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Hyphal tip growth and cytoplasmic characters of *Conidiobolus coronatus* (Zoopagomycota, Entomophthoromycotina)

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ABSTRACT
Characteristics of hyphal structure and growth can provide insights into the mechanisms of polarized growth and support investigations of fungal phylogeny. To assist with the resolution of evolutionary relationships of the zygomycetes, the authors used comparative bioimaging methods (light [LM] and transmission electron [TEM] microscopy) to describe selected subcellular characters of hyphal tips of *Conidiobolus coronatus*. Growing hyphae of *C. coronatus* contain Spitzenkörper (Spk). Spk are most commonly present in hyphae of Dikarya (Ascomycota and Basidiomycota) and are rarely reported in zygomycete hyphae, which possess an apical vesicle crescent (AVC). Such findings raise questions regarding the evolution of the Spk and its relationship with the AVC. Descriptions of additional subcellular characters (e.g., mitotic-phase spindle pole bodies, cytoplasmic behavior, organelle structure) are also presented.

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Hyphal growth; light microscopy; phylogeny; Spitzenkörper; transmission electron microscopy; zygomycetes

INTRODUCTION

Zygomycetes comprise a large and understudied group of fungi. They include plant symbionts, animal pathogens, and decomposers of organic compounds and are used in the production of organic acids and fermentation of foods. These fungi were thought to be an early-diverging lineage of the chytrids (Chytridiomycota and Blastocladiomycota) giving rise to terrestrial growth and a thallus made up of nonflagellated, filamentous hyphae that exhibit constitutive polarized cell growth, which characterizes most terrestrial fungi today. However, zygomycetes represent a polyphyletic group (Hibbett et al. 2007). The previous delineation of the phylum Zygomycota has been discarded and split into two phyla, the Mucoromycota and the Zoopagomycota (Spatafora et al. 2016). Mucoromycota includes the subphyla Mucoromycotina, Mortierellomycotina, and Glomeromycotina and is a sister group to Dikarya. This group includes plant pathogens, species that form symbiotic relationships with plants, and species exploited for industrial processes. The Zoopagomycota contains the subphyla Entomophthoromycotina, Kickxellomycotina, and Zoopagomycotina, whose members are noted for their associations with insects and other fungi and few have associations with living plants (see Spatafora et al. 2016 and references therein).

The growing fungal hypha is a dynamic and complex cell, requiring, among many things, the synthesis of secretory vesicles and their precise delivery to and fusion with the apical plasma membrane in order to deliver the proteins, membrane, and cell wall components required for growth. Many organelles and inclusions found within the cytoplasm of fungal hyphae are similar to those present in other heterotrophic eukaryotic cells. However, due to their constitutive polarized mode of growth, tube-like morphology, and diverse ecological interactions, some cytoplasmic components of hyphae are unique to the fungi and can exhibit structural plasticity across the fungi. Such subcellular characters are of particular interest in understanding aspects of hyphal growth and behaviors (Roberson et al. 2010) and can be used as indicators of evolutionary relationships (Celio et al. 2006; Hibbett et al. 2007; McLaughlin et al. 2009, 2015; Fisher and Roberson 2016).

Using light microscopy (LM) and/or transmission electron microscopy (TEM) techniques, we have observed and documented subcellular characteristics and growth dynamics of hyphal tips in *Conidiobolus coronatus*. The genus *Conidiobolus* is one of three clades within the Entomophthoromycotina, which also includes the *Basidiobolus* clade and the Entomophthoraceae clade (Gryganskyi et al. 2012). Phylogentically, the *Conidiobolus* clade is positioned between the basal Basidiobolaceae and the more highly derived Entomophthoraceae. The *Conidiobolus* clade appears distinct from the other two clades based upon

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both molecular data and its primarily saprobic nature (Gryganskyi et al. 2012). Our current research showed the presence of a phase-dark Spitzenkörper (Spk) in actively growing hyphae of *C. coronatus*, and when observed using TEM, a spherical aggregation of secretory vesicles was noted. A Spk, a character of fungi in the Dikarya, has only been observed in one other member of the zygomycetous fungi—*Basidiobolus*, a sister group to *Conidiobolus* (Roberson et al. 2011).

