blastocladiella emersonii Cantino & Hyatt	BLAS	1	0	1	2	?	?	?	0	0	?	?	Lessie and Lovett, 1968
Catenaria anguillulae Sorokin	BLAS	1	0	1	2	?	?	0	0	4	1	1	Ichida and Fuller, 1968
Coemansia reversa Tiegh. & G.Le Monn.	KICK	0	2	1	4	3	1	1	0	?	0	?	This study
Entomophaga aulicae (Reichardt) Humber	ENTO	0	4	0	5	4	1	1	0	0	0	3	Murrin et al., 1984, 1988
Harpochytrium hedinii Wille	MONO	1	0	1	3	?	0	2	2	0	1	?	Whisler and Travland, 1973
Monoblepharella sp.	MONO	1	0	1	3	2	0	0	2	0	1	2	Dolan and Fuller, 1985
Mucor hiemalis Wehmer	MUCO	0	1	0	?	?	?	1	0	0	0	3	McCully and Robinow, 1973
Pandora neoaphidis (Remaud. & Hennebert) Humber	ENTO	0	2	0	0	1	1	1	0	0	0	3	Butt and Beckett, 1984a, b
Phlyctochytrium irregulare Koch	CHYT	1	0	1	3	2	0	2	2	?	1	?	McNitt, 1973
Phycomyces blakesleeanus Burgeff	MUCO	0	1	0	?	?	?	?	0	?	0	3	Franke and Reau, 1973; Heath, 1986
Pilobolus kleinii Tiegh.	MUCO	0	1	0	?	?	?	1	0	0	0	3	Bland and Lunney, 1975; Heath, 1986
Polyphagus euglenae Nowakowski	CHYT	1	0	1	3	2	0	2	2	?	1	2	Roychoudhury and Powell, 1991
Powellomyces variabilis Longcore, D. J. S. Barr & Desauln.	CHYT	1	0	1	3	2	0	2	1	2	1	0	Powell, 1975
Rhizophydium sphaerotheca Zopf	CHYT	1	0	1	3	2	0	0	2	?	1	?	Powell, 1980
Zygorhynchus moelleri Vuill.	MUCO	0	1	0	?	?	?	?	0	0	0	3	Heath and Rethoret, 1982; Heath, 1986

room temperature (−20°C for 2 h, −4°C for 2 h, room temperature for 30 min) and then washed three times with acetone. Samples were slowly infiltrated for embedding following the infiltration steps for chemical fixation. Embedding, polymerization, cell selection, sectioning, poststaining, and imaging were as in Celio et al. (2007) except that polymerization was carried out at 70°C for 14 h, and uranyl acetate formulation was 2% w/v in 50% ethanol.

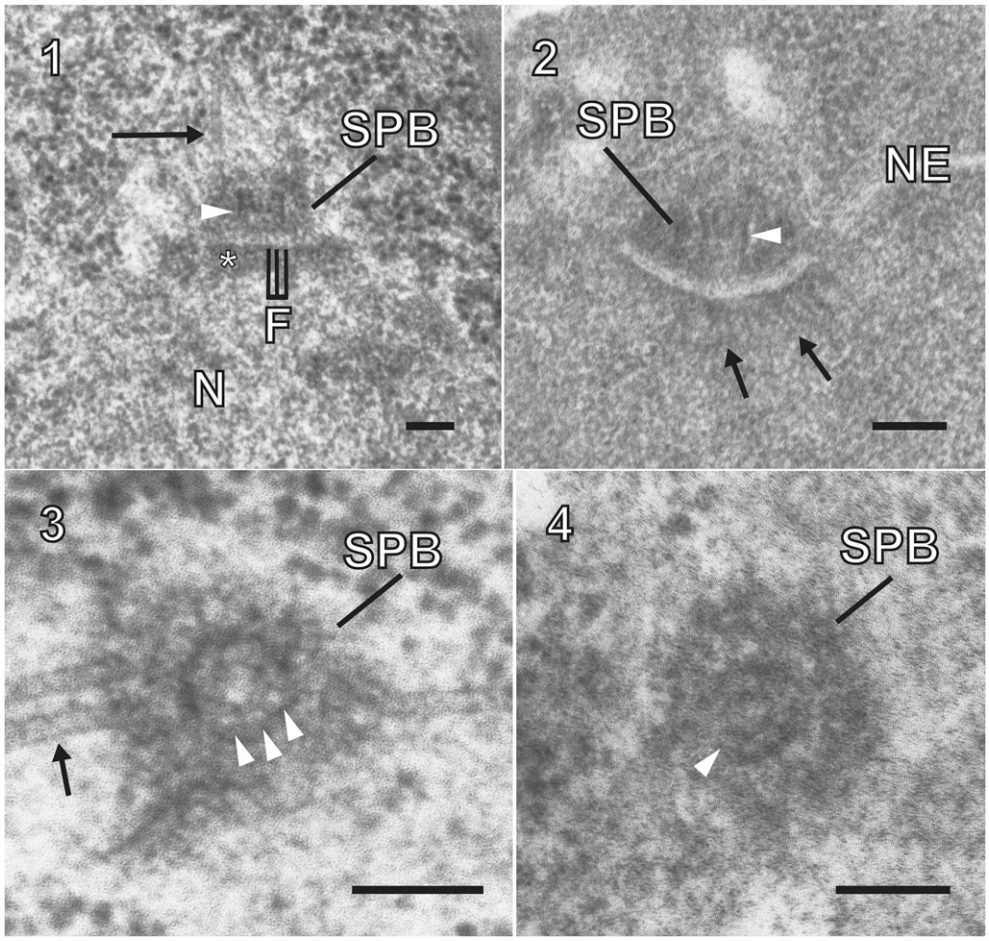
An electronic version of Markham's rotation (Markham et al., 1963) of cross sections of the cylinder in the SPB was done in Adobe Illustrator CS4 v. 14 (Adobe Systems, San Jose, California, USA) to enhance the periodicity of the SPB. This was done by importing images of the same cross section into the same position, setting the opacity level of each to 100/*n*, and rotating the first one by 360/*n*, the second by 2(360/*n*), the third by 3(360/*n*), etc., until the desired number of rotations (*n*) was reached. This was done for 7 to 10 rotations. Advantages of using digital images in a graphic software design program such as Illustrator over 1/*n* exposures of the original negative rotated *n* times around the center of the symmetrical object of interest, as originally described for this technique, was the ease of precision of centering, of rotating *n* degrees, and of achieving the desired percent opacity for each stack of images.

