Microbiological insights into ecology and taphonomy of prehistoric wetlands.

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Abstract

In the course of this dissertation, I present investigations of the microbial constituents of fossil plants preserved at an anatomical level of detail, and detail the results of an ecological survey of root-endogenous fungi within the cosmopolitan emergent macrophyte, *Typha*. These studies together elucidate processes in the taphonomy of fossil plants. Biostratinomy is addressed through descriptions of saprotrophic communities within the Eocene Princeton Chert mire assemblage, and within a Carboniferous fern which previous studies had suggested contained fossilized actinobacteria. Re-investigation of the 'actinobacteria' suggests instead that the structures are disordered ferrous dolomites, raising implications for the contribution of sulfate-reducing bacteria to the early-diagenesis mineralization of plants preserved in carbonaceous concretions. The fossilized remains of saprotrophic and putatively endophytic fungi within roots of *in-situ* plants from the Princeton Chert also provide insight into early diagenesis. Some of the fungi described herein are preserved in several co-occurring developmental phases, providing evidence that early phases of silicification in this assemblage were rapid. As the Princeton Chert is not a hot-spring sinter deposit, these data conflict with prior hypotheses for the preservation of this peat-forming wetland assemblage. Understanding the microbial paleoecology of this system, and other wetland assemblages that constitute paleobotanical *Konservat-lagerstätten*, will provide important foundations upon which to improve hypotheses of plant-microbe interactions in the fossil record. Research into fossil plant-microbe interactions must, however, be conducted with reference to appropriate biogeochemical analogues. The concluding component of this dissertation establishes that endogenous fungi in contemporary wetland plant roots are affected by persistent inundation. Although the constituents of root-endogenous communities do not appear to change between inundated roots and those growing in

subaerially-exposed soils, their incidence within roots does differ. These data offer clear implications for assessing the probable ecology of *in-situ* fossil plants that hosted endogenous microbial communities during life.

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When my mother was in grade school, she acquired the paperback *Search for a Living Fossil*, a narrative account of the discovery of the [first] living coelacanth, *Latimeria chalumnae* Smith, off the Comoros Islands. It opens with Marjorie Courtenay-Latimer poking about the docks for ichthyological specimens – female, outsider, scientist, and intensely curious. Plenty of people who come to paleontology cite *Jurassic Park*, or seeing dinosaurs in museums. I trace my own enthusiasm to a much-battered, age-worn green and purple schoolbook. Coming to KU was coming full circle, in a sense: Leaning over the coelacanth tank in the BI, and marvelling as JLB Smith himself might have, was the culmination of years of enthusiastic reading in pop science paleontology and geology. And in terms of reading, another serendipitous moment presented itself, when I had the opportunity to learn molecular phylogenetics from Mark Holder, whose analysis placing the Indonesian coelacanth was the first scientific paper I'd ever read. I can still hardly believe that one of my science heroes agreed to serve on my committee. I am also exceedingly grateful to Jennifer Roberts, whose geomicrobiology course filled an important gap in my knowledge and changed the way I conceptualized permineralization. Ari Jumpponen provided valuable critical feedback in the course of my research, and Leonard Krishtalka has been an enthusiastic supporter who constantly encouraged me to think big and conceptualize the 'story' in my work.

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Chapter 1: Introduction

Interactions between plants and microbes are a cornerstone of life on earth, and untangling the origins of these syndromes and these organisms' shared evolutionary history remain key questions in botany, mycology, and paleontology alike. Plant-microbe interactions span the gamut of obligate mutualism through commensalism, parasitism and pathogenicity, and are fundamental to biogeochemical systems, driving carbon and phosphorus cycling, weathering, and soil formation (Beerling and Berner 2005, Leake et al. 2008, Steemans 2010, Kenrick et al. 2012). 450 million years ago, plant-fungal mutualisms drove early land plant evolution (Pirozynski and Malloch 1975, Lambers et al. 2009, Humphreys et al. 2010, Wang et al. 2010, Bidartondo et al. 2011, Kenrick et al. 2012, Feijen et al. 2017), and today root-endemic fungi and bacteria are major ecological drivers in land plant communities (Leake et al. 2008, Smith and Read 2008, Johnson 2010). Yet, there is a paucity of fossil evidence from critical portions of the geological record (Butterfield 2015, Kenrick et al. 2012, Selosse et al. 2015, Wellman and Strother 2015, Gerrienne et al. 2016, Smith 2016). As a consequence, we develop inferential hypotheses about plant-microbe interactions in the fossil record, through phylogenetic comparisons (Wang et al. 2010, Delaux et al. 2015, Delwiche and Cooper 2015), and through reference to *Konservat-lagerstätten*, fossil assemblages in which organisms exhibit exceptional preservation.

Konservat-lagerstätten that entomb the fossil record of plant-microbe interactions are heavily biased toward wetland assemblages. Wetland or marginal assemblages comprise not only the overwhelming majority of paleobotanical localities but in fact *all* fossil assemblages in which mutualistic or commensal microbes are preserved in association with in-situ rooting systems (Stubblefield 1987, Remy et al. 1994, Phipps and Taylor 1996, LePage et al. 1997,

Stockey et al. 2001, Klymiuk et al. 2013a, 2013b). One of the most famous *Konservat-lagerstätten* wetland assemblages, the Devonian (407 Ma) Rhynie Chert, has long been regarded as a fossil analogue for the earliest terrestrial communities (Remy et al. 1994, Boyce 2009, Strullu-Derrien et al. 2017, Brundrett et al. 2018, Field et al. 2018). Despite post-dating the origin of land plants by at least 70 Ma, the Rhynie plants and microbes that lived along margins of geothermally-sourced pools (Rice et al. 2002, Channing and Edwards 2003, 2009, Fayers and Trewin 2004) serve as a benchmark for ongoing experimental work in understanding the cryptogamic groundcovers which may have typified the earliest land plant communities (Mitchell et al. 2016, Graham et al. 2017, Field et al. 2015a, 2015b). From the coal swamps of the Carboniferous, to the Princeton Chert, *Konservat-lagerstätten* that inform our understanding of most of the fossil record of plant-microbe interactions is biased towards plants living in, or near, inundated soils, yet the ecology of these interactions has been consistently interpreted (Taylor and Osborn 1996, Taylor and Taylor 2000, Klymiuk et al. 2013a, 2013b) in accordance with models of plant-microbe interaction developed in subaerially-exposed soil systems (Smith and Read 2008, Rodriguez et al. 2009, Johnson 2010, Newsham et al. 2011). I propose that it is necessary to understand the fossil record of plant-microbe interactions from a stance informed by reference to wetland soils and processes, and that this perspective will not only better constrain our hypotheses for plant-microbe interactions in the fossil record, but enable new insights into plant taphonomy.

Taphonomy, the paleontological discipline established by Efremov in 1940, is concerned with explicating post-mortem fates of organisms and their potential for recruitment into the fossil record. Microbes play critical roles in all taphonomic pathways, as agents of degradation, and, ironically, preservation. Within wetland soils, fungi are the principal agents of decomposition

(Thormann 2006, Gessner et al. 2007, Thormann and Rice 2007) in oxidized or aerobic portions of the soil, while facultative aerobic, microaerophilic, and anaerobic microbial metabolisms drive pore water reduction-oxidation chemistry (Seo and DeLaune 2010, Lin et al. 2012, Lispon et al. 2013), with consequences for the stoichiometric stability of minerals that fossilize plants. The precipitation of many iron, carbonate, phosphate, sulfur, silicate, and clay minerals is bacteriogenically mediated (Ferris et al. 1987; Beveridge et al. 1989, Ferris 1993, Konhauser 1998, Bazylinski and Frankel 2003, Briggs 2003, Martin et al. 2004, Konhauser et al. 2011, Darroch et al. 2012), and fossilization can occur when minerals nucleate on microbial cell surfaces or biofilms (Popa et al. 2004, Maclean et al. 2008). Indeed, biofilms are probably integral to preservation of leaves as compression-impression fossils (O'Brien et al. 2002, Spicer 1977, Dunn et al. 1997). Complete high-fidelity replacement of organics, as in pyritized Devonian and Eocene fossil plants (Allison 1988, Stewart and Rothwell 1993, Grimes et al. 2001, Brock et al. 2006) owes to sulfate reducing bacteria (SRB), which utilise sulfate as a terminal electron acceptor under anaerobic conditions, producing H_2S as a metabolic by-product, which reacts with dissolved iron and precipitates as iron sulfide (Frankel and Bazylinski, 2003). Sulfate reducing bacteria may also be implicated in the early-diagensis mineralization of fossil plants permineralized in carbonate concretions (Chapter 2, Klymiuk et al. 2013c), the 'coal balls' and 'nodules' that contain plant fossils preserved at a cellular level of detail (Stewart and Rothwell 1993, Klymiuk and Stockey 2011).