MATERIALS AND METHODS

Cultures.—*Conidiobolus coronatus* (NRRL 24638) was maintained on 1% *w/v* malt extract agar (MEA; BD Difco, Franklin Lakes, New Jersey) at 23 C.

Preparation of cultures for light microscopy.—For low-magnification mycelial growth observations, Petri dishes containing 2–3-d-old cultures of *C. coronatus* on MEA were visualized using a Nikon SMZ-2T stereo scope (Nikon Instruments, Melville, New York) coupled to an Olympus DP72 camera and imaged with Olympus CellSens software (Olympus, Tokyo, Japan).

Culture slides were prepared by applying either a thin layer of potato dextrose broth (BD Difco) and 15% gelatin or malt extract–yeast extract (MEYE; malt extract 0.24%, yeast extract 0.24%, peptone 0.4%, dextrose 0.8%) broth and 15% gelatin over one surface of sterilized microscope slides. Hyphae were inoculated onto the surface of the coated slides, and the samples grown for 12 h in a moist chamber at 23 C. After placing coverslips (no. 1.5) over the colony's leading edge, hyphae were imaged using an Axioskop light microscope (Carl Zeiss, Thornwood, New Jersey) equipped with differential interference contrast (DIC) and phase-contrast (PC) optics using plan-neofluar 100×/1.3 numerical aperture (NA) (oil immersion) objective and 0.9 NA or 1.4 NA (oil immersion) condenser lens. The microscope was coupled to a Hamamatsu Orca Flash 4.0 digital camera (Hamamatsu Photonics, Hamamatsu City, Japan) and images captured using HCImage Live (Hamamatsu Photonics). Data were processed and analyzed using ImageJ (National Institutes of Health, Bethesda, Maryland) and Adobe Photoshop 8.0 (Adobe Systems, San Jose, California).

Preparation of cultures for transmission electron microscopy.—MEYE surfaces were overlaid with a deionized and sterilized dialysis membrane. Dialysis membranes were deionized by boiling in 0.1 M EDTA for 1 h, followed by washing in five to six changes of deionized water with continuous stirring for 8 h at room temperature. Membrane surfaces were inoculated with hyphae, and after sufficient growth, hyphal tips and membranes were trimmed into 5 × 7 mm pieces and placed back of the surface of the agar for a 30–60-min recovery period before being plunge-frozen in liquid nitrogen–cooled condensed propane following the methods of Howard and O'Donnell (1987). After plunge freezing, samples were transferred to a precooled freeze-substitution fluid (−85 C) of 1% glutaraldehyde and 1% tannic acid in acetone (Fields et al. 1997). After being held for 48–72 h at −85 C, cells were washed three times in cold acetone (−85 C) and transferred to a solution of cold (−85 C) 1% OsO_4 in acetone for 1 h. The samples were warmed slowly to 23 C according to this regimen: 2 h at −15 C, 2 h at 4 C, and then to room temperature (RT). Cells were left at RT for 30–45 min, washed three times with acetone, infiltrated in 25% increments with Spurr's resin (Spurr 1969) and acetone to 100% resin, flat-embedded between Teflon-coated glass slides, and polymerized at 65 C for 24 h. Flat-embedded hyphae were imaged and selected for the lack of visible freeze damage using 100× phase-contrast optics (FIG. 4B) and sectioned using a Leica Ultracut R Ultramicrotome (Leica Microsystems, Bannockburn, Illinois). Ultrathin sections (70 nm thick) were collected on formvar-coated copper slot grids and post-stained for 10 min in 2% uranyl acetate in 50% ethanol and for 5 min in lead citrate. Sections were then examined on a JEOL 1200EX (JEOL, Tokyo, Japan) TEM equipped with a SIA L3C charge-coupled device (CCD) camera (SIA, Duluth, Georgia). Micrographs were analyzed using Adobe Photoshop 8.0 (Adobe Systems) and Image J (National Institutes of Health).

