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Paleomycology of the Princeton Chert I. Fossil hyphomycetes associated with the early Eocene aquatic angiosperm, *Eorhiza arnoldii*

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Abstract: The Eocene (~ 48.7 Ma, Ypresian–Luteian) Princeton Chert of British Columbia, Canada, has long been recognized as a significant paleobotanical locality, and a diverse assemblage of anatomically preserved fossil plants has been extensively documented. Co-occurring fossil fungi also have been observed, but the full scope of their diversity has yet to be comprehensively assessed. Here, we present the first of a series of investigations of fossilized fungi associated with the silicified plants of the Princeton Chert. This report focuses on saprotrophic, facultative-aquatic hyphomycetes observed in cortical aerenchyma tissue of an enigmatic angiosperm, *Eorhiza arnoldii*. Our use of paleontological thin sections provides the opportunity to observe and infer developmental features, making it possible to more accurately attribute two hyphomycetes that were observed in previous studies. These comprise multi-septate, holothallic, chlamydospore-like phragmococonidia most similar to extant *Xylomyces giganteus* and basipetal phragmospore-like chains of amerspores like those of extant *Thielaviopsis basicola*. We also describe a third hyphomycete that previously has not been recognized from this locality; bisepitate, chlamydosporic phragmoconidia are distinguished by darkly melanized, inflated apical cells and are morphologically similar to *Brachysporiella rhizoidea* or *Culcitalna achraspora*.

Key words: *Brachysporiella*, *Culcitalna*, Eocene, fossil fungi, Princeton Chert, *Thielaviopsis*, *Xylomyces*

INTRODUCTION

Although several Eocene deposits are known from western Canada's Okanagan Highlands, the majority of these sites contain fossil plants preserved only as compressions. By contrast, the fossil plants of the Princeton Chert have been anatomically preserved (such that a cellular level of detail is available for study) within a succession of silicified, coal-forming peats. Because microbial biota associated with plant tissues also are subject to permineralization (Taylor et al. 2005, Dotzler et al. 2008), the Princeton Chert constitutes not only a pre-eminent paleobotanical locality but also an important opportunity to examine a microbial assemblage in the context of a well described and highly diverse Eocene flora.

The floristic components of the peat-forming Princeton mire have been intensively documented over the past 30 y, and the described flora includes several filicalean ferns (Basinger and Rothwell 1977, Stockey et al. 1999, Karafit et al. 2006, Smith et al. 2006) and three conifers, two of which have been reconstructed as whole plants (Rothwell and Basinger 1979, Stockey 1984, Klymiuk et al. 2011). Angiosperms, however, comprise most of the taxonomic diversity and include fruits, seeds and vegetative organs attributed to basal angiosperms and magnoliids (Cevallos-Ferriz and Stockey 1989, 1990a; Smith and Stockey 2007; Little et al. 2009), monocots (Erwin 1987; Cevallos-Ferriz and Stockey 1988a; Erwin and Stockey 1989, 1991, 1994; Smith and Stockey 2003) and core eudicots (Basinger 1976, Cevallos-Ferriz and Stockey 1988b, 1990b, 1991; Erwin and Stockey 1990; Cevallos-Ferriz et al. 1993; Pigg et al. 1993; Stockey et al. 1998; Little and Stockey 2003). Several flowering plants also cannot be confidently placed in systematic context, including the rhizomatous vegetative axes of an emergent or aquatic dicot, *Eorhiza arnoldii* Robison et Person (1973, Stockey and Pigg 1994).