In the second chapter of this dissertation, I suggest that bacteriogenically-mediated precipitation of disordered dolomite may be a key factor in the early diagenesis of plants permineralized in carbonate concretions. In Chapter 2, I present a reinvestigation of structures originally interpreted as the earliest representatives of Actinomycetes (Actinobacteria) in

association with vascular plants (Smoot and Taylor, 1983). Actinomycetes are high guanine-cytosine content Gram-positive bacteria (Embley and Stackebrandt 1984, Stackebrandt and Woese 1981) that function as saprotrophs within the rhizosphere (Jaatinen et al. 2008, Aliasgharzad et al. 2010). Within plants, some are nitrogen-fixing mutualists (Reddell and Bowen 1985, Sellstedt et al. 1986) while others may confer resistance to pathogenic fungi (Conn et al. 2008). Their fossil record is sparse, and predominantly Cenozoic (Waggoner 1993, 1994a, 1994b; Wilkinson 2003, Fostowicz-Frelik and Frelik 2010, Poinar 2011, Saint Martin et al. 2012). Re-examination of the structures described by Smoot and Taylor (1983) provides little support for considering them actinomycete bacteria. Instead, I suggest that these structures are authigenic biomimetic carbonates precipitated in conjunction with anaerobic degradation of plant cell material. The structures are likely disordered ferrous dolomites, which I've inferred from critical examination of morphology, monochromatic luminescence mapping, and energy dispersive X-ray spectrometry, which identified magnesium, calcium, and iron within the biomimetic structures. The precipitation of these pseudofossils was likely biologically mediated by sulfate-reducing bacteria (e.g., Wright and Wacey 2004, 2005), and may also have involved organomineralization around carboxyl-rich organic polymers (e.g., Roberts et al. 2013). Understanding how microbes contribute to hydrogeochemistry can assist us in developing testable hypotheses for early stages in plant fossil diagenesis, improve our perceptions of potential information loss in the fossil record, and permit more sophisticated models for putative depositional environments.

Understanding microbes as the agents of decay also enables insight into taphonomic controls and processes. In the third, fourth, and fifth chapters of this dissertation, I present descriptions of predominantly saprotrophic microfungi from the Princeton Chert *Konservat-*

lagerstätten, one of the most intensively documented floras the Eocene (48.7 Ma) Thermal Maximum (Pigg and Stockey 1996, Pigg and DeVore 2016). The permineralized plants of the Princeton Chert have proven a rich paleomycological resource (LePage et al. 1994, Currah et al. 1997, LePage et al. 1997, Stockey et al. 2001, Klymiuk et al. 2011), offering a rare opportunity to understand a diverse community of fossil saprotrophic fungi within *in-situ* wetland plants (Stockey and Pigg, 1994, Cevallos-Ferriz et al. 1991). Investigations of the rhizomes of the extinct angiosperm *Eorhiza arnoldii* Robison et Person (1973) and the aquatic fern *Dennsteadtiopsis aerenchymata* Arnold et Daugherty (1964), yielded microfungi fossilized in growth and developmental context, providing key features necessary for identification of anamorphic fungi (asexual 'molds'). As such, I have been able to clarify and expand descriptions for two saprotrophs previously observed (Chapter 3, Robison and Person 1973, LePage et al. 1994, Klymiuk et al. 2013a), describe two additional saprotrophs (Chapter 3, 5, Klymiuk et al. 2013a, Klymiuk 2016), and have described the first putative dark septate endophytes (DSE) in the fossil record (Chapter 4, Klymiuk et al. 2013b). DSE are a globally ubiquitous informal assemblage composed predominantly of ascomycetes (Addy et al. 2005), which proliferate asymptomatically within the living tissue of their hosts (Jumpponen and Trappe 1998); they may be mutualists, or become weak pathogens and saptrophs upon host senescence (Schulz and Boyle 2005, Rodriguez et al. 2009, Newsham 2011, Mandyam et al. 2013). In addition to improving our understanding of the composition of the saprotrophic component of this paleoecosystem, the studies presented here (Chapters 3–5) provide insight into the timing of permineralization: Articulated spores in multiple developmental stages suggest that some stages of silicification of the Princeton mire were exceedingly rapid, despite the fact that this succession is not associated with geothermal activity (Mustoe 2011), which accounts for

rapid silicification in other *Konservat-lagerstätten* wetland assemblages like the Devonian Rhynie Chert (Rice et al. 2002) and the Jurassic Deseado Massif (Massini et al. 2016).

It has been a largely-unexamined paradox that prehistoric wetland assemblages constitute our most important fossil records (Channing and Edwards 2013) for the interactions of plants and microbes, yet our understanding of these systems has been rooted in fully terrestrial paradigms. Inundated soils constitute biologically hostile environments (Vepraskas and Faulkner 2001, Reddy and Delaune 2008, Mitsch et al. 2009, Pezeshki and Delaune 2012), where reduction-oxidation dynamics are established through the interplay of microbial decay of accumulated organic carbon (Vepraskas and Faulkner 2001), and oxygen diffusion from plant roots (Flessa 1994, Aldridge and Ganf 2003). Strict and facultative aerobes, including saprotrophic fungi and chytrids (Emerson and Natvig 1981, Dighton et al. 2005, Thormann 2006, Thormann and Rice 2007) rapidly deplete pore-water oxygen; thereafter, anaerobic microbial respiration successively depletes the next most energetically favourable metal cation, and the soil becomes increasingly electronegative (Vepraskas and Faulkner 2001, Canfield et al. 2004, Alewell et al. 2008, Lipson et al. 2013, Sims et al. 2013, Lin et al. 2012, 2014). Reducing conditions and root-zone anoxia are typical of wetland systems, affecting the bioavailability of limiting nutrients, as well as increasing phytotoxic compounds (Ponnamperuma 1984, Pezeshki 2001, Weis and Weis 2004, Reddy and Delaune 2008). Historically, root-zone anoxia was thought to prevent microbes from inhabiting the roots of wetlands (Khan and Belik 1995), but we now recognize that these communities are ubiquitous and diverse (Søndergaard and Laegaard 1979, Cook and Lefor 1998, de Marins et al. 2009, Sudová et al. 2011, Kohout et al. 2012, Sandburg et al. 2014, Zhouying et al. 2016, Ramírez-Viga et al. 2018). Although it has become apparent that wetland plants host

all major groups of root-endogenous microbes, the extent to which these communities parallel those in subaerially-exposed soils has been only minimally investigated.

Understanding plant-microbe interactions in modern wetlands may vastly improve our understanding of the paleoecology of fossil wetland successions. In an ecological investigation (Chapter 6) of fungal community structure in contemporary wetlands, I examined how a) the incidence of morphological structures paralleling those available in the fossil record, and b) community composition of culturable endogenous fungi changed with respect to inundation. Wetland plants employ physiological and anatomical adaptations to mitigate root-zone anoxia (Armstong 1979, Jackson and Armstrong 1999, Strand 2002, Gibbs and Greenway 2003, Greenway and Gibbs 2003, Evans 2004, Colmer and Voesneck 2009), so I hypothesized that **H_0:** environments within well-aerated roots may closely resemble roots in subaerially-exposed soils, or **H_A:** endogenous microbes may be in direct competition with plant cells for diminishing oxygen resources (Bedford et al. 1991, Kludze and Delaune 1996, Chabbi et al. 2000, Matsui and Tsuchiya 2006). The focal organism, reedmace (*Typha* spp.) has been used as a model plant in inundation studies (Ray and Inoue 2006, Inoue and Tsuchiya 2009), and has distributions of cortical aerenchyma similar to that observed in the extinct dicot, *Eorhiza arnoldii*, samples of which were intensely colonized by saprotrophic fungi and putative endophytes (Klymiuk et al. 2013a, 2013b, Klymiuk 2016). Results of this study suggest that all aerobically-respiring fungi respond to inundation in similar ways; trends previously identified for extant AMF (Clayton and Bagyaraj 1984, Tanner and Clayton 1985, Khan and Belik 1995, Miller 2000) probably hold for other groups of endogenous fungi. This research sets the stage for continued investigation of ecological drivers structuring communities of endogenous microbes in contemporary wetlands. Such studies will provide much-needed references from which to develop *aktuopalaontologische*

experiments for wetland plant taphonomy, and to more appropriately frame our interpretations of plant-fungal interactions observed in the fossil record.