RESULTS

Light microscopy.—Hyphae of *C. coronatus* were approximately 5.4 μm (SD = 0.7 μm; n = 22) diam, with infrequent septa, and exhibited a growth rate of 1.6 μm/min (SD = 0.3 μm/min; n = 28) (FIGS. 1, 2; SUPPLEMENTARY MOVIES 1, 2). Growing hyphae maintained a cytoplasmic organization that was divided into three regions (I, II, III) based on cytoplasmic content (FIG. 2A).

Region I extended back from the hyphal apex approximately 2.0–3.0 μm. A well-defined Spk dominated the cytoplasm of region I. The Spk was a homogeneously dense, spheroid structure that lacked a differentiated central core. The Spk exhibited small, rapid changes in position during hyphal growth

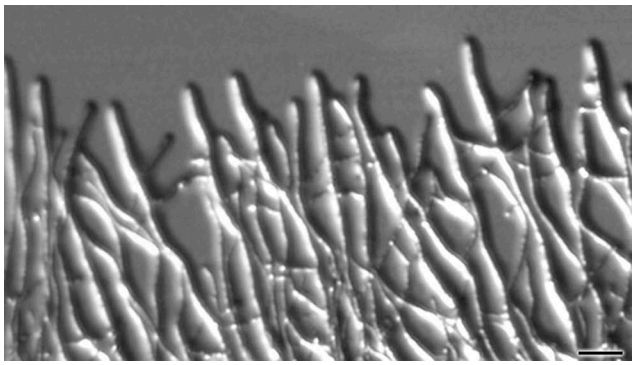

Figure 1. Hyphal tip cells of *C. coronatus* growing on semisolid agar. Bar = 50 µm.

(SUPPLEMENTARY MOVIE 2). Repositioning of the Spk within the apical dome (FIG. 2D, F) appeared to influence the direction of hyphal growth direction. Spitzenkörper of *C. coronatus* were unstable during live-cell observations, having a tendency to migrate from the tip in a retrograde direction and then disassemble, resulting in the cessation of growth.

Region II extended 10–20 µm behind region I and contained abundant mitochondria, multivesicular bodies (MVBs) (FIG. 2A), and Golgi body equivalents based on TEM observations (FIG. 3A). Mitochondria were aligned predominantly parallel to the long axis of the hypha. Mitochondria and MVBs moved forward with the advancing cytoplasm in a bulk-flow manner and also exhibited rapid anterograde and retrograde movements characterized by frequent starts and stops (i.e., saltatory motility) (Rebhun 1972). Saltatory motility was independent of the overall cytoplasmic migration and movements of other adjacent cytoplasmic components. Mitochondria often exhibited rapid anterograde movements into region I, briefly coming in close proximity to the Spk, and quickly retreating into region II (FIG. 2A, F).

Region III was distinguished from region II by the presence of multiple nuclei and vacuoles, as well as mitochondria and MVBs (FIG. 2A). Nuclei and vacuoles exhibited bulk-flow motility; however, rapid saltatory movements of vacuoles were observed. The cytoplasm of hyphae that grew at reduced rates or not at all lacked both an Spk and the cytoplasmic order described above. In these hyphae, nuclei and vacuoles were often observed in regions I and II.

Transmission electron microscopy.—Cryopreparation protocols provided good to excellent