In contrast to the flora, fungal diversity within the chert has been less comprehensively assessed; most were recognized due to their symbiotic or pathogenic relationships with vascular plants. The coralloid roots of the two dominant conifers hosted arbuscular mycorrhizae and ectomycorrhizae (LePage et al. 1997, Stockey et al. 2001) and several fungi parasitized angiosperms. These include a tar-spot infestation on leaves of the palm, *Uhlia allenbyensis* Erwin et Stockey (Currah et al. 1997), loculate pseudoparenchymatous mycelia associated with sepals, seeds, and

fruits of *Decodon allenbyensis* Cevallos-Ferriz & Stockey and an *Ascochyta*-like pycnidial fungus found within some fruits and seeds of *Princetonia allenbyense* Stockey (LePage et al. 1994). Studies also indicated the presence of a smut associated with floral remains (Currah and Stockey 1991, LePage et al. 1994), but the putative teliospores are now recognized as pollen of *Saururus tuckeri* Smith et Stockey (Saururaceae; Smith and Stockey 2007).

Less emphasis has been placed on fungi occupying predominantly saprotrophic niches, although LePage et al. (1994) proposed that dense sclerotia observed in seeds of the nymphaeaceous dicot *Allenbya collinsonae* Cevallos-Ferriz et Stockey (1989) might have affinities with *Alternaria* Nees, an anamorph genus that includes both parasitic and saprotrophic species. These authors also suggested that fungi inhabiting the aerenchymatous tissues of *Eorhiza arnoldii* were saprotrophic, citing the presence of several species as an indication that the host tissue was moribund. Conidia occurring in *E. arnoldii* were the first fungi described from the Princeton Chert (Robison and Person 1973) and originally were identified as septate hyphae that formed arthric conidia and phragmospores. LePage et al. (1994) interpreted the former as pleurogenous “cercosporoid” phragmospores and did not differentiate them from the second conidial morphology observed by Robison and Person (1973). By examining additional specimens of *Eorhiza* rhizomes, we are able to elucidate further details of growth and development for both microfungi, allowing a more confident attribution of these fossils to extant lineages. We also recognize a third hyphomycetous anamorph that has not been previously observed in the Princeton Chert. These microfungi indicate that *E. arnoldii* was colonized by several fungi before permineralization, and they provide new insight into both the early diagenesis of this fossil plant and its paleoecological context, in addition to expanding our understanding of fungal diversity during the early Eocene.

MATERIALS AND METHODS

Fungi described in this study occur within cortical tissues of the extinct aquatic angiosperm *Eorhiza arnoldii*, which occurs within many of the individual bedding planes that comprise the Princeton Chert locality of southern British Columbia, Canada (UTM 10U 678057 5472372; 49°22'40"N, 120°32'48"W). The locality is a single inclined exposure that crops out along the east bank of the Similkameen River and is composed of at least 49 layers of chert interbedded with sub-bituminous coal and carbonaceous shale. The 7.5 m thick deposit occurs within the informally named Ashnola Shale, the uppermost unit of the Allenby Formation (Fm) of the Princeton Group (Read 1987, 2000; Mustoe 2011). A

volcanic ash within Layer No. 22 of the chert has been radiometrically dated as 48.7 Ma (Smith and Stockey 2007); the age of the locality is therefore latest Ypresian or earliest Lutetian.

Slabs of chert containing *Eorhiza* specimens were selectively sectioned into 3–5 cm² samples, which were mounted on glass slides with Hillquist Two Part mounting medium (Hillquist, USA). Serial paleontological thin sections were cut with a Buehler Petrothin[®]; sections were 50–150 µm thick. Photomicrographs were captured directly from the rock surface under oil immersion, with a Leica DC500 CCD attached to a Leica DM5000B transmitted-light compound microscope. Serial photomicrographs of the same specimen at different focal planes were compiled into composite focal-stacked images, produced by selectively erasing specific areas to reveal three dimensionality of the specimen as is visible under transmitted light (after Bercovici et al. 2009). Image processing was performed in Adobe Photoshop CS5 12.1. Specimens and slides are deposited in the Paleobotanical Collections, Natural History Museum and Biodiversity Institute, University of Kansas (Lawrence) (KUPB), under specimen accession numbers 17030 C_{bot} 001, 17035 E_{top} # 001, 002; 17035 E_{bot} #001 and 17037 F_{bot} #001.