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Chapter 2: Reinvestigating Carboniferous 'actinomycetes' — authigenic formation of biomimetic carbonates provides insight into early diagenesis of permineralized plants

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Abstract

Paleoecological interactions among fossil microorganisms have garnered significant interest within the paleobotanical community; however, an understanding of the early diagenesis of associated plant material is of critical importance when assessing putative body fossils of fungi and bacteria. Structures preserved within permineralized petioles of the Carboniferous fern *Botryopteris tridentata* Felix (Scott) have been interpreted as the earliest remains of Actinobacteria found in association with vascular plants, but re-examination of the specimens indicates instead that these biomimetic structures (BMS) are authigenic carbonate minerals. Using spinning disk confocal microscopy, we generated monochromatic luminescence maps of BMS found within the phloem cells of *Botryopteris*. Luminescence was captured at wavelengths of 665 nm, consistent with an interpretation of these structures as disordered dolomites, an inference subsequently corroborated with energy-dispersive X-ray spectrometry (SEM-EDS). The presence of high-magnesium carbonates within *Botryopteris* is suggestive of an early anaerobic stage of plant tissue degradation characterized by metabolic activities of sulfate-reducing bacteria. Anaerobic biodegradation may also have been performed by chytridiomycetes, and we interpret larger (5–8 μm) unicells found within the specimens as fossils of chytrid zoosporangia. Understanding microbial contribution to the early diagenesis of plants

preserved within calcium carbonate concretions (coal balls) is dependent upon both characterizing diversity of microbial communities within fossil plants, and elucidating the geomicrobiological parameters of mineralization. As such, this study underscores the necessity of integrating geomicrobiology with plant taphonomy in investigations of the microbial component of ancient ecosystems.

Introduction

For nearly half a century, microbial fossils have been the focus of intense interest among researchers who have attempted both to understand the early conditions under which life evolved, and to characterize the fossil record of bacteria (e.g., Barghoorn and Tyler 1965, Barghoorn and Schopf 1966, Knoll 1982, Schopf 1993, Wacey et al. 2011). As a result, we are moving towards a more sophisticated understanding of hydrogeochemical conditions under which bacteria may fossilize (e.g., Van Lith et al. 2003, Lalonde et al. 2005, García-Vallès et al. 2008, Dupraz et al. 2009), and some of the abiogenic processes that can mimic bacterial morphologies (Hofmann 1972, García-Ruiz 1994, Lowe 1994, Grotzinger and Rothman 1996, García-Ruiz et al. 2002, 2003; McLoughlin et al. 2008). As living hosts or decaying substrates, plant tissues constitute a physical and biochemical landscape in which distinct microbial ecosystems have evolved (Bianciotto et al. 1996, Lodwig et al. 2003, Kotsyurbenko et al. 2004, Bouwmeester et al. 2007). The interactions between microbes and vascular plants have a deep evolutionary history (Pirozynski and Malloch 1975, Bateman et al. 1998, Tomescu et al. 2006, Wang et al. 2010), and understanding how such interactions have developed and changed throughout the Phanerozoic is a rapidly expanding field of paleobotanical research (Beimforde et al. 2011, Bidartondo et al. 2011, Massini et al. 2012). A reinvestigation of structures originally interpreted as the earliest representatives of Actinomycetes (Actinobacteria) in association with

vascular plants (Smoot and Taylor 1983) underscores the necessity of approaching some putative microbes with an understanding of early diagenetic processes that can produce biomimetic structures (BMS).

The actinomycetes are physiologically diverse, high G-C (guanine-cytosine) content Gram-positive bacteria (Stackebrandt and Woese 1981, Embley and Stackebrandt 1984, Fox and Stackebrandt 1988), many of which occur as branching septate filaments that are morphologically reminiscent of fungal hyphae, although generally much smaller (Waksman 1950, Lechevalier and Lechevalier 1967). Actinomycetes are known to play a vital role in the ecology of plant communities. Within the rhizosphere, they function as saprobes (Goodfellow 1983, Jaatinen et al. 2008; Aliasgharzad et al. 2010), and many also have intimate associations with vascular plants, such as the nitrogen-fixing mutualist *Frankia* (Reddell and Bowen 1985, Sellstedt et al. 1986). Endophytically, some actinomycetes occur as plant pathogens or parasites, but most have a commensal relationship with their hosts, conferring resistance to pathogenic fungi (Goodfellow 1983, Doumbou et al. 2001, Taechowisan et al. 2003, Conn et al. 2008). Understanding the evolution of such mutualistic associations may offer insight into the early evolution of terrestrial plants, and may also allow us to better conceptualize ecological constraints within ancient plant communities.

At present, however, the fossil record for actinomycetes is sparse, and predominantly Cenozoic (Waggoner 1993, 1994a, 1994b; Wilkinson 2003, Fostowicz-Frelik and Frelik 2010, Poinar 2011, Saint Martin et al. 2012). The oldest known records of filamentous bacteria in association with plant tissue are specimens described from within three-dimensionally permineralized cells of a Pennsylvanian fern, *Botryopteris tridentata* (Felix) Scott (Smoot and Taylor 1983). A re-examination of these specimens, however, demonstrates there is little

support for considering them actinomycete bacteria. Instead, we suggest that these structures are authigenic carbonate minerals, formed in conjunction with the anaerobic degradation of plant cell material.

While there is no evidence of actinomycete remains within these Carboniferous specimens, the *Botryopteris* tissue is not devoid of microbial fossils, and we reinterpret larger spherical unicells (Smoot and Taylor 1983, fig. 8) as chytridiomycete zoosporangia. Chytrids (Chytridiomycota) are morphologically simple fungi (James et al. 2006a, 2006b) that occupy environmental niches in polar to tropical terrestrial, freshwater, estuarine, and marine ecosystems (Powell 1993, Barr 2001, Hibbett et al. 2007), where they function as saprotrophs, bio-eroders, parasites, mutualists, and pathogens (Karling 1977, Gleasen et al. 2008, Kilpatrick et al. 2009). Chytrids exhibit a plesiomorphic form of reproduction where motile, flagellated spores (zoospores) are borne in larger, saclike structures termed zoosporangia (James et al. 2006a), and representatives of these life cycle stages are well known in the chytrid fossil record (e.g., Millay and Taylor 1978, Taylor et al. 1992, Trewin et al. 2003, Krings et al. 2009a, 2009b; Massini et al. 2012). The body fossils we interpret as zoosporangia provide additional evidence for the role of these fungi as saprotrophs within ancient environments.

The microbial paleoecology of plant tissues is a field of inquiry that is gaining significant momentum, and these studies continue to offer insight into the interactions between microbes and the plants that both host them and act as substrates (e.g., Taylor and Krings 2010, Krings et al. 2011, 2012; Harper et al. 2012). As has been indicated by other studies (Buick et al. 1990, Van Zuilen et al. 2002, Brasier et al. 2005, Schopf 2004), our reinvestigation of putative actinomycete remains demonstrates the necessity of appreciating abiogenic processes that can produce microscopic pseudofossils. In this study, we illustrate that critical examination of such

structures can reveal features suggestive of abiogenicity, and utilize both monochromatic luminescence mapping and energy-dispersive X-ray spectrometry (SEM-EDS) to characterize their mineralogy. In addition to suggesting methods by which to identify legitimate microbial body fossils from biomimetic carbonates, our results indicate that comprehensive investigation of degrading plant tissue in a geomicrobiological context will further our understanding of taphonomic processes that lead to both information loss and preservation in the paleobotanical record.

Materials and Methods

Material investigated in this study occurs within calcium carbonate concretions, commonly known as coal balls, which were collected from the Lewis Creek locality (37° 0'0.35"N, 83°17'34.39"W) of Leslie County, Kentucky, USA. The concretions, within which plant remains are anatomically preserved, are associated with the Copland (Taylor) coal (Smoot and Taylor 1983). The Copland coal is considered the uppermost unit of the Moscovian (Middle Pennsylvanian) Hyden Formation (Fm) of the Breathitt Group (Chesnut et al. 1996, Greb et al. 1999), and is overlain by shales of the Magoffin Member of the Four Corners Fm (Schopf 1961, Smoot and Taylor 1983, Chesnut et al., 1996).

The specimens were originally prepared using the cellulose acetate peel technique (Joy et al. 1956), and resultant sections were mounted on microscope slides using xylene-soluble Harleco (EMD Millipore) Synthetic Resin (Smoot and Taylor 1983). Additional material was prepared for scanning electron microscopy (SEM) by etching portions of the phloem tissue (Smoot 1979), and in this reinvestigation, these original SEM micrographs are refigured. In addition to photomicrographing specimens from slides prepared by Smoot and Taylor (1983), we also mounted several acetate peels for SEM-EDS analysis; these comprised serial sections #4,

#13, #19, #25 from specimen 6809 D_{side}. All specimens and slides are deposited in the Paleobotanical Collections, Natural History Museum and Biodiversity Institute, University of Kansas, (Lawrence, KS). Slide accessions comprise 7523, 7525, 7532, 7554, 7556, 7563, and 7566, and were made from accessioned specimens 6592 C top 7, 6592 D side 11, 6592 D side 15, 6809 D3 side B 22, 6809 D3 side B 29, 6809 D3 side B 43, and 6809 D3 side B 50. In the course of our investigations, we also reexamined specimens originally figured as 'mycorrhiza' (Andrews and Lenz 1943, fig. 6), using slides from the Henry N. Andrews collection, deposited at the George Safford Torrey Herbarium, Department of Ecology and Evolutionary Biology, Storrs, Connecticut.