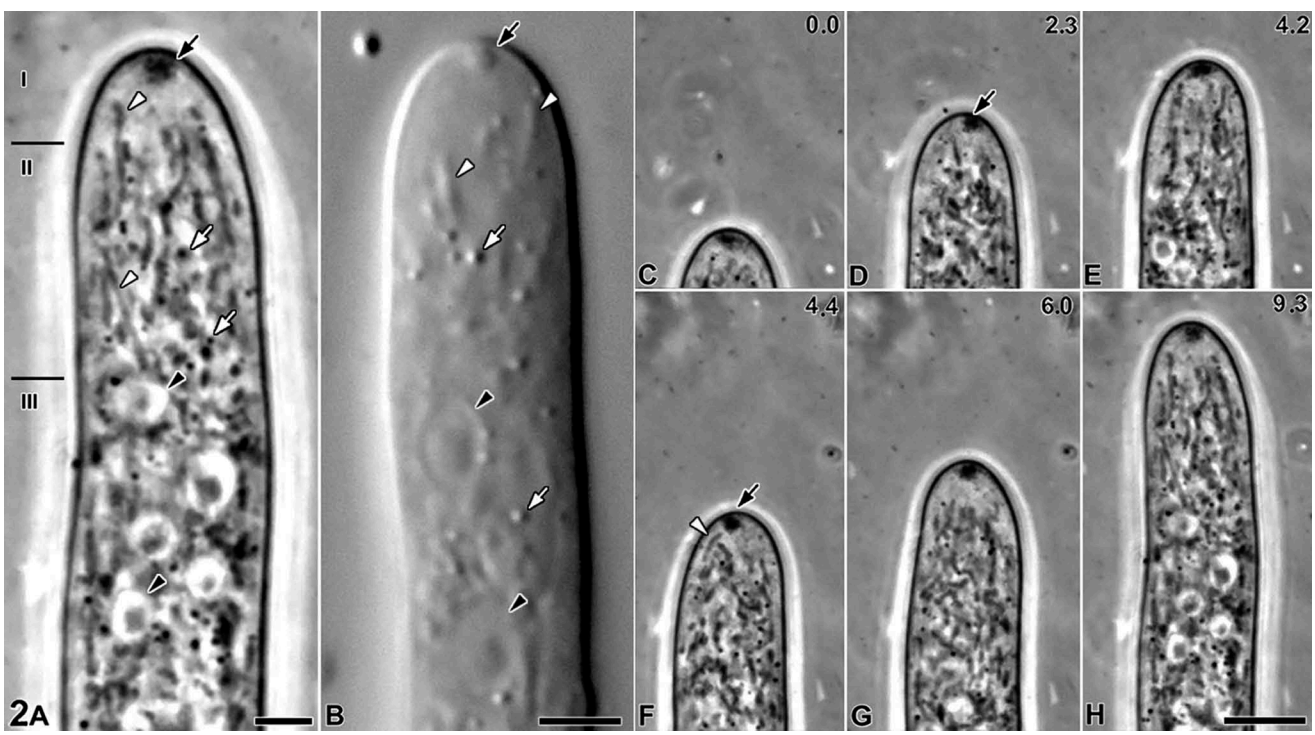

Figure 2. A, B. Phase-contrast (A) and DIC (B) light microscopy illustrating Spk (black arrows), nuclei (black arrowheads) with phase-dark nucleoli, mitochondria (white arrowheads), multivesicular bodies (white arrows), and hyphal regions (I, II, III). C–H. Phase-contrast light microscopy of growing hypha. Spitzenkörper exhibits slight change in position (D, F; arrows) followed by comparable change in direction of growth (E, G). Mitochondrion (arrowhead). Time (min.s) in upper right. Bars: A = 1.7 µm; B = 2.5 µm; C–H = 4.0 µm.