***Character evolution analysis*—**Character states for nuclear division and SPB characters were updated on the AFTOL Structural and Biochemical Database (http://aftol.umn.edu/) and a NEXUS file for Chytridiomycota, Blastocladiomycota, Monoblepharidomycota, Entomophthoromycota, and zygomycotan fungi downloaded for parsimony analysis in the program Mesquite v. 2.75 (Maddison and Maddison, 2008), using a phylogenetic backbone assembled with guidance from a multigene analysis of Gryganskyi et al. (2012: fig. 3), James et al. (2006a, b), and Stajich et al. (2009) with placement of some species based on Gryganskyi et al. (2013). An additional character (Table 1, character 3) was added to the analysis. Uninformative characters and character states were eliminated, characters and character states renumbered, and a final character list (Table 1) and data matrix (Table 2) produced.

RESULTS

***Mitosis*—**Approximately 60 nuclei were analyzed for this study. Determining the early stages of mitosis was problematic. Interphase ($N = 15$) was defined as the stage when a single SPB was present on the cytoplasmic side of the nuclear envelope, chromatin showed limited condensation and no association with the SPB, and the nucleolus was in a central or peripheral position within the nucleus. At late interphase–prophase ($N = 17$) short intranuclear microtubules and/or condensed chromatin were associated with the SPB. Late interphase–prophase was more easily recognized when the SPB had duplicated and two SPBs were evident in close proximity on the outer nuclear surface ($N = 7$). An additional 19 nuclei could not be assigned to a particular stage.

At interphase, the SPB consisted of two components: a cytoplasmic, electron-dense sphere containing a cylindrical structure with microtubules oriented approximately perpendicular to the nucleus, and an electron-dense intranuclear component appressed to the nuclear envelope (Figs. 1, 2). Microtubules within the SPB were about 23 nm in diameter, approximately the same diameter as microtubules associated with the SPB surface (Fig. 3). The cylinder was approximately 68–71 nm in height ($N = 2$) and 80–100 nm in width at its midpoint ($N = 4$), while the overall height of the SPB was 150 nm as measured from the nuclear envelope. A ring of approximately nine microtubules was detected in tangential sections through the SPB, but no micrograph showed all nine microtubules clearly (Figs. 3, 4). Since the SPB was symmetrical, we could take advantage of Markham's rotation (with 7–10 rotations) to reinforce the image of the cylindrical structure in tangential sections and gauge the results for strength of rotation at the lowest possible periodicity (7 rotations) and increasing rotations past the point where the number of microtubules was most distinct, with the least amount of noise. Reinforcement was strongest at nine rotations, which suggests nine microtubules per SPB. The microtubules were not truly parallel but were closer together at their nuclear