All digital images were captured with a Leica DC500 CCD attached to a Leica DM5000B transmitted-light compound microscope and minimally processed using Adobe Photoshop CS5 12.1. Multiple photomicrographs of the same specimen, taken at different focal planes, were compiled (after Bercovici et al. 2009) to produce the composite images in Figure 6. Focal stacking was performed in Adobe Photoshop CS4 11.0.2 by erasing specific areas to reveal three dimensionality of the specimen as is visible under transmitted light. Measurements were performed in ImageJ 1.43u (W.S. Rasband, U. S. National Institutes of Health, Bethesda, Abràmoff et al. 2004).

Spinning disk confocal microscopy was performed using an Olympus IX71 microscope equipped with a Yokagawa CSU10 spinning disk confocal illumination system. Excitation was performed with a 641 nm Coherent solid-state laser. Emission was collected using a Semrock longpass 665 nm filter, and image capture was performed with a Hamamatsu 512 x 512 back-thinned electron multiplying CCD (quantum efficiency ~94%). Images were captured using

Slidebook 5.0 (Intelligent Imaging Innovations, Denver, CO) and were pseudocolored green with ImageJ 1.45s, to optimize visibility.

Elemental composition of structures of interest was assessed using SEM-EDS. Select specimens, as previously indicated, were coated with 15 nm Au, using a Quorum EMS 150T ES. Scanning electron microscopy was performed using a Carl Zeis LEO 1550 Field Emission Scanning Electron Microscope with an Everhart-Thornley detector. Spectrometry was conducted at 15 kV, and spectra were collected with an EDAX SiLi detector, using the collection package Genesis (EDAX Inc., Mahwah, New Jersey, USA).

Results

Biomimetic structures. Smoot and Taylor (1983, p. 2252) originally described inclusions within some petioles of the Pennsylvanian fern *Botryopteris tridentata* (Figs. 1A–B) as 'smooth, knobby filaments and spheres'. They suggested that some (Fig. 1B–E) represent body fossils of filamentous bacteria most similar to extant actinomycetes, whereas others are dried mucilage or cytoplasm (Figs. 1F–L). The inclusions are distributed within cells interpreted as phloem mucilage cells (Fig. 1A); cells that contain inclusions with a 'bacterial' morphology are adjacent to those in which acicular or amorphous inclusions occur (Figs. 1A–B).

The 0.5–1.0 μm spore-like structures are spheroidal, but are neither isodiametric nor consistent in size (Fig. 1C–E). Furthermore, by shifting the focal plane, the so-called filaments are revealed as dense aggregates of spheroids (Figs. 1D–E). Additionally, the spheroidal structures occur as interwoven lattices, a morphology that is inconsistent with the manner in which the coccoidal spores of extant actinomycetes are borne (Table S1). Smoot and Taylor (1983) originally interpreted reticulate aggregations of acicular precipitates (Figs.1F–L) as dried mucilage or coagulated cytoplasm, but these structures grade into pyriform and spheroidal

morphologies (Figs. 1G–1H), consistent with those interpreted as bacterial body fossils. Moreover, patterns within the reticulate aggregates which superficially resemble septa (Fig. 1I, arrows) are in fact fractures (Figs. 1J–L). Some cells also contain larger, 2.0–4.0 μm, spheroidal or oblate structures (Fig. 1M), which are irregular, amorphous, translucent aggregations that are in close proximity with both acicular and spheroidal structures (Fig. 1M, arrows).

Laser excitation of three types of inclusions (Fig. 2A–C) in *Botryopteris* resulted in emission that was captured at 665 nm; the monochromatic map of luminescence observed with spinning disk confocal illumination (Fig. 2D–F) indicates that despite very differing morphologies, these biomimetic structures contain similar activating and sensitizing cations. Luminescence was not observed at wavelengths typically associated with organic polymers or calcium carbonate, and could not be detected using standard epifluorescence techniques.

Elemental composition of inclusions was characterized with comparison to plant cell walls and intracellular calcium carbonate cement (Fig. 3). Calcium (Ca) and magnesium (Mg) are present in both cell walls and cement, although the spectral signature for Ca is more robust; furthermore Ca is highly represented within the intracellular cement, in accordance with expectations (Figs. 3A–B). Typical spectra associated with inclusions have peaks for both Ca and Mg that are of similar magnitude (Fig. 3C). Iron (Fe) was not observed in the plant cell walls, but a slight peak at ~6.04 keV was observed in intracellular cement (Fig. 3B), consistent with the Kα shell of Fe. Within inclusions, spectral peaks concordant with both the Lα and Kα electron shells of Fe are evident (Fig. 3C). Iron sulfide, or pyrite, is also present within some of the *Botryopteris* tissues (Fig. 4). It occurs as small, 1–2 μm euhedral crystals that are typically surrounded by a rind of translucent mineral (Fig. 4, inset), containing both Ca and Mg.

Cell wall microstructure. Slender, scalalike protrusions, 1 μm wide, occur in the intercellular spaces between some phloem cells when viewed in longitudinal section (Fig. 5A). The projections are continuous with the cell walls, which may be shrunken into the lumen (Fig. 5B). Where projections do not extend entirely across the intercellular space, they may be terminated by spheroidal masses, some of which have a bi-lobed, or slightly dumbbell-shaped morphology (Fig. 5C, arrow). Mineral halos may be observed around some of the projections (Fig. 5B, inset).

Microbial body fossils. Smoot and Taylor (1983, fig. 8) originally figured large (5.0–8.0 μm diameter) spherical structures within phloem mucilage cells of *Botryopteris* (Fig. 6A–B), but did not discuss their possible affinities. Re-examination of these fossils indicates that they represent the remains of chytridiomycete zoosporangia. Each has a single pore, 1.0–2.0 μm in diameter (Fig. 6B) and there are minute, amorphous precipitates encrusting the exterior surface of the fungal cell wall. Individual precipitates vary significantly in size, and they are continuous with similar subspheroidal precipitates lining the plant cell tissue beneath the fungal remains (Fig. 6B). The discovery of additional fungal remains, namely a cluster of unicells in close association with a fern spore adjacent to the *Botryopteris* petiole (Fig. 6C), provides additional features not observed in the original study. These unicells are identical to those figured by Smoot and Taylor (1983); they range in diameter from 5–8 μm, and a faint collar surrounds the 1.0 μm diameter discharge pore (Fig. 6D). Additionally, an operculum is visible on a single zoosporangium (Fig. 6D, upper arrow), and a 0.7 μm diameter zoospore is present near a discharge pore (Fig. 6D, lower arrow).

Discussion

Structures do not represent Actinomycetes. Actinomycetes are filamentous bacteria, the taxonomic affinities of which were uncertain prior to comparative approaches employing 16S rRNA, owing to similarities between their morphology and colonial development, with that of the anamorphic phases of some true fungi (Waksman 1950, Stackebrandt and Woese 1981, Fox and Stackebrandt 1987). Actinomycetes are now classified within Actinomycetales, the largest of five orders comprising the Phylum Actinobacteria, which is sister to low G-C, Gram-positive bacteria (Embley and Stackebrandt 1994, Stackebrandt et al. 1997, Zhi et al. 2009). Actinomycete colonies grow via the production of dense vegetative thalli or 'substrate mycelia,' with chains of spores produced via fragmentation of so-called aerial hyphae (Lechevalier and Lechevalier 1967). Almost all actinobacteria have coccoidal or bacilloid spores and branching filaments that are 0.5-1.0 μm in diameter (Lechevalier and Lechevalier 1967, Goodfellow 1983). In extant actinomycetes, filament morphology can vary greatly, ranging from straight, flexuose, or fascicled to mono- and biverticillate; these latter forms may be further elaborated by the presence or absence of spirals, loops, and hooks (Lawton et al. 1989). The shape and arrangement of aerial filaments are commonly utilized in identification of actinobacteria (Hunter-Cevera and Eveleigh 1990).

Morphologically, the Carboniferous structures described by Smoot and Taylor (1983) are similar to extant actinomycetes in terms of the size of individual 'coccoid' elements. By contrast, structures preserved within the *Botryopteris* phloem lack filaments entirely; structures previously interpreted as filaments are in fact aggregates of spheroids. Although of similar size to coccoid spores, these spheroids are neither isodiametric, nor consistent in size. Furthermore, as spore production is most frequently accomplished through septation of aerial filaments (Lechevalier and Lechevalier 1967), the latticelike morphology of the Carboniferous structures is

problematic to an interpretation of these structures as ‘sporulated’ actinobacterial colonies, as no extant actinomycetes produce interwoven filaments.