RESULTS

Type I.—Conidia of a hyphomycetous anamorph have developed preferentially within locules, or intercellular spaces, of cortical aerenchyma, (FIG. 1A), often with more than 50 individual mitospores in similar orientation. The macroconidia are dematiaceous or darkly pigmented smooth-walled, cylindrical and phragmosporic, 75–125 µm long and 7–10 µm diam, with as many as 30–35 transverse septa (FIG. 1A, B). Observation of subtending hyphae at conidial apices and bases (FIG. 1B, at arrows) indicates that conidiogenesis was holothallic. Although some conidial cells exhibit constriction or contraction of the conidial wall (FIG. 1C, at arrow), these features are not regular and do not occur in most conidia (FIG. 1B, D). Furthermore, because constricted intercalary cells exhibit walls that are otherwise of similar thickness to adjacent cells, it is probable that constriction represents a preservational effect, as opposed to indicating alternate-arthric conidiogenesis. Instead, conidiogenesis appears to have been thallic solitary (FIG. 1D, E, F), with individual multi-septate chlamydosporous conidia produced from sparsely branched, 2.5–3.0 µm diam, micronematous conidiophores (FIG. 1E, F, G), indistinct from mycelial hyphae (FIG. 1F). Conidial secession is rhexolytic (FIG. 1E, F), and dispersed conidia may bear remnants of the subtending cell (FIG. 1F, lower arrow).

Type II.—This hyphomycetous anamorph exhibits propagation via unequally pigmented, apically dema-