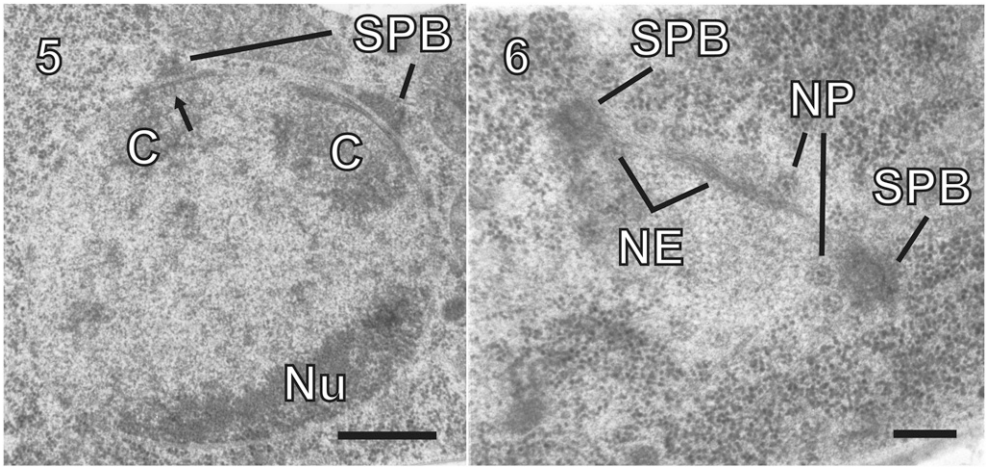
Figs. 1–4. Spindle pole bodies (SPBs) at interphase in sections radial (Figs. 1, 2) and tangential (Figs. 3, 4) to the nucleus observed after cryofixation and freeze substitution (Figs. 1, 3) and chemical fixation (Figs. 2, 4). **1.** Microtubules (arrow) associated with the interphase SPB radiate into the cytoplasm. Cylindrical structure (arrowhead) within SPB with electron-dense, plate-like structure at center of cylinder parallel to the long axis of the arrowhead. Intranuclear component (asterisk) forming an electron-dense band within nucleus (N) adjacent to the SPB. Fine filaments (F) appear to extend through the nuclear envelope that separates the extra- and intranuclear SPB components. **2.** SPB with cylinder (arrowhead) appressed to the intact nuclear envelope (NE) with intranuclear component forming a thin band at which short microtubules (arrows) terminate. **3.** Extranuclear SPB component with astral microtubules terminating at its margin and three microtubules visible within cylinder (arrowheads). **4.** Extranuclear SPB component sectioned through cylinder (arrowhead) with microtubules. Scale bars = 0.1 μm.

end (Figs. 1, 2, 9). Internally the cylindrical structure showed some differentiation in radial sections after cryofixation with freeze-substitution or chemical fixation in that a plate-like structure was observed near its nuclear end (Figs. 1, 2, 9, arrowhead). Fine filaments appeared to extend from the base of the SPB through the nuclear envelope (Fig. 1). Microtubules radiated into the cytoplasm from the SPB surface (Figs. 1, 3).

The SPB duplicated at late interphase–prophase, and the paired SPBs separated on the intact nuclear envelope (Figs. 5, 6). From each spindle pole, astral microtubules appeared to radiate from the SPBs, many in a plane tangential to the nucleus, but no microtubules connected the separating SPBs. A groove or indentation in the nuclear envelope extended between the two poles (Fig. 6). Short microtubules were present on the intranuclear SPB components within the nucleus beneath the extranuclear SPB components (Fig. 5), and heterochromatin aggregated beneath the separating SPBs. The nucleolus formed an extensive band along the nuclear envelope distant from the SPB.

Spindles with chromatin arranged near their center made metaphase–anaphase easy to recognize (N = 6). Nuclear division appeared to be intranuclear with interpolar and kinetochore microtubules interspersed with the condensed chromatin (Fig. 7). Chromosomes aligned at the center of the spindle, but a well-defined metaphase plate appeared to be absent (Fig. 7). Rather, the chromosomes were arranged in a broad band across the mid region of the spindle. The nuclear envelope was intact beneath the external SPBs (N = 3) at metaphase–anaphase (Figs. 8, 9). Kinetochores were somewhat electron-dense and appeared as slightly flared ends of the microtubules (Fig. 10). The nucleolus retained a peripheral position within the nucleus or was also in a pocket in the nucleus (Fig. 7). Late stages in nuclear division were not observed.

***Character evolution analysis*—**Presence of a centriole and associated material in the SPB (character states 1-1, 2-0) (Table 1) is plesiomorphic in Fungi, and these character states have been lost (1-0) in the common ancestor to zygomycetes and Dikarya; however, where the transition to a ring-like SPB (2-2) or a SPB with a small amount of extranuclear material (2-1) occurred in zygomycetes is unresolved by Mesquite at the branch joining the Mucoromycotina clade and the Kickxellomycotina-Entomophthoromycota clades (Fig. 11). A ring of



Figs. 5, 6. Duplicated spindle pole bodies (SPBs) at prophase separating on the nuclear envelope (NE) in radial (Fig. 5) and tangential (Fig. 6) section; both freeze-substituted. **5.** Heterochromatin (C) within nucleus aggregated beneath the separating SPBs, short microtubules (arrow), and nucleolus (Nu) forming a band distant from the SPBs. **6.** Indentation in nuclear envelope (NE) extends between the separating SPBs. Nuclear pores (NP). Scale bars = 0.5 μm.