These Carboniferous structures likewise do not resemble other fossils that have been ascribed to Actinobacteria. Although these bacteria are thought to be exceptionally ancient (Embley and Stackebrandt, 1994, Ventura et al. 2007), their fossil record is sparse. Most body fossils of actinomycetes have been described from amber (Girard and Adl 2011). These include: coccoid spores borne on substrate filaments with simple branching; described from fecal pellets of beetles preserved in Oligocene–Miocene Dominican amber (Poinar 2011); 1.0–2.0 μm coccoid spores from Eocence amber from Washington State (Waggoner 1993); and dark, blue-black 1.0–4.0 μm long filaments with spores 1.0 μm in diameter that are preserved in Eocene–Oligocene Dominican amber (Waggoner 1994a). Older records include filaments up to 6.0 μm long, with 1.0 μm coccoid spores, described from Cretaceous amber, where they occur in association with other prokaryotes (Waggoner 1994b; Saint Martin et al. 2012). The Carboniferous structures described by Smoot and Taylor (1983) were thought to have been the earliest record of actinomycetes in association with vascular plant tissue. As these structures closely resemble neither extant Actinobacteria, nor any of the known fossil actinomycetes (all of which exhibit both filaments, and spores, the latter of which are of uniform diameter and morphology within a specimen), we henceforth refer to them as biomimetic structures (BMS). Thus, a streptomycetelike actinomycete (Wilkinson 2003) from the Eocene (Wilkinson 2003) can now be said to constitute the oldest evidence of Actinobacteria in direct association with plant tissues.

The spheroidal BMS originally interpreted as actinomycetes are in close spatial association with acicular to amorphous structures that Smoot and Taylor (1983) interpreted as

dried mucilage or cytoplasm (Smoot and Taylor 1983). These stalactitic, translucent precipitates can form dense meshes similar to the spheroidal BMS (i.e., Fig. 1F), and in some cases, those that have a filamentous morphology appear to be septate. Close examination, however, reveals that putative septa are fractures ergo these structures are also merely biomimetic. Additionally, the fractures indicate that the substance these BMS are composed of was frangible prior to the permineralization of surrounding plant cells. Because sub-botryoidal and spheroidal 'spore-like' masses are sometimes continuous with acicular BMS (i.e., Figs. 1G, 1H), and filament-like BMS may be found in proximity to larger, oblate or amorphous structures, it is likely that all are composed of the same substance, which we suggest is an authigenic carbonate mineral.

Compositional characterization of the biomimetic structures. Despite their differing morphologies, the BMS preserved within these *Botryopteris* specimens appear to be composed of the same substance, as evidenced by similarities in luminescence (Fig. 2). Luminescence, inclusive of both fluorescence and phosphorescence, is a well-known feature of some organic compounds and many minerals. Luminescence occurs when the electrons of specific trace dopants within the crystal lattice have been excited to a higher energy level, and release a photon upon relaxation to a lower energy state (Marfunin 1979, Gaft et al. 2005). Many of these activator dopants have characteristic emission spectra that can aid in identification of minerals (Gaft et al. 2005, McRae and Wilson 2008). The most common activators are the transition metals Mn^{2+}, Mn^{4+}, Sn^{2+}, Pb^{2+},and Fe^{3+} (Gorobets and Rogojine 2002, Gotze 2002). While several rare earth elements can act as activators (Marfunin 1979, Machel and Burton 1991), luminescence in carbonates is most often attributed to Mn^{2+} cations, as they easily substitute for Ca^{2+} and Mg^{2+} cations, resulting in emission within red wavelengths (Waychunus 1988, El Ali et al., 1993, Gotze, 2002).

Where Mn^{2+} has replaced Mg^{2+} in dolomite, Ca(Mg)CO3, emission spectra peak at 661 nm (McRae and Wilson 2008); it is therefore likely that luminescence observed in these BMS results from interactions between Mn^{2+} and sensitizer dopants in a disordered dolomitic mineral species. The intensity of emission was low, however, such that the BMS appeared nonluminescent using standard epifluorescence microscopy techniques. Although luminescence quenching likely resulted from the presence of Fe^{2+} cations, a variety of factors can result in nonradiative decay, including imperfections in crystal lattice (Marfunin 1979, Gaft et al. 2005). The interactions between quenching dopants, sensitizers, and lattice structure complicate the positive mineralogical identification of carbonates through cathodoluminescent techniques alone (Marfunin 1979, Machel 1985, Machel and Burton 1991, Gaft et al. 2005), but corroboration of the chemical composition of BMS using SEM-EDS indicates that luminescence mapping may be a useful proxy. SEM-EDS analyses indicate the presence of Ca, Mg, and Fe ions within BMS, substantiating the inductive inference that these mineral inclusions are a species of disordered ferrous dolomite.

Biomimetic features of cell wall microstructure. Scalalike protrusions are present within the intercellular spaces of phloem cells, and aspects of their morphology superficially resemble filamentous bacteria or fungi. Comparison with microanatomy of extant plants (e.g., Carr and Carr 1975), however, indicates instead that these features represent intercellular pectic protuberances (IPP). Variously termed pectic filaments, thickenings, scala, strands, and projections, IPP have been noted in the intercellular spaces of eudicots, monocots, and pteridophytes (Carlquist 1956, 1957; Carr and Carr 1975, Potgieter and van Wyk 1992, Veys et al. 1999, Leroux et al. 2007). IPP are composed predominantly of polysaccharides rich in galacturonic acid and in some ferns, may also contain proteins and callose (Veys et al. 1999,

Leroux et al. 2007). They are generally thought to form from middle lamella pectins during the expansion of cells, but may also be laid down later, sometimes in response to stress or wounding (Carlquist 1956, Carr and Carr 1975, Potgieter and van Wyk 1992). The structures observed between phloem cells of *Botryopteris* (Fig. 5) mark the first identification of IPP in a fossil fern; however, similar structures were figured by Williamson and Scott (1894, fig. 31 A-C) in *Calamites*, and a reinvestigation of other Carboniferous fossils is likely to yield further examples.

The IPP between *Botryopteris* phloem cells do differ from the pectic scalae of extant ferns in two respects. First, the regular, scala-like IPP observed in Botryopteris are slightly larger in diameter than those described in other plants (Carr and Carr 1975, Potgieter and van Wyk 1992), and secondly, individual strands are sometimes terminated by spheroidal masses reminiscent of BMS seen elsewhere in the specimen (c.f. Figs. 1H, 5C). The difference in size likely resulted from mineral nucleation upon the original pectin strands (i.e., Fig. 5B, inset), and the spheroidal to sub-botryoidal masses that occur on some strands appear morphologically congruent with disordered dolomite that has been synthesized in the presence of sulfides (Zhang et al. 2010).

Processes of authigenic mineralization. Many common sulfate, silicate, and carbonate minerals can be precipitated in the presence of organic polymers, or as a result of biological processes. The production of authigenic minerals strictly through abiogenic processes is termed organomineralization, while biologically mediated mineral precipitation is identified as biomineralization (Trichet and Defarge 1995). These processes differ in that organomineralization encompasses mineral precipitation in the presence of carbonaceous polymers, whereas biomineralization occurs in the presence of living cells (Trichet and Defarge

1995). Biomineralization is subdivided into mineral formation that is either directly controlled, as in the case of magnetite crystals formed within magnetotactic, microaerophilic bacteria (Bazylinski 1996), or passively induced, which may occur by mineral nucleation on microbial surfaces or extracellular polymeric substances (Ferris et al. 1987, Thompson and Ferris 1990, Fortin et al. 1997, Leveille et al. 2000, Van Lith et al. 2003), or by metabolic processes that alter local hydrochemistry permitting stoichiometric precipitation (Lovley and Phillips 1986, Lovley et al. 1987, Roh et al. 2003, Straub et al. 2004). Biomimetic carbonates within *Botryopteris* may have resulted from a combination of both organomineralization and passive biomineralization.

Although many minerals passively nucleate on microbiogenic surfaces, most can also be precipitated through strictly abiogenic processes, including a number of biomimetic carbonates (Reitner 2004). Humification reactions have been implicated in abiotic precipitation of rhodochrosite, $MnCO_3$, (Hardie et al. 2009), and biomimetic crystals have been experimentally grown in aqueous solutions containing pectin, cellulose ethers, and xanthan (Butler et al. 2008, Zhang et al. 2009, Yang and Xu 2011), and on Langmuir monolayers of stearic acid (Chen et al. 2009). As noted by Smoot and Taylor (1983), the Carboniferous BMS are restricted to phloem cells of *Botryopteris tridentata* (see Fig. 1A), which may indicate that similar soluble organic polymers played a role in mineral nucleation. Similarly, SEM-EDS analyses suggest the presence of carbonate minerals in association with plant cell walls, and components of the cell wall microstructure (the intercellular pectic protuberances) appear to have been nucleation sites for authigenic minerals.