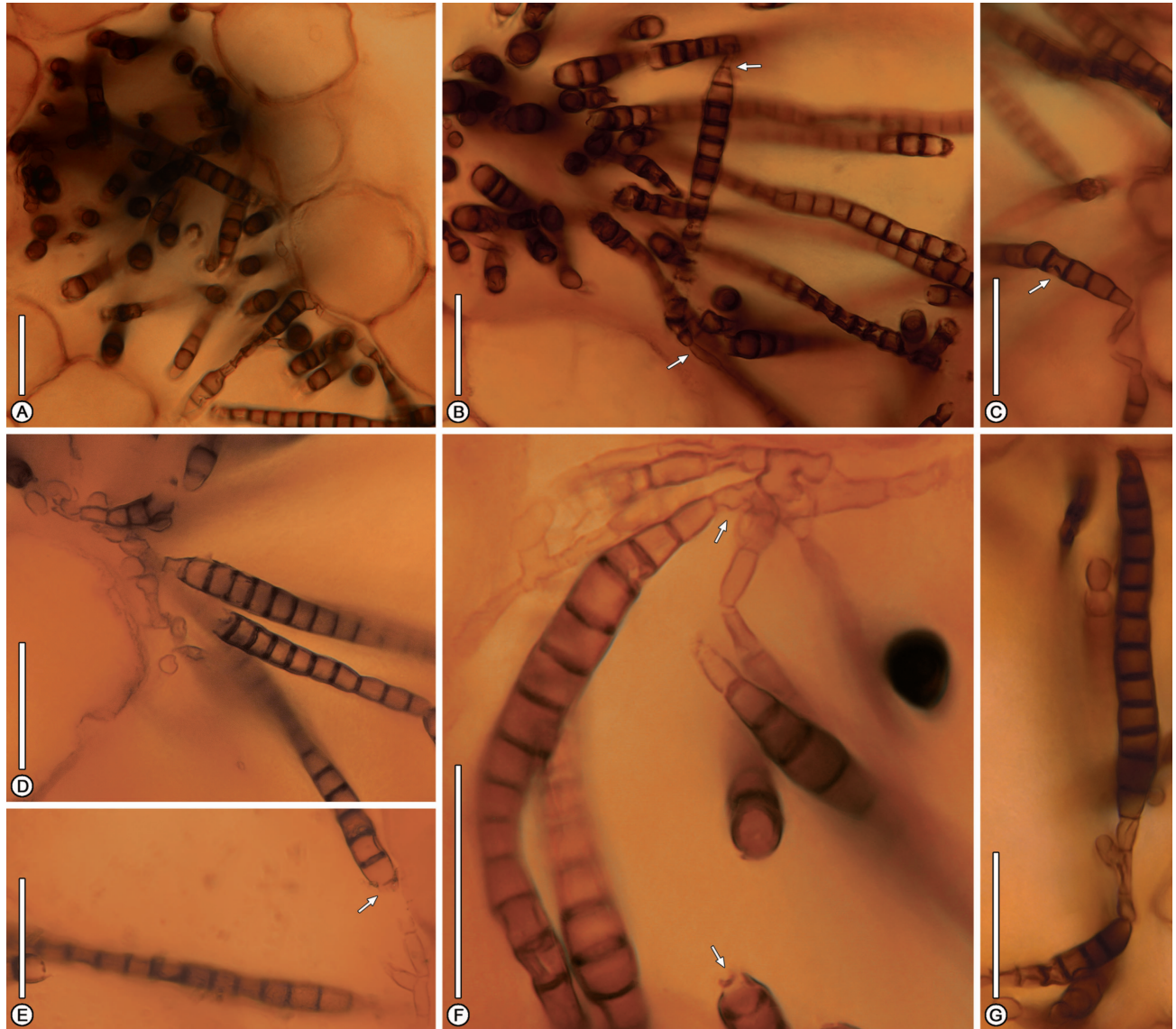


FIG. 1. Type I fossil hyphomycete. A,B. Holothallic multiseptate chlamydospores occurring within intercellular spaces in cortical aerenchyma of the Middle Eocene vascular plant *Eorhiza arnoldii*. Note subtending hyphae in B (arrows). C. Chlamydospores attached to hyphae; an intercalary cell (arrow) exhibits shrunken internal cell walls. D–G. Chlamydospores attached to branching mycelia; arrows indicate sites of rhexolytic secession and remnant of torn hyphal cell. A, G: 17037 F_{bot} #001; B, C, D, E, F: 17035 E_{bot} #001. Bars = 25 μ m.

tiaceous chains of amspores resembling clavate phragmospores; basipetal holoblastic chains are 15–25 μ m long and 8 μ m diam, with 3–6 transverse septa (FIG. 2A, B). Simple septal pores are present between successive cells (FIG. 2B, inset). The conidiogenous cells are 4 μ m diam and elongated, 5–9 μ m long; some are ampulliform (FIG. 2A, at left arrow), although most are doliiform. Seccession is schizolytic, sometimes occurring at the base of conidiogenous cells, which may remain attached to the dispersed spores (FIG. 2A, at right arrow). The full extent of conidiophore morphology is not visible, but conidia may have been

borne from a number of short, terminal branches, an inference supported by the distribution of conidia preserved in or near growth position (FIG. 2A, C). It also appears that multiple conidia were produced from the same conidiogenous cell (FIG. 2C, at arrow), and the conidiogenous locus is therefore indeterminate.

Type III.—The 20–25 μ m long biseptate, dematiaceous, pyriform phragmospores of this hyphomycetous anamorph (FIG. 2D–G) are characterized by an apical cell that is deeply pigmented when mature (FIG. 2D, cf. F), and markedly inflated, up to 15 μ m diam.