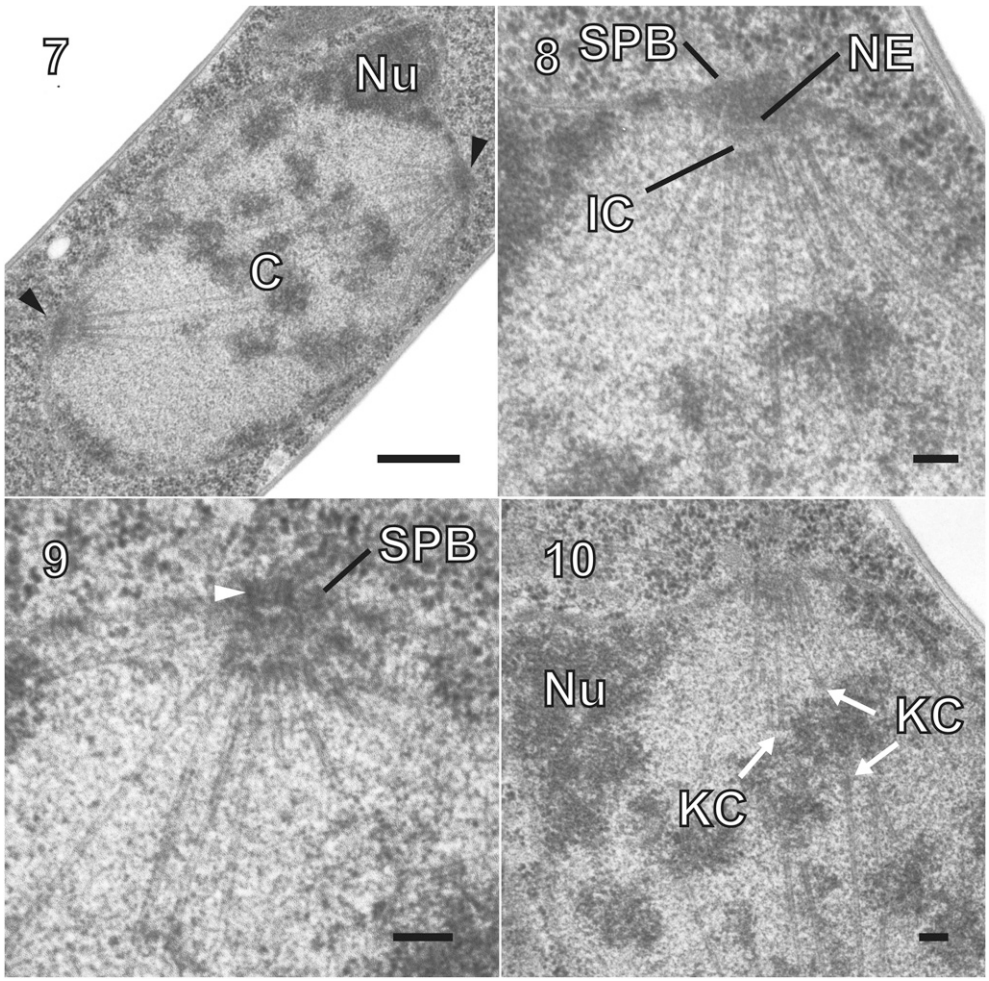
microtubules within the SPB (3-1) is plesiomorphic and found in *C. reversa* (Kickxellomycotina) and *Basidiobolus* (Entomophthoromycota) and the Chytridiomycota and other centriole-containing fungi. This character state (3-0) has been lost independently in Mucoromycotina and one clade of the Entomophthoromycota (*Ancylistes*, *Pandora*, and *Entomophaga* clade). These genera each show a different form of the SPB, a plaque or disc (2-3), a ring (2-2), or a bar (2-4), respectively, with two independent losses of the ring-like SPB in *Ancylistes* and *Entomophaga* (2-3, 2-4).

The detailed forms of the SPB at interphase–prophase (character 4) and at metaphase–anaphase (character 5) are incompletely known in some clades. In some cases, the character states seem to be autapomorphic within a clade (Blastocladiomycota, 4-2; Chytridiomycota, and Monoblepharidomycota, 4-3, 5-2; *Coemansia reversa*, Kickxellomycotina, 4-4, 5-3), but in the better-studied Entomophthoromycota clade a range in diversity of SPB forms (4-0, 4-1, 4-5; 5-0, 5-1, 5-4) is observed at these mitotic stages. The spindle development site is intranuclear (6-1) in *C. reversa* and most Entomophthoromycota, while in Chytridiomycota and Monoblepharidomycota the spindle develops in a gap in the nuclear envelope (6-0); missing data make evolution of this character unclear. Migration of the duplicated SPBs to the spindle poles occurs before spindle formation in the Blastocladiomycota, Chytridiomycota, and Monoblepharidomycota clades (7-0) but during spindle formation (7-1) in the zygomycete clades. The plesiomorphic condition for the timing of SPB migration is unclear (7-0, 7-2). Whether a closed or open nuclear envelope at the poles at metaphase (character 8) is plesiomorphic is unresolved. An opening at the poles is shared by Chytridiomycota and Monoblepharidomycota (8-1, 8-2), but a closed nuclear envelope (8-0) is shared by Blastocladiomycota and the zygomycetes with a loss of this character state in *Basidiobolus ranarum* (8-3).