While organomineralization processes may have contributed to permineralization of cell walls, microbial contribution to authigenic mineral formation is implicit in the presence of Mg^{2+}-rich carbonates that form biomimetic structures, as naturally occurring dolomite is known to

readily form at low temperatures only in the presence of microbial activity (Vasconcelos et al. 1995, Wright and Wacey 2004). Bacteriogenic dolomites typically have a distinctive dumbbell-shaped morphology (e.g., Warthmann et al. 2000, Van Lith et al. 2003) similar to some of the spheroidal BMS and the spheroidal masses associated with some IPP. Dolomite is most often, although not exclusively, formed by microbes employing anaerobic sulfate reduction (Warthman et al. 2000, Roberts et al. 2004, Wright and Wacey 2005, Sánchez-Román et al. 2008). The occurrence of pyrite (Fig. 4) within the *Botryopteris* petiole provides additional evidence for the presence of anaerobic sulfate reduction, as euhedral pyrite grains like those present within *Botryopteris* are considered evidence for early diagenetic mineralization resulting from the metabolic activities of sulfate-reducing bacteria (Grimes et al. 2002, McKay and Longstaffe 2002). These pyrite crystals are encrusted with amorphous Mg-enriched carbonates, indicating that mineralization throughout the plant tissue was protracted, and likely progressed through several stages.

We hypothesize that early diagenesis mineralization of the decaying *Botryopteris* tissues began in an anoxic setting, where the metabolic activities of anaerobic sulfate-reducing bacteria facilitated stoichiometric precipitation of disordered ferrous dolomite; soluble organic polymers derived from humic reactions of phloem tissue may have functioned as initial mineral nuclei. Local changes in pore-water chemistry resulting from sulfate reduction may also have favored the precipitation of Mg^{2+}-enriched carbonates along cell walls, the microstructural components of which appear to have acted as nucleation sites. As bacterial proliferation declined, calcium carbonate precipitation was favored, filling cell lumens and intercellular spaces. Thus, individual permineralized plants may be microcosms of the concretions within which they are preserved, as the formation of Carboniferous coal balls is suggested to have occurred in multiple

stages (Scott and Rex 1985, Scott et al. 1996, Diettrich et al. 2000, 2001; Boyce et al. 2001, Scott and Collinson 2003).

Comprehensive investigation of the stages and processes involved in microscale mineralization necessitates an understanding that degrading plant substrates constitute highly localized chemical environments, resulting from combinations of pore-water chemistry, temperature, substrate composition, and the interactions within and among saprotrophic assemblages. Such microbial assemblages are themselves influenced by these extrinsic environmental factors, in addition to oxygen and metal cation availability (Eriksson et al. 1990, Jenkins and Suberkropp 1995, Robertson et al. 2000, Zhou et al. 2002, Kravachenko and Sirin 2007). Characterizing the microbial paleoecology of fossil plant substrates, then, is an obvious first step in understanding early diagenetic processes at the tissue level, as the activities of saprotrophic organisms were intimately tied to the chemical regimes under which mineralization occurred.

Chytridiomycete fossils indicate diversity of saprotrophic assemblage. Of the many saprotrophic organisms identified in modern rhizospheres, fungi, like bacteria, are ubiquitous agents of biodegradation and nutrient cycling (Goodfellow 1983, Newell 1996, Dighton et al. 2005). The association of legitimate microbial remains with degraded *Botryopteris* tissue indicates that the anaerobic degradation of these plant remains need not have been accomplished by sulfate-reducing bacteria alone, as some extant free-living chytrids also engage in anaerobic fermentation (Emerson and Natvig 1981).

Although figured by Smoot and Taylor (1983), the unicells preserved within some *Botryopteris* cells were not attributed to a microbial group. Here, we interpret them as holocarpic, monocentric chytridiomycete zoosporangia. Although small, the size of these fossils

accords with that of some other fossil chytridiomycete zoosporangia (Taylor et al. 1992, Krings et al. 2009a, 2009b). Traditional classifications of chytrids that employ morphological and developmental features of zoosporangia are known to be artificial, and these characters are acknowledged to be of little use in higher-level taxonomy (Blackwell et al. 2006, James et al. 2006b). The thallus is holocarpic, and the presence of an annular collar provides evidence that zoospores were released via an operculate discharge pore as in other monocentric chytrids, in which thallus development occurs through enlargement of an encysted zoospore (Beakes et al. 1992). We are unable to characterize aspects of zoospore development and morphology (i.e., flagellae), however, precluding further identification of the fossils at this time.

The external surfaces of the zoosporangia are covered by fine precipitates, which are continuous with those that occur on the surface of associated plant cell walls. As the zoosporangial walls of extant chytrids are typically smooth (Longcore 1995), these precipitates are unlikely to reflect cell ornamentation, and we interpret them instead as authigenic minerals. As precipitates are less prevalent near the discharge pore, local mineralization probably commenced prior to removal of the operculum by zoospore discharge. As such, these fossils not only provide evidence for the diversity of microbes involved in the taphonomy of fossil plants, but demonstrate that some stages of mineralization were likely to have been synchronous with microbial proliferation.

Further research. Biomimetic structures similar to those examined in *Botryopteris tridentata* may be ubiquitous in permineralized plants. For instance, Rothwell and Taylor (1972) have noted small dark masses that are similar in size and arrangement to the BMS identified here, as have Andrews and Lenz (1943, fig. 6), who describe such structures as mycorrhizal haustoria. During this study, we reexamined these latter structures, and found that they are

visually indistinguishable from those preserved in the phloem of *Botryopteris*. Similarly, material which has been identified as possible callose in a number of Carboniferous seed ferns, including *Callistophyton boyssetii* (Renault) Rothwell (1980), *Schopfiastrum decussatum* Andrews (1945), *Medullosa pandurata* Stewart (1951), and *Callistophyton poroxyloides* Delevoryas et Morgan (1954), may in fact be additional examples of bacteriogenic ferrous dolomite, and this possibility bears reinvestigation. Because many of these figured specimens were prepared as cellulose acetate peels, the use of monochromatic luminescence mapping may be preferable as a nondestructive alternative to SEM-EDS compositional analyses.

Conclusion

Expanding the known microbial fossil record in association with plant remains is essential to furthering our understanding of the diverse roles of microbes in ancient ecosystems, but as this study demonstrates, the biogenicity of putative body fossils must be definitively established, as early diagenetic processes can produce biomimetic pseudofossils. Inclusions within the phloem of a Carboniferous fern, *Botryopteris tridentata*, although originally interpreted as filamentous actinomycete bacteria, are in actuality disordered ferrous dolomites. The carbonate mineralogy of these inclusions was inferred from critical examination of morphology and monochromatic luminescence mapping, and was corroborated by SEM-EDS, which identified magnesium, calcium, and iron within the biomimetic structures. The precipitation of these structures within cell lumens is likely to have been biologically mediated by sulfate-reducing bacteria, and may also have involved organomineralization around nuclei of soluble organic polymers. The presence of similar mineral morphologies in association with intercellular pectic protuberances suggests that the chemical and biological regimes that resulted in the biomimetic structures also contributed to permineralization of the surrounding plant tissue.

Although we hypothesize that the metabolic activities of anaerobic sulfate-reducing bacteria were of primary importance in producing local hydrochemical environments favoring the precipitation of dolomite over calcite (and thereby contributing to the preservation of plant tissue), it should be noted that other microbial remains—to wit, uniporate cells which we interpret as holocapric chytridiomycete zoosporangia—are also present within these plant tissues. As such, the suite of anaerobic microorganisms involved in microbial preconditioning of plant tissues prior to, and concurrent with, the precipitation of authigenic minerals was likely to have been diverse. Further characterization of biomimetic carbonates in association with permineralized plants may provide insight into the microbial paleoecology of these ancient environments, by allowing more sophisticated inferences as to the metabolic strategies of associated fossil microbes. Such investigations are also likely to afford novel opportunities to characterize the hydrogeochemistry of early stages of diagenesis, thereby expanding our understanding of taphonomic controls and processes within the paleobotanical record.