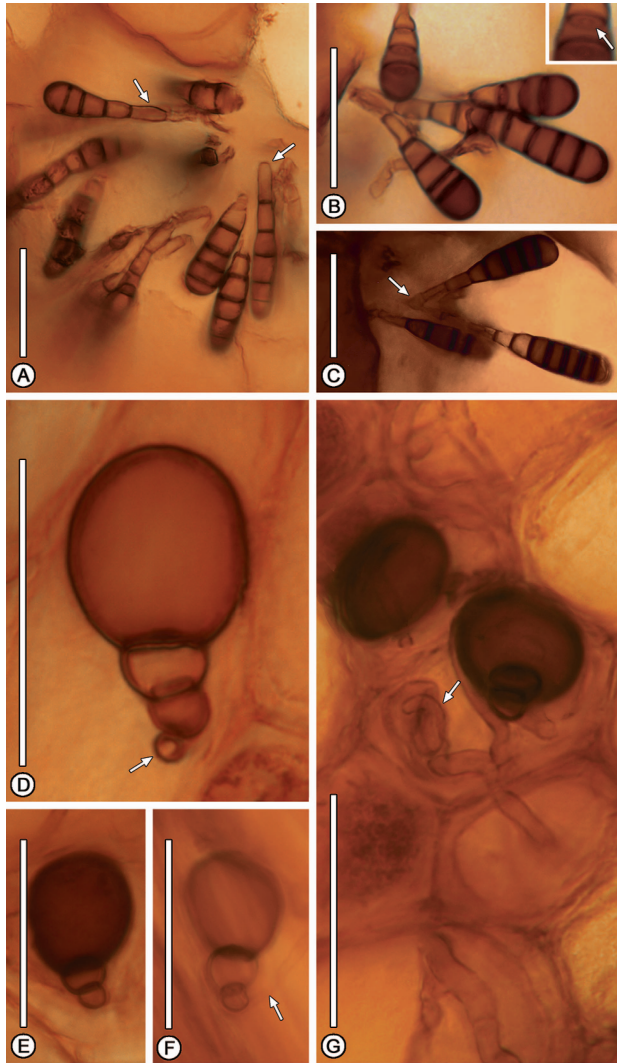


FIG. 2. A–C. Type II fossil hyphomycete. Apically pigmented, phragmospore-like aleuriospores with simple pores (B, inset, at arrow) produced from typically doliiform but occasionally ampulliform (A, at left arrow) conidiogenous cells. Dispersed chains of conidia can appear caudate as a result of schizolytic secession at the base of conidiogenous cells (A, at right arrow). Some conidiogenous cells appear indeterminate (C, arrow). D–G. Type III fossil hyphomycete. Apically inflated, bisepitate phragmosporic chlamydo-spores produced from gracile conidiophores (D, F, at arrows); note possible “rhizoidal” growth of associated hypha (G, at arrow). A: 17035 E_{top} #002; B, C: 17030 C_{bot} #001; D, E, F, G: 17035 E_{bot} #001. Bars = 25 μ m.

Conidiogenesis is acrogenous and monoblastic. Conidiogenous cells are isodiametric, 5 μ m wide, globose (FIG. 2D, F), and are retained at the base of the dispersed conidium (FIG. 2E) as a consequence of schizolytic secession from the micronematous conidiophore (FIG. 2D, F at arrows). Some hyphae in close association with spores produce curved branches

oriented toward associated assimilative mycelia, the hyphal diameters are 2.5–3 μ m (FIG. 2G, at arrow).

DISCUSSION

In their description of the aquatic angiosperm *Eorhiza arnoldii*, Robison and Person (1973) noted that the material contained abundant fungal remains, including assimilative mycelia and conidia. Subsequent to these early investigations, the cellulose acetate peel technique was modified for use with hydrofluoric acid (Basinger and Rothwell 1977), permitting rapid and extensive exploration of the flora. This may have occurred at the cost of observing the full extent of paleomicrobial diversity, because the peel technique is not optimal for recovery of fungal remains (Taylor et al. 2011). Our re-investigation of *Eorhiza arnoldii* with paleontological thin sections supports previous assessments of microbial diversity in the Princeton Chert (Robison and Person 1973, LePage et al. 1994) and has made it possible to elucidate developmental features of anamorphs previously known only from dispersed conidia. Although fossil conidia are frequently attributed to palynological form genera, this practice has been criticized for Cenozoic specimens, because many can be attributed to extant lineages (Pirozynski 1976, Pirozynski and Weresub 1979). Therefore, the aim of this study is twofold: In addition to describing these anamorphs in more detail than previously possible, we also seek to identify their probable context among extant fungi.