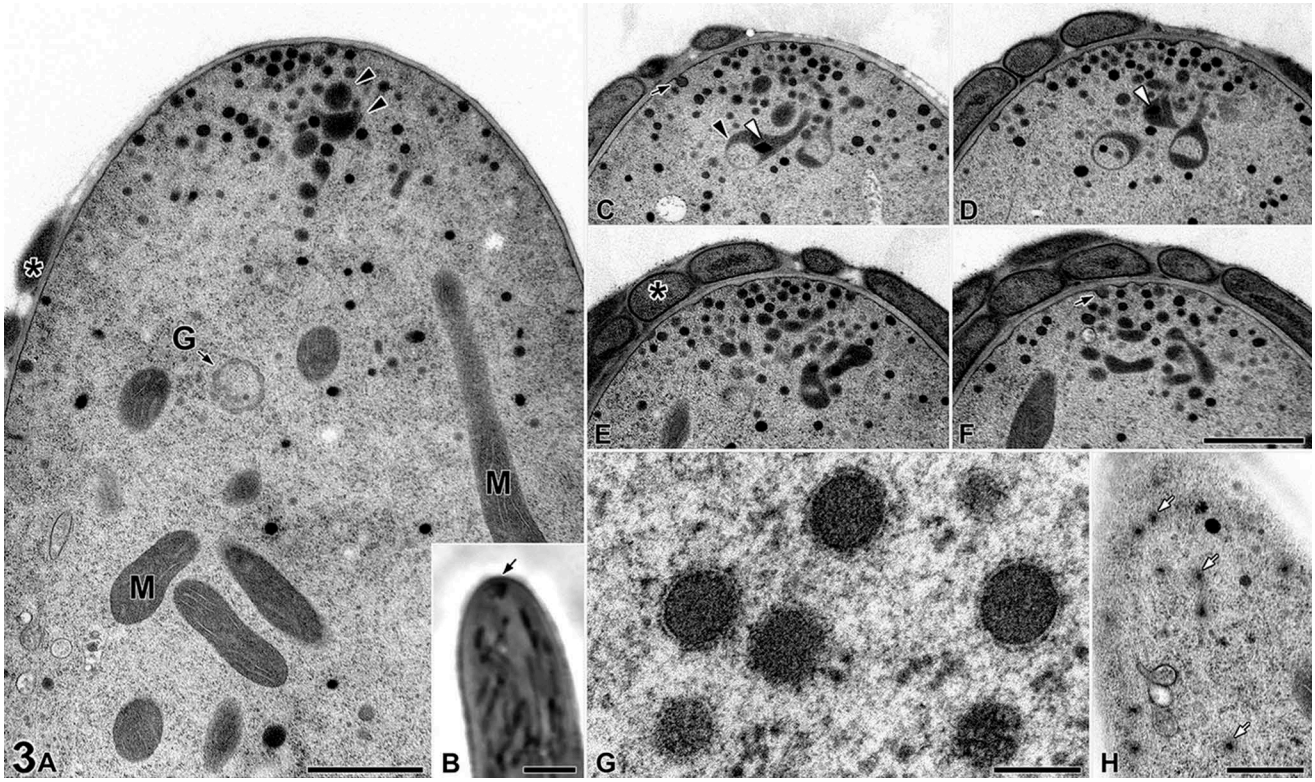

Figure 3. Transmission electron microscopy. A. Hyphal regions I and II. The accumulation of secretory vesicles at the hyphal apex is organized into a Spk subtending the plasma membrane. Amorphous membrane-bound bodies (arrowheads), mitochondria (M), Golgi body equivalent (G), bacterium (asterisk). B. Phase-contrast light microscopy of fixed and resin-embedded hyphae shown in A, C, and D. Spk (arrow). C–F. Series of four consecutive sections from of hypha shown in A. Amorphous membrane-bound bodies (black arrowheads) with crystal-like inclusions (white arrowheads), exocytotic events (arrows), bacterium (asterisk). G. Macrovesicles. H. Dense fibrous patches (arrows). Bars: A = 1.0 µm; B = 2.5 µm; C–F = 1.0 µm; G = 100 nm; H = 500 nm.

ultrastructural preservation of *C. coronatus* hyphae (FIGS. 3, 4). It is noteworthy that cultures of some TEM fixations contained sparse bacterial contamination (FIGS. 3, 4) and that these cultures exhibited the best cryofixation characteristics (i.e., reduced freeze damage). Typical fungal organelles and inclusions were identified (e.g., Golgi body equivalents, mitochondria, vacuoles, and endoplasmic reticulum). The Spk was observed as an aggregation of vesicles in region I (FIG. 3A–F). Spitzenkörper vesicles appeared less well-organized than anticipated based on LM observations (FIGS. 2, 3B). A central core within the Spk was not apparent, corroborating LM data. Sites of vesicle exocytosis were routinely noted along the apical plasma membrane (FIG. 3C, F). Two populations of macrovesicles were documented, both containing a homogeneous electron-dense, granular lumen bound by a well-defined unit membrane and differentiated by their sizes (FIG. 3G). The population of larger macrovesicles averaged 102 nm (SD = 16 nm; n = 110) diam, and the smaller vesicles averaged 76 nm (SD = 11 nm; n = 49) diam. A third vesicle class was infrequently noted that averaged 38 nm (SD = 3.2 nm; n = 9) diam (i.e., microvesicles). Dense fibrous patches containing an electron-opaque core were present in the cortex of the cytoplasm near the interface of regions I and II (FIG. 3H). These were likely filasomes (i.e., actin- and actin-associated protein–coated microvesicles; Hoch and Howard 1980; Howard 1981), but a clear microvesicle core could not be resolved. Commonly present among vesicles in the Spk were amorphous membrane-bound bodies with electron-dense, granular contents and on occasion electron-opaque, crystal-like inclusions (FIG. 3A, C–F). These inclusions were also observed in samples that were free of bacterial contamination and could be detected using phase-contrast LM (not shown).