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Figures

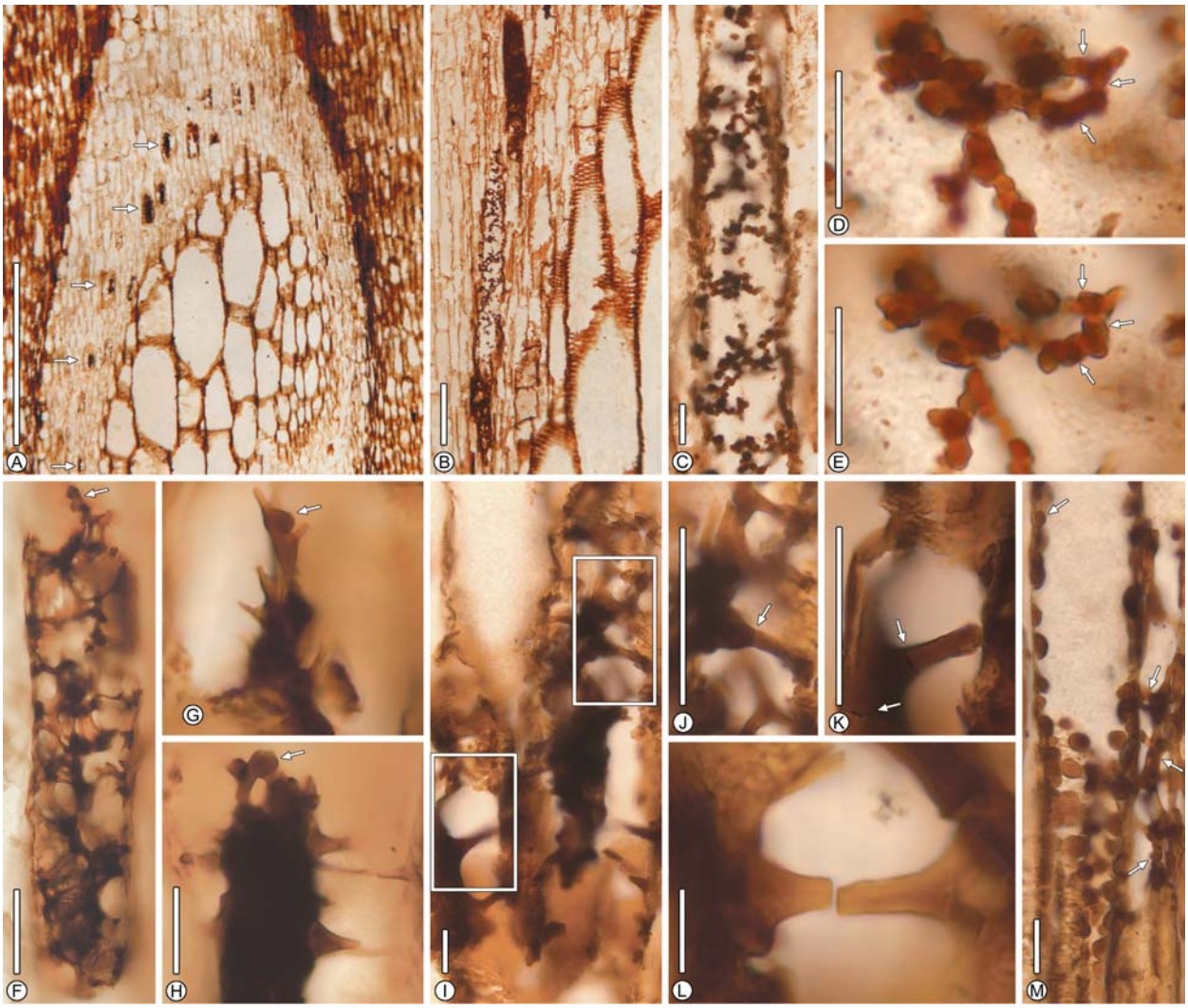

Figure 1: Photomicrographs of biomimetic structures (BMS) preserved within phloem mucilage cells of *Botryopteris tridentata*. A) Oblique transverse section of *Botryopteris tridentata* petiole; arrows = BMS, Slide No. 7523, scale

500 μm. B) BMS originally interpreted as actinobacteria; Slide No. 7563, scale 100 μm. C) Higher magnification of BMS originally interpreted as actinobacteria; Slide No. 7563. D–E) Spheroidal BMS viewed in different focal planes to demonstrate optical illusion of filamentlike morphology. Note that putative filaments are composed of aggregated spheroids (arrows); Slide No. 7556. F-H) Acicular aggregated BMS originally interpreted as dried mucilage or protoplasm. Arrows denote spheroids similar to the 'actinobacteria-like' BMS; F = Slide No. 7525, G–H = Slide No. 7523. I) Acicular BMS with features resembling septa. Upper box = Fig. J; lower box = Fig. K; Slide No. 7556. J–K) Magnification of putative septa in acicular BMS demonstrates that these features are fractures (arrows); Slide No. 7556. L) Fractured BMS; Slide No. 7523, scale M) Spheroidal to oblate BMS, in association with spheroidal (upper- and lowermost arrows) and acicular BMS (medial arrows); Slide No. 7554, all scales C–M 10 μm.

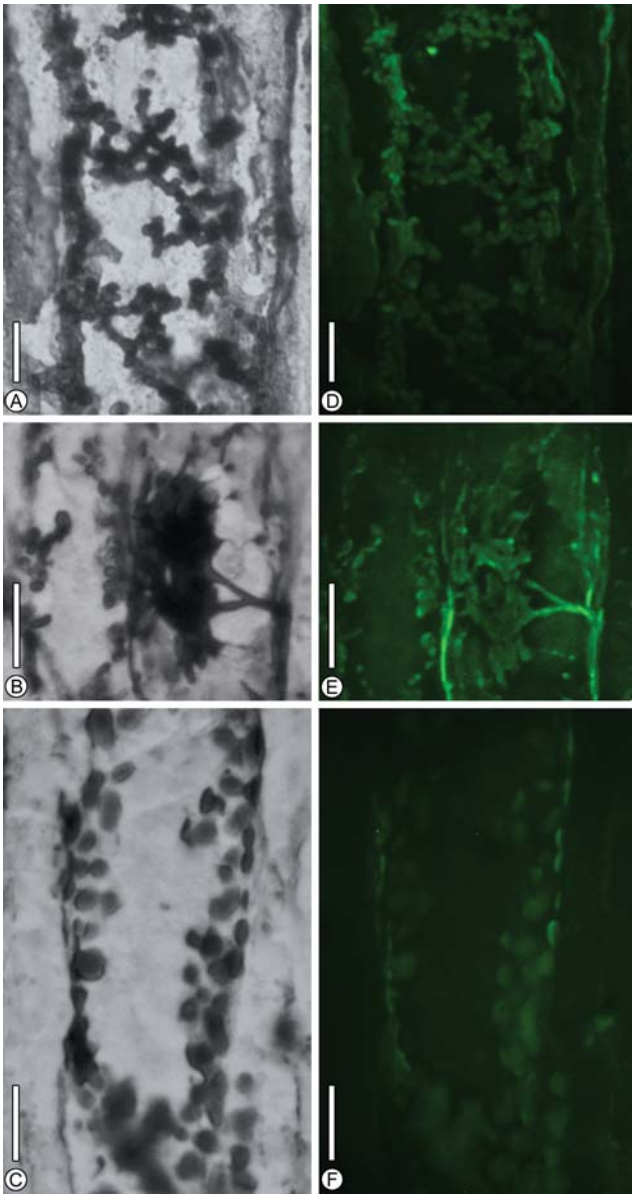

Figure 2: Monochromatic mapping of luminescence in biomimetic structures. A–C) Bright field image of BMS corresponding to monochromatic maps of luminescence. D–F) Monochromatic maps of emission spectra captured at 665 nm. A, D = Slide No. 7563; B, E = Slide No. 7523; C, F = Slide No. 7523, all scales 15 µm.

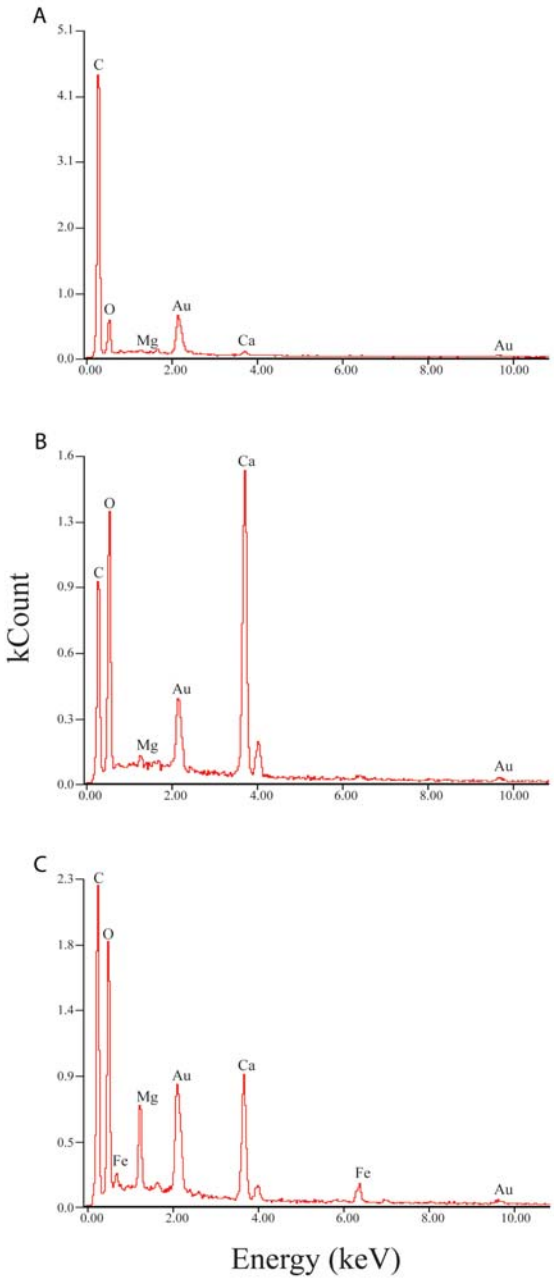

Figure 3: Representative spectra from scanning electron microscope energy-dispersive X-ray spectrometry (SEM-EDS) of cellulose acetate peels of *Botryopteris tridentata*. A) Cell wall. B) Intracellular cement. C) Spheroidal biomimetic structure.