DISCUSSION

Our results provide additional support for the hypothesized derivation of the SPB from the centrosome of the common ancestor of flagellate and terrestrial fungi. *Coemansia reversa* possesses a sixth type of zygomycetous SPB, and the third type that suggests a reduced centriolar component. The ring of nine microtubules in *C. reversa* closely parallels the centriolar structure of fungi that make motile cells, Blastocladiomycota, Chytridiomycota, and Monoblepharidomycota clades (Ichida and Fuller, 1968; Lessie and Lovett, 1968; McNitt, 1973; Whisler and Travland, 1973; Olson, 1974; Powell, 1975, 1980; Dolan and Fuller, 1985; Roychoudhury and Powell, 1991), but the nine microtubules are not in triplets, and the central structure parallel to the microtubules is absent in *C. reversa* (Fig. 12). Similar changes in centriolar structure have been reported in other eukaryotes with the loss of the evolutionary constraints associated with flagella function (Carvalho-Santos et al., 2011). While nine microtubules were not observed within a single tangential section of a *C. reversa* SPB, use of Markham's rotation supported the conclusion that the ring contains nine microtubules. The 11 microtubules in SPBs of *Basidiobolus* are perpendicular to the nuclear envelope (Fig. 12; McKerracher and Heath, 1985; Roberson et al., 2011); however, in *C. reversa* they diverged slightly outward from the nuclear envelope, which may explain why it is difficult to visualize all nine microtubules at once and why attempts to obtain a clearer image of the microtubules using a tilting stage in the electron microscope were not successful. The cylindrical SPB of *Pandora neoaphidis* lacks microtubules, and the sides of the cylinder diverge outward from the nuclear envelope (Fig. 12; Butt and Beckett, 1984a), which is similar to the arrangement of the microtubules and cylinder in *C. reversa*. These results strongly support the hypothesis of centriolar loss in the evolution of the SPBs of zygomycetes with various degrees of modification of the centriole in different taxa in the Entomophthoromycota clade.

Although the deep roots of the zygomycetes are not fully resolved, molecular phylogenetic analysis separates them into two major subdivisions: (1) Entomophthoromycota, Kickxellomycotina, and Zoopagomycotina and (2) Mucoromycotina, Mortierellomycotina, and Glomeromycota (Gryganskyi et al., 2012). Spindle pole body and nuclear division data are available for only three clades, Entomophthoromycota, Kickxellomycotina, and Mucoromycotina. Major structural differences between these two subdivisions are suggested by the unusual features of nuclear division and the SPB with its very small



Figs. 7–10. Metaphase–anaphase nucleus, freeze-substituted. **7.** Spindle microtubules extend between the spindle pole bodies (SPBs) (arrowheads) with chromosomes (C) loosely arranged across the mid region. Nucleolus (Nu) is in a pocket on one side of the nucleus and along the edge of the nucleus. **8.** Intact nuclear envelope (NE) separates the extranuclear SPB component from the intranuclear component (IC) and the spindle microtubules. **9.** Serial section adjacent to Fig. 8 showing the cylindrical structure within the SPB (arrowhead) and spindle microtubules. **10.** Kinetochore microtubules interspersed with chromosomes near the center of the spindle with single and paired kinetochores (KC) visible. Nucleolus (Nu) is in a pocket in the nuclear envelope. Scale bars for Fig. 7 = 0.5 μm and for Figs. 8–10 = 0.1 μm.

extranuclear component in Mucoromycotina (Heath and Rethoret, 1982) (Fig. 12) and the more typical fungal nuclear division in *C. reversa* and Entomophthoromycota (*Ancylistes*, *Basidiobolus*, *Entomophaga*, and *Pandora*) (see discussion below). The variations in SPB form and nuclear division characters in the Entomophthoromycota clade are reported in a large number of species within the phylum (Gryganskyi et al., 2012: supplemental table S5), but the data to support these conclusions are unpublished.

The phylogenetic placement of *Olpidium*, an obligate root parasite, has been controversial in analyzing SPB evolution. It produces motile cells and has a unicellular thallus like many flagellated fungi, but when rRNA genes and a single protein-coding gene for an *Olpidum* species were added to a six-gene analysis of all major groups of fungi it grouped with *Basidiobolus* in the zygomycetes (James et al., 2006a). A subsequent analysis of four protein-coding genes of four *Olpidium* species showed that they grouped in a well-supported clade only with zygomycetes, thus making it the only group of fungi with motile cells among the hypha-forming, nonflagellated fungi (Sekimoto et al., 2011). In a recent multigene study, it grouped with flagellated fungi, but as sister to zygomycetes (Gryganskyi et al., 2012). *Olpidium*'s placement within, rather than sister to, the zygomycete clades would indicate that there was more than one loss of the flagellum in the origin of the zygomycetes (James et al., 2006a).