Affinities of type I.—The cylindrical macroconidia redescribed here originally were identified as thallic-arthric conidia (Robison and Person 1973). In their review of Princeton Chert fungi, LePage et al. (1994) suggested that the long, multiseptate conidia were produced pleurogenously, and they attributed these conidia to the genus *Cercospora* Fres. It is now apparent that the conidia were produced via holothallic conidiogenesis, wherein existing hyphae are transformed into conidia by production of transverse septa, enlargement and subsequent melanization. Several conidia exhibit attachment immediately adjacent to branches in the subtending hyphae (e.g. FIG. 1E, F), and some also exhibit attachment to distal hyphae (e.g. FIG. 1B, at arrows).

Although Robison and Person (1973) suggested that these spores might disaggregate as arthrospores, there is evidence only for rhexolytic secession of the entire conidium from the subtending hypha. Dispersed spores are common within the aerenchyma of *Eorhiza* and are invariably long, with no evidence for subsequent alternate-arthric disaggregation. Consequently, it is unlikely that these fossils have close

affinity with extant genera like *Eriocercospora* Rak. Kumar, A.N. Rai et Kamal ex U. Braun, in which some schizolytically abscising thalloblastic conidia subsequently break into smaller arthrospores (Braun 1998). Similarly, although developing conidia of *Ampulliferina* B. Sutton resemble the fossil spores, they subsequently break into oblong didymospores (Ellis 1971). *Rhexoampullifera* P.M. Kirk also produces short, cylindrical conidia through thallic-arthric conidiogenesis, but in this genus intercalary cells within a conidial chain remain thin-walled and act as the site of rhexolytic secession (Kirk 1982).

Elongate, cylindrical phragmoconidia with darkly pigmented, smooth surfaces are typically attributed to the palynological form genus *Scolecospirites* Lange & Smith (Lange and Smith 1971, Kalgutkar and Jansonius 2000). A number of extant genera produce conidia consistent with this morphology but can be differentiated from the fossils by morphological variations in their conidiogenous cells and conidiophores, which reflect developmental sequences incongruent with those of these fossils. For instance, conidiogenesis in *Gangliophora* Subram. and *Phragmoconidium* G.F. Sepúlveda, Pereira-Carvalho & Dianese occurs at fixed, enteroblastic loci (Subramanian 1992, Pereira-Carvalho et al. 2009), as do phragmoconidia of *Fusichalara* Hughes & Nag Raj (1973), which are further distinguished by extremely long “collarettes” of hyphal cell wall surrounding the conidiogenous locus.

The holothallic conidia described here are most appropriately attributed to the ascomycete *Xylomyces* Goos, Brooks & Lamore (Dothideomycetes: Jahnuales: Aliquandostipitaceae), a genus of saprotrophic aquatic hyphomycetes that produce thick-walled, dematiaceous, multiseptate chlamydo-spores (Goos et al. 1977, Goh et al. 1997, Sivichai et al. 2011). The resistant spores of *Xylomyces* are produced by intercalary hyphal septation, which we infer to have been the mode of conidiogenesis in these fossil fungi (FIG. 1B), and subsequent melanization. Of the eight described species of *Xylomyces*, most occur in freshwater and produce 3–7 septate conidia (Goos et al. 1977, Goh et al. 1997, Hyde and Goh 1999). However, the chlamydo-spores of *X. chlamydo-sporus* Goos, Brooks, & Lamore may have 14 septa, while those of *X. giganteus* Goh, Ho, Hyde & Sui possess up to 26 septa (Goos et al. 1977, Goh et al. 1997). The fossils described here are most similar to *X. giganteus*, although we have not been able to observe irregular longitudinal striations that typically occur on chlamydo-spore surfaces (Goh et al. 1997) owing to opacity of the chert matrix; nor have we observed intercalary germination in the specimens presently available to us.