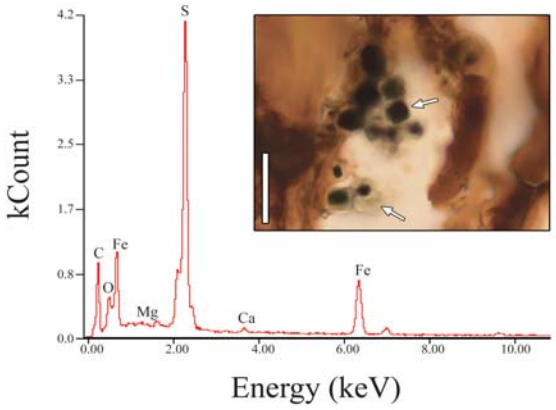

Figure 4: Representative SEM-EDS spectrum of pyrite within *Botryopteris tridentata*. Inset, photomicrograph of euhedral pyrite (upper arrow) encrusted with carbonate minerals (lower arrow); Slide No. 7563. Scale 10 μm.

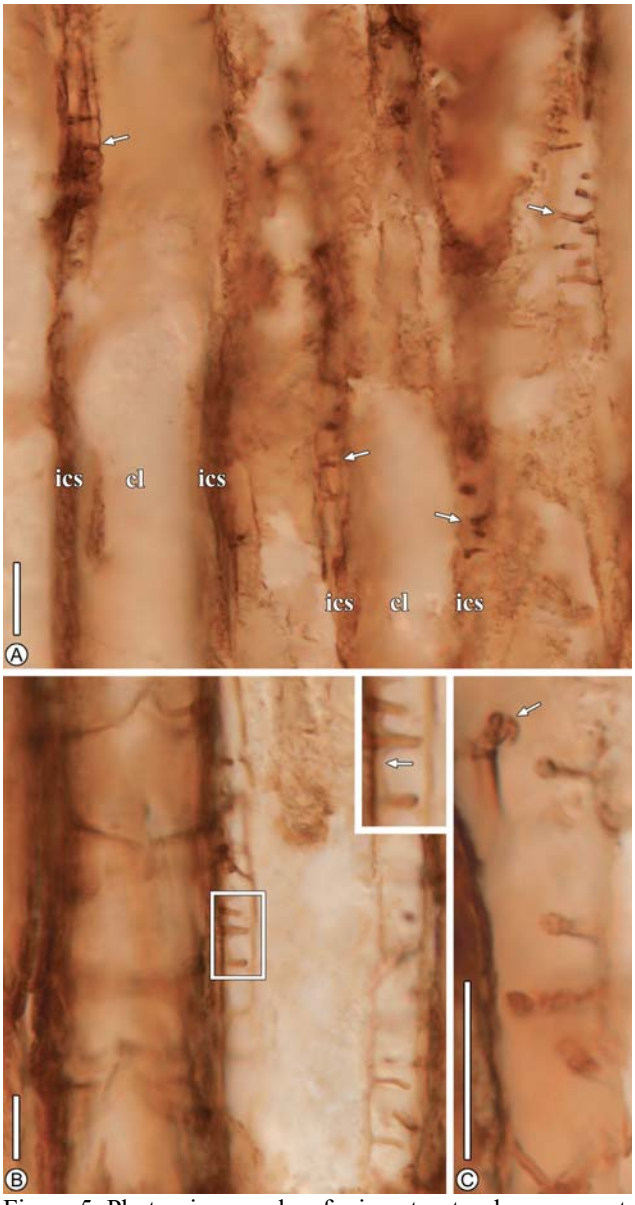

Figure 5: Photomicrographs of microstructural components of degraded phloem cells. A) Scala-like structures (arrows), interpreted as intercellular pectic protuberances (IPP), cl = cell lumen, ics = intercellular space; Slide No. 7523. B) Distribution of IPP in degraded middle lamella of mucilage cell (right) adjacent to phloem parenchyma cells (left). Magnification (inset) shows a mineral halo surrounding some IPP (arrow); Slide No. 7523. C) IPP with subtended by terminal masses morphologically similar to spheroidal BMS (arrow); Slide No. 7523, all scales 10 µm.

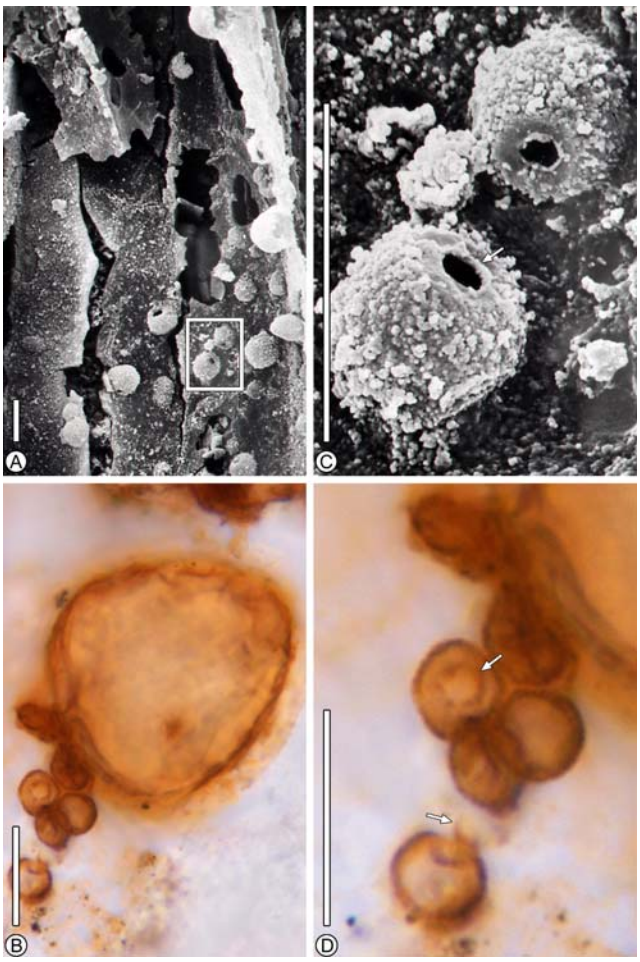

Figure 6: Microbial body fossils associated with *Botryopteris tridentata*. A) Reimaged SEM micrograph of zoosporangia in degraded phloem cells; Box = Fig. 3B. B) Magnification of Figure 3A. Note collar surrounding pore (arrow) and mineral precipitates on cell surfaces. C) Photomicrograph of zoosporangia clustered adjacent to fern spore near *Botryopteris* petiole; Slide No. 7523. D) Magnification of clustered cells. Note operculum (upper arrow) and discharged zoospore (bottom arrow); Slide No. 7523, all scales 10 µm.

Chapter 3: Fossil hyphomycetes associated with the early Eocene aquatic angiosperm, *Eorhiza arnoldii*

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Abstract

The Eocene (~48.7 Ma, Ypresian–Lutetian) 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 have also 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 multiseptate, holothallic, chlamydospore-like phragmoconidia most similar to extant *Xylomyces giganteus*, and basipetal phragmospore-like chains of amerospores like those of extant *Thielaviopsis basicola*. We also describe a third hyphomycete that has not been previously recognized from this locality; biseptate, chlamydosporic phragmoconidia are distinguished by darkly melanized, inflated apical cells, and are morphologically similar to *Brachysporiella rhizoidea* or *Culcitalna achraspora.*

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 are also subject to permineralization (Taylor et al. 2005, Dotzler et al. 2008), the Princeton Chert constitutes not only a preeminent 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 thirty years, 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 (Stockey 1984, Rothwell and Basinger 1979, 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 (Cevallos-Ferriz and Stockey 1988a, Erwin, 1987, Erwin and Stockey1989, 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). There are also several flowering plants that 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). Previous 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 tuckerae* 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) may 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 were originally 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 prior to 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 #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 cm^2 samples, which were then mounted on glass slides using Hillquist Two Part mounting medium (Hillquist, USA). Serial paleontological thin sections were cut using a Buehler Petrothin®; sections ranged in thickness from 50–150 μm. Photomicrographs were captured directly from the rock surface under oil immersion, using 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, KS) (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 preferentially developed within locules, or intercellular spaces, of cortical aerenchyma, (Fig. 7A), 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 in length and 7–10 μm in diameter, with as many as 30–35 transverse septa (Figs. 7A, 7B). Observation of subtending hyphae at conidial apices and bases (