A closed nuclear envelope during mitosis, which is also associated with an intranuclear SPB component, is a characteristic that links the zygomycete and Blastocladiomycota clades with the exception of *Basidiobolus* spp., which have lost these character states (Ichida and Fuller, 1968; Lessie and Lovett, 1968; Tanaka, 1970; Sun and Bowen, 1972; Franke and Reau, 1973; McCully and Robinow, 1973; Gull and Trinci, 1974; Olson, 1974; Bland and Lunney, 1975; Moorman, 1976; Heath and Rethoret, 1982; Butt and Beckett, 1984a, b; Murrin et al., 1984, 1988; McKerracher and Heath, 1985). These character states differ from other fungi with motile cells, i.e., Chytridiomycota and Monoblepharidomycota clades, in which the nuclear envelope is open, spindle development occurs in a gap in the nuclear envelope, and an intranuclear SPB component is

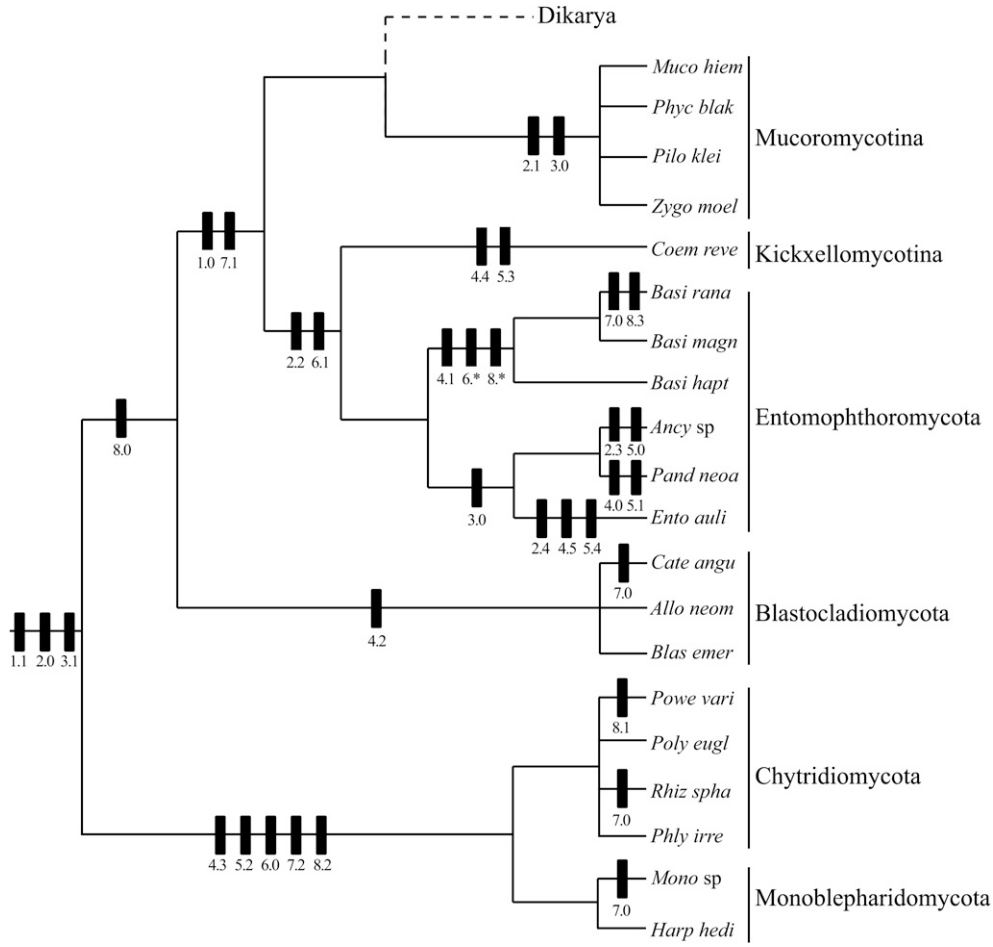


Fig. 11. Character analysis tree from Mesquite showing ancestral state reconstructions. Numbers show character state changes for nuclear and spindle pole body (SPB) characters (Table 1). Genus and species names (see Table 2) abbreviated to first four letters. Tree shows three clades of zygomycetes (Mucoromycotina, Kickxellomycotina, Entomophthoromycota), three clades of traditional Chytridiomycota (Blastocladiomycota, Chytridiomycota, Monoblepharidomycota) and the position of the Dikarya, which includes the sister clades, Ascomycota and Basidiomycota. An intact metaphase nuclear envelope (8-0) and microtubules in the SPB arranged in a circle perpendicular to the nuclear envelope (3-1) are found in the Blastocladiomycota clade with motile cells and Kickxellomycotina and Entomophthoromycota clades. Changes in the basic organization (character states) of the SPB can be followed in character 2. The Mucoromycotina clade has an unusual, small extranuclear SPB (2-1), while Kickxellomycotina and Entomophthoromycota clades have a variety of larger SPB forms (2-2, 2-3, 2-4). New information is noted on the branch leading to *Coemansia* where the SPB is a ring containing microtubules with centriolar 9-fold symmetry during interphase/prophase (4-4) and metaphase/anaphase (5-3). For other character states, see Table 1. * Character state data missing.

absent (McNitt, 1973; Whisler and Travland, 1973; Powell, 1975, 1980; Dolan and Fuller, 1985; Roychoudhury and Powell, 1991). The formation of an intranuclear component, which acts as a MTOC, is associated with a closed nuclear envelope. These characters provide synapomorphies for zygomycetes and Blastocladiomycota.