Affinities of type II.—Although Robison and Person (1973) observed these conidia, they grouped them with the Type I (*Xylomyces giganteus*-like) chlamydo-spores and attributed them to the palynological form genus *Multicellaesporites* Elsik (Sheffy and Dilcher 1971). Because more specimens are now available for study, it is apparent that the Type II conidia are distinct from the Type I chlamydo-spores, in that conidiogenesis in Type II is holoblastic and the branching conidiogenous cell is terminal upon micronematous conidiophores. Several extant genera produce terminal cylindrical to clavate phragmo-spores from sympodial conidiogenous cells. It is possible to exclude *Mariellottia* Shoemaker, because the conidiogenous cells are cicatrized (Shoemaker 1998, Ellis 1971). *Rhodoveronaea* Arzanlou, W. Gams & Crous and *Eriocercospora* Deighton have hyaline to lightly pigmented conidia (Deighton 1969, Ellis 1971, Arzanlou et al. 2007), and while the conidia of *Brachysporiellina* Subram. & Bhat (Subramanian and Bhat 1987, Leão-Ferreira et al. 2008) are dematiaceous, they also are inflated apically, and the indeterminate conidiogenous cells are denticulate. As such, the fossil spores described here are clearly not attributable to these genera.

Instead, we consider these fossil fungi to be most similar to *Thielaviopsis* Went, in which basipetal chains of doliiform amero-spores are produced from a weakly sympodial or branching conidiogenous cell (Ellis 1971). A synanamorph frequently found in close spatial association (sometimes even arising from the same mycelium) produces narrow, doliiform to cylindrical enteroblastic hyaline amero-spores from obvious phialides (Nag Raj and Kendrick 1975). The dematiaceous conidia, in comparison, are aleurio-sporic, and because the cells do not readily undergo schizolytic secession, they often remain attached to hyphae in chains resembling phragmo-spores (Ellis 1971). Conidiogenesis of aleurio-spores from indeterminate loci in *Thielaviopsis* closely resembles the condition seen in some fossil specimens (e.g. FIG. 2C). Of the four extant species of *Thielaviopsis* that produce aleurioconidia, the fossils most closely resemble *T. basicola* (Berk. & Br.) Ferr., because aleurioconidia of other species are globose and solitary (Nag Raj and Kendrick 1975, Paulin-Mahady et al. 2002). However, there is as yet no evidence of synanamorph phialides or endoconidia in association with the fossils, and secession of the individual conidia from basipetal chains has not been observed.

Affinities of type III.—The final hyphomycete described in this study has not been previously recognized within the Princeton Chert. It is similar to the palynological form genus *Brachysporisporites*

Lange & Smith (Lange and Smith 1971, Kalgutkar and Jansonius 2000). Relatively few extant fungi produce phragmospores that are as distinctively inflated and apically pigmented as the fossils described here. Some species of *Brachysporiellina* and *Acaracybiopsis* J. Mena, A. Hern. Gut. & Mercado are morphologically similar, but in the former conidia are produced from acropleurogenous or sympodial conidiogenous cells, and in the latter conidiogenous cells are percurrent (Subramanian and Bhat 1987, Mena-Portales et al. 1999, Leão-Ferreira et al. 2008), while the fossils are solitary and terminal.

The apical inflation of these fossil conidia is similar to that of both *Brachysporiella rhizoidea* (V. Rao & de Hoog) W.P. Wu, and *B. setosa* (Berk. & M.A. Curtis) M.B. Ellis. Lengthy percurrent conidiophores like those of *B. setosa* have not been observed in the fossils, but one specimen (FIG. 2G) may exhibit “rhizoidal” mycelial branching similar to *B. rhizoidea* (Rao and de Hoog 1986), although Wu and Zhuang (2005) consider this character to be of minor taxonomic value. Although suggestive, the fossils currently available to us are not oriented in such a way as to allow observation of the entire conidiophore, and basal cells of the fossil conidia are more inflated than those of *B. rhizoidea*. In this latter respect, the fossils are more comparable to chlamydospores of *Culcitalna achraspora* Meyers & R.T. Moore (Sordariomycetes: Microascales: Halosphaeriaceae).