Nuclei were spherical (2.1 µm diam; n = 10) to slightly oblong in shape and contained a single prominent, centrally located nucleolus with little heterochromatin (FIGS. 2A, B, 4A, B). Membranous elements (i.e., nuclear lamina) were present in the nucleoplasm and

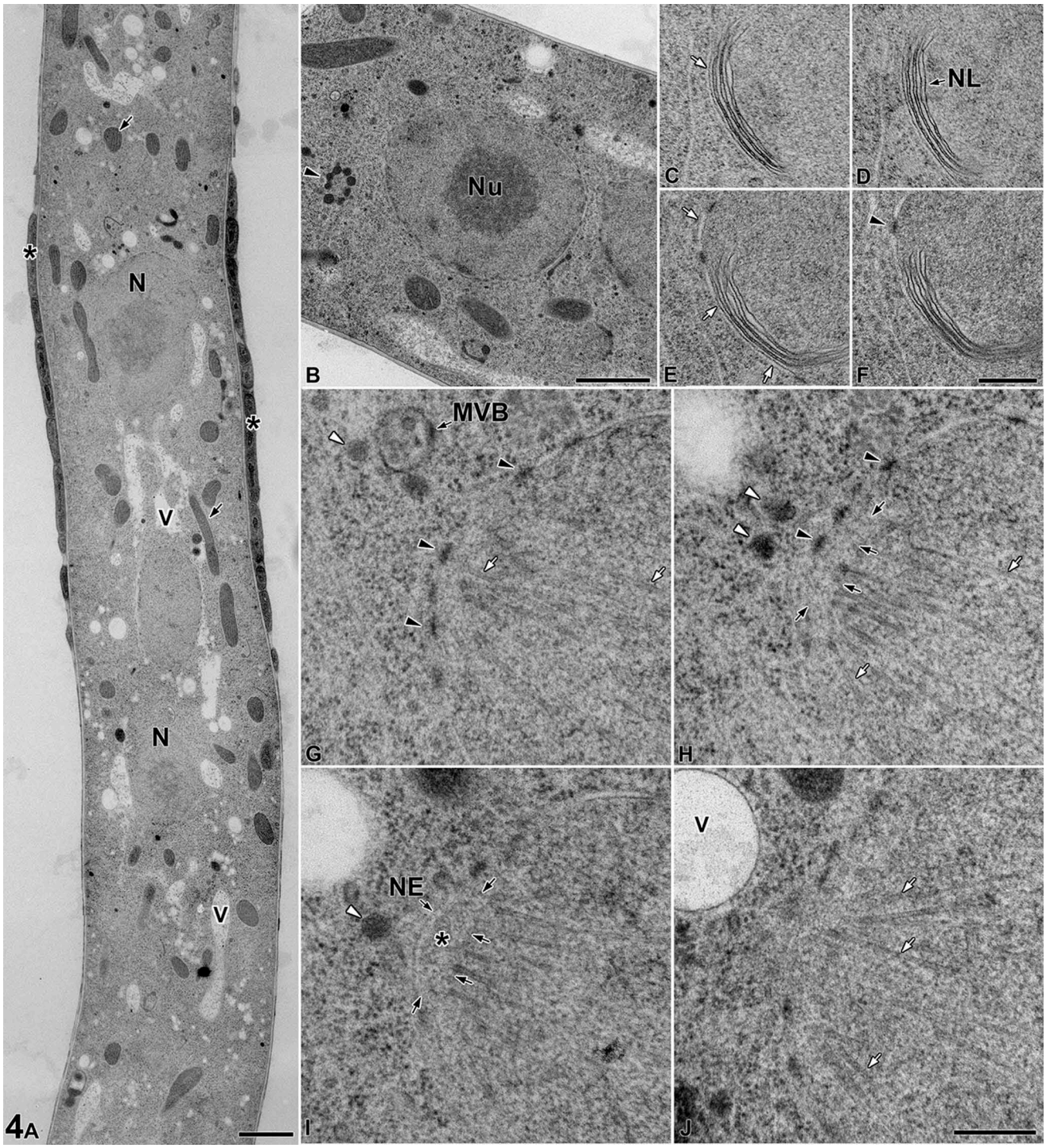

Figure 4. Transmission electron microscopy. A. Region III of hyphal cell. Nuclei (N), vacuoles (V), mitochondria (arrows), bacteria (asterisk). B. Nucleus with prominent nucleolus (Nu), Golgi body equivalent (arrowhead). C–F. Four consecutive sections through nuclear lamellae (NL). Nuclear envelope (arrows), nuclear pore complex (arrowhead). G–J. Four consecutive sections through portion of mitotic spindle pole. Intranuclear component of SPB is outlined (black arrows, asterisk). Microtubules (white arrows), nuclear envelope (NE), nuclear pore complexes (black arrowheads), spherical electron-dense inclusions (white arrowheads), multivesicular body (MVB), vacuole (V). A = 1.5 µm; B = 1.0 µm; C–F = 270 nm; G–J = 100 nm; H = 250 nm.

were organized into four to five cisternal stacks near the inner surface of the nuclear envelope (FIG. 4C–F). Cisternae contained an electron transparent to dense lumen. Membrane connectivity was not observed between the cisternal stacks or with the nuclear envelope. No interphase spindle pole body (SPB) was

detected. Spindle microtubules were abundant during mitosis, although astral microtubules were scant (FIG. 4G–J). The nuclear spindle of *C. coronatus* was eccentric during prophase (SUPPLEMENTAL FIG. 1). Extranuclear SPB components were not recognized, although spherical electron-dense inclusions of unknown origin or function were noted. An intranuclear component of the SPB was identified as a region of nucleoplasm just beneath the nuclear envelope from which microtubules appeared to emanate. The nuclear envelope and nucleolus remained intact during mitosis (FIG. 4G–J; SUPPLEMENTAL FIG. 2).