There are a number of nuclear division characters that could be usefully compared among these fungi when late nuclear division data are obtained for *C. reversa*. These data include whether a metaphase plate, defined as the presence of a well-defined equatorial array of chromosomes at metaphase (Heath, 1980), is formed, and the fate of the nucleolus and nuclear envelope during mitosis (Table 1, characters 9–11; Appendix S1, see Supplemental Data with the online version of this article). In *C. reversa* a metaphase plate appears to be absent, but additional data from later stages in mitosis are desirable to confirm it. A metaphase plate is typically absent in other zygomycetes, except for *Basidiobolus*, but present in Blastocladiomycota, Chytridiomycota, and Monoblepharidomycota clades (Ichida and Fuller, 1968; Tanaka, 1970; Sun and Bowen, 1972; McNitt, 1973; Whisler and Travland, 1973; Gull and Trinci, 1974; Powell, 1975, 1980; Dolan and Fuller, 1985; Heath, 1986; Roychoudhury and Powell, 1991). Typically, chromosomes are loosely gathered near the center of the spindle at metaphase in many fungi, including *C. reversa*, but the situation is quite different in the Mucoromycotina clade where an unusual mechanism of mitosis has been proposed in which the kinetochore microtubules remain short and the chromosomes are attached to the spindle poles throughout division (Heath and Rethoret, 1982). Thus, in Mucoromycotina, the chromosomes are separated by the elongating spindle microtubules without a metaphase plate or clustering of the chromosomes near the center of the spindle. The nucleolus in *C. reversa* is usually persistent at least through metaphase–anaphase, but whether it is discarded after metaphase as in most Blastocladiomycota, Chytridiomycota, and Monoblepharidomycota or is persistent as in Mucoromycotina and Entomophthoromycota, with the exception of *Basidiobolus*, is unknown (Ichida and Fuller, 1968; Tanaka,