Culcitalna achraspora produces 2–3-septate phragmospore chlamydospores, in which each cell is inflated, and the most distal cell is deeply pigmented (Meyers and Moore 1960). The phragmospores are borne on micronematous conidiophores that are typically so highly reduced that spores can appear to be borne from hyphae, although longer conidiophores can occur (Meyers and Moore 1960, Seifert et al. 2011). Because the chlamydospores of *Culcitalna* can exhibit intercalary branching, observation of this character in a fossil specimen would let us more conclusively attribute this hyphomycete to the genus. Although *Culcitalna* is often regarded as a marine hyphomycete (e.g. Abdel-Wahab 2011), Meyers and Moore (1960) indicated there was no difficulty culturing it on artificial medium prepared with distilled water. Therefore, the occurrence of a *Culcitalna*-like hyphomycete within the tissues of *Eorhiza*, which had an aquatic or emergent habit, would not be particularly surprising.

This preliminary investigation of fungal diversity within the exquisitely preserved plants of the Princeton Chert indicates that this Eocene mire will prove a significant resource for paleomycologists. By preparing samples of chert in paleontological thin section, we have been able to observe developmental features

of several anamorphic fungi preserved within the cortical aerenchyma of *Eorhiza arnoldii*. As a result, we have been able to better attribute two hyphomycetes described in Robison and Person et al. (1973) and LePage et al. (1994) and have observed chlamydospores not previously identified within the Princeton Chert. All three are attributable to extant lineages, and one appears morphologically congruent with an extant species.

The fossil chlamydospores that we suggest are most similar to *Xylomyces giganteus* are of particular interest as calibration points in molecular divergence hypotheses. Because a holomorphic concept linking the teleomorph *Jahnula aquatica* (Kirschst.) Kirschst. with its anamorph, *X. chlamydosporus*, has been established (Sivichai et al. 2011), it is probable that *X. giganteus* also has its teleomorph among the ~ 15 species of *Jahnula* Kirschst. (Hyde and Wong 1999, Pang et al. 2002, Pinruan et al. 2002, Raja and Shearer 2006, Raja et al. 2008, Sivichai and Boonyeun 2010) or else within closely related members of the Jahnulales (Pang et al. 2002). If so, the presence of an *X. giganteus*-like species within the early Eocene provides a stratigraphically well constrained minimum calibration record for this order of lignicolous freshwater saprotrophs.

The three hyphomycetes illustrated in this study also provide additional insight into the paleoecological and taphonomic context of the *Eorhiza* plant. LePage et al. (1994) suggested that many *Eorhiza* specimens were moribund, in that several fungal anamorphs are present within most specimens. In addition, we have observed extensive mycelial proliferation, both inter- and intracellularly, with no evidence of host response. The presence of *Thielaviopsis*-like conidia suggests that *Eorhiza* might have been infected by a pathogenic fungus in life, because these fungi commonly occur as root pathogens (Paulin-Mahady et al. 2002). We suggest that the other two hyphomycetes are most comparable to genera that are facultative aquatic hyphomycetes and consistently occur on submerged substrates (Meyers and Moore 1960, Rao and de Hoog 1986, Goh and Hyde 1996, Shearer et al. 2007), indicating post-mortem colonization of *Eorhiza* in an inundated setting. Because fossil conidia were produced preferentially within intercellular spaces of the cortical aerenchyma, the host tissue probably was colonized quickly before becoming so degraded as to be waterlogged. By inference, this also suggests that the earliest stages of subsequent permineralization likewise occurred within a short temporal span.

Continuing investigations of mycological diversity in association with the silicified plants of this Eocene mire are likely to provide additional specimens of the

fungi described here. In addition to providing calibration points, the discovery of fossil exemplars of extant lineages will continue to expand our understanding of microbial contributions to the paleoecology of the Princeton Chert.

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