DISCUSSION

Although the Spitzenkörper (Spk) remains a defining feature of the Dikarya, two exceptions outside the Dikarya are documented: *Allomyces macrogynus* (Chytridiomycota; Vargas et al. 1993) and *Basidiobolus* (Entomophthoromycotina; Roberson et al. 2011). Based on LM and TEM observations presented here, we present a third exception, *Conidiobolus coronatus*. Both the documented morphology of the apical vesicle aggregation and its behavior warrant the label of Spk. The Spk of *C. coronatus* are spherically organized inclusions in region I of growing hyphae. When viewed with PC optics, the Spk appears as a phase-dark structure with no phase-light central core present. Although Spk display considerable variability and nine patterns of morphological organization are described in the Dikarya (López-Franco and Bracker 1996), most contain a phase-light center surrounded or partially surrounded by a phase-dark vesicle aggregation. All members of the Mucoromycota and Kickxellomycotina studied to date lack Spk but instead contain an apical vesicle crescent (AVC) (Girbardt 1969; Grove and Bracker 1970; Bentivenga et al. 2013; Fisher and Roberson 2016). The AVC has a less complex arrangement of vesicles than the Spk and in total appears as a thin, phase-dark crescent subtending the apical plasma membrane when viewed with phase-contrast optics (Fisher and Roberson 2016). Using TEM, the AVC most often appears as a loose aggregation of vesicles within a narrow zone beneath the apical plasma membrane.