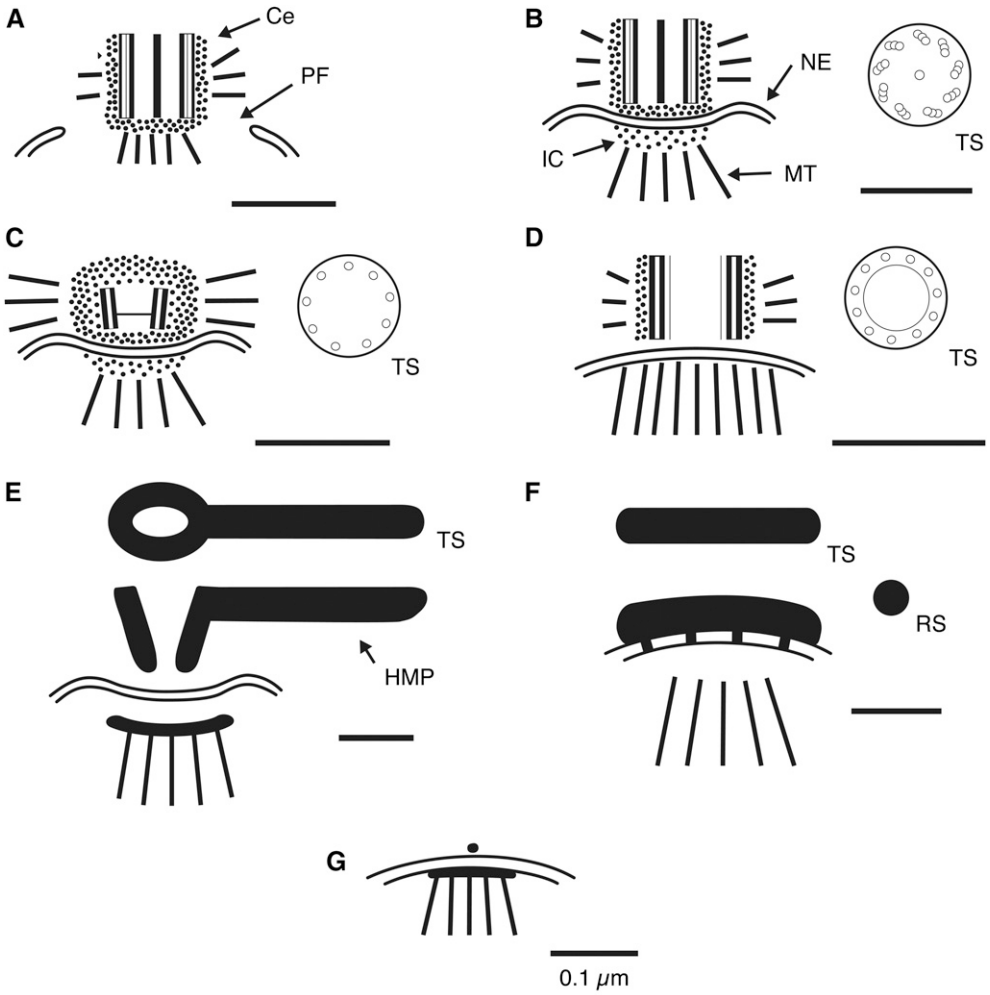


Fig. 12. Drawings of the spindle pole body (SPB) and nuclear envelope relationships at metaphase–anaphase in traditional Chytridiomycota (A, B) and zygomycetes (C–G). (A) Chytridiomycota (Powell, 1980); (B) Blastocladiomycota (Ichida and Fuller, 1968); (C) Kickxellomycotina (this paper); (D–F) Entomophthoromycota (D, McKerracher and Heath, 1985; E, Butt and Beckett, 1984a; F, Murrin et al., 1984); and (G) Mucoromycotina (McCully and Robinow, 1973) in radial section. (F) *Entomophaga aulicae*, radial section, RS across the long axis of the bar-shaped SPB. Ce, centriole; HMP, half middle piece; IC, intranuclear component of the SPB; MT, microtubule; NE, nuclear envelope; PF, polar fenestra; TS, tangential section to the nucleus. Bars = 0.25 μm except G.

1970; Sun and Bowen, 1972; Franke and Reau, 1973; McCully and Robinow, 1973; Gull and Trinci, 1974; Olson, 1974; Powell, 1975; Bland and Lunney, 1975; Moorman, 1976; Heath and Rethoret, 1982; Butt and Beckett, 1984b; Dolan and Fuller, 1985; Murrin et al., 1988; Roychoudhury and Powell, 1991). The nuclear envelope is typically retained during nuclear division with a median constriction or fragmentation at telophase in these clades, i.e., the plesiomorphic state, with some variation among genera (Ichida and Fuller, 1968; Lessie and Lovett, 1968; Tanaka, 1970; Sun and Bowen, 1972; McCully and Robinow, 1973; Whisler and Travland, 1973; Gull and Trinci, 1974; Olson, 1974; Bland and Lunney, 1975; Powell, 1975; Moorman, 1976; Heath and Rethoret, 1982; Butt and Beckett, 1984b; Dolan and Fuller, 1985; Murrin et al., 1988).

The multiple types of SPBs in the Entomophthoromycota clade indicate that it is not possible to generalize on SPB form for the Kickxellomycotina clade. There is an incomplete report of a SPB in Kickxellomycotina for *Harpella melusinae* L. Léger & Duboscq with extra- and intranuclear SPB components and a semicircle of eight to nine tubular or particulate substructures lying adjacent to or part of the extranuclear component (Reichle and Lichtwardt, 1972). This structure may represent an alternate form of the SPB with microtubules, but needs additional study to be understood. A number of taxa need to be analyzed in each clade before it is possible to conclude that a SPB type is characteristic for the clade.

In summary, the SPB in *C. reversa* appears to be more closely related to the SPB of fungi that produce motile cells than previously reported SPBs of zygomycetes and to represent a stage in evolution from the SPB of fungi with motile cells to that of the Dikarya and some zygomycetes in which no morphological trace of a microtubular structure is found within the SPB. The absence of a microtubular structure within the SPB of Dikarya (Celio et al., 2006), Mucoromycotina (Franke and Reau, 1973; McCully and Robinow, 1973; Bland and Lunney, 1975; Heath and Rethoret, 1982), and some Entomophthoromycota (Moorman, 1976; Butt and Beckett, 1984a; Murrin et al., 1984) supports at least two independent origins of this SPB type within the Fungi. With the

sequencing of the *C. reversa* genome, it may be possible in the future to relate the SPB form in *C. reversa* to the centriole and centrosome of Chytridiomycota-like fungi and understand the progression of genomic changes that have led to the SPB types found in zygomycetes and Dikarya. The dramatic differences in SPB and nuclear division characters between the Kickxellomycotina plus Entomophthoromycota clades and the Mucoromycotina clade agrees with the molecular phylogenetic data that divide zygomycetes into two major subdivisions (Gryganskyi et al., 2012), but data for these characters from three unstudied clades are needed to confirm it. These missing data are also needed to understand character evolution in the Dikarya. The available nuclear division and SPB data raise the question of a closer relationship between the Dikarya (Ascomycota and Basidiomycota) clade and the Kickxellomycotina and Entomophthoromycota clades than with the Mucoromycotina clade as determined by molecular phylogenetic analysis (James et al., 2006a; White et al., 2006; Sekimoto et al., 2011; Gryganskyi et al., 2012), i.e., this placement may be more parsimonious based on structural characters. Zygomycetes are, therefore, a critical group in need of much greater structural and molecular analysis to understand character evolution and synapomorphies for terrestrial fungi. Analysis of the SPB in *C. reversa* adds to the diversity of structures that suggest a gradual loss of centriolar components in the evolution of fungal SPBs during the radiation of the zygomycetes and Dikarya.

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