The presence of the Spk is directly correlated with the rate of growth of *C. coronatus* hyphae. Hyphae that do not contain a Spk exhibit reduced or negligible growth. Our data suggest that as the Spk of *C. coronatus* shifts position within the hyphal apex, a change in the direction of growth follows, although additional investigation is warranted on this issue. The relationship between the position of the Spk and the direction of hyphal growth occurs in numerous hyphae of the Dikarya (Bartnicki-Garcia et al. 1995; López-Franco and Bracker 1996; Bracker et al. 1997; Riquelme et al. 1998; Wright et al. 2007) and in *Basidiobolus* spp. (Roberson et al. 2011). Evidence of this behavioral relationship is lacking for Spk of *A. macrogynus*. Although the AVC observed in other zygomycetous fungi is associated with hyphal growth (e.g., optimal hyphal growth occurs when an AVC is present), no evidence currently suggests that a change in the position of the AVC precedes the direction of hyphal growth (Fisher and Roberson 2016); instead, these events appear to occur at the same time.

Two populations of macrovesicles are present in *C. coronatus* and are defined by their average diameters (102 and 72 nm). Both populations of vesicles contained a uniformly dense, granular lumen similar to that documented in macrovesicles of the Dikarya (Howard 1981; Roberson and Fuller 1988) and Kickxellomycotina (Fisher and Roberson 2016). Vesicles of *Basidiobolus* spp. contain an electron-dense core surrounded by a less dense, fibrous zone with a distinct, delimiting membrane (Roberson et al. 2011) similar to what is reported in Mucoromycotina and Mortierellomycotina (Grove and Bracker 1970; Fisher and Roberson 2016).

Interphase nuclei of *C. coronatus* contain a single prominent nucleolus with little heterochromatin in the nucleoplasm. Similar observations are described in *Basidiobolus* spp. (McKerracher and Heath 1985; Roberson et al. 2011). The significance of membranous cisternae located in the nuclei of *C. coronatus* is unknown. This accumulation of membrane may represent a novel mechanism of providing required membrane during mitosis, although no connections between the nuclear envelope and the cisternal membranes were noted. Such accumulations of intranuclear membrane are not observed in other eukaryotes. However, a somewhat comparable observation is reported in *Entophlyctis* spp. (= *Geranomyces*) where new nuclear envelope membrane forms from original envelope during telophase (Powell 1975).

The SPB morphology varies significantly among clades of the Zoopagomycota, whereas only one type is reported in the Mucoromycotina, and no data exist for the Glomeromycotina, Mortierellomycotina, and Zoopagomycotina (McLaughlin et al. 2015). An interphase SPB was not observed in *C. coronatus* hyphae. This supports the observation reported in a personal communication attributed to Robinow (Heath 1977). During prophase, the nuclear spindle of *C. coronatus* is eccentric, like those seen in *Entomophaga aulicae* (Entomophthoromycotina; Murrin et al. 1988). As mitosis progresses, the spindle expands throughout the entire nucleus and an intranuclear component of the SPB is positioned at the spindles poles in close association with the inner surface of the nuclear

envelope. Abundant spindle mitochondria emanated from the intranuclear component, suggesting its role as a microtubule-organizing center. Similar observations are reported in *Coemansia reversa* (McLaughlin et al. 2015) and in the Blastocladiomycota (Ichida and Fuller 1968). This character, and the fact that nuclear envelope remains intact during mitosis, suggests a relationship between these two fungal groups.

The hyphal apex of *C. coronatus* contains a spherical, apical inclusion we have determined to be a Spk based upon both its physical characteristics and behavior in the growing hypha. *Conidiobolus coronatus* is only the second zygomycetous fungus in which a Spk is identified, and only the third identified outside of the Dikarya. Currently, it is difficult to present conclusive statements regarding the evolution of the Spk and the AVC. Has the Spk evolved independently multiple times over the course of fungal evolution? Or, have fungi such as *Allomyces, Basidiobolus, Conidiobolus*, and the Dikarya retained a form of the ancestral condition and the Spk in fact has been lost multiple times (i.e., Mucoromycota and Kickxellomycotina)? The more widespread occurrence of the Spk throughout the Fungi suggests that the Spk confers a selective advantage over the AVC; however, a clear understanding of what this advantage remains uncertain. Probing molecular characteristics of polarity proteins and their genes in selected members of the zygomycetes and Dikarya could help elucidate these questions.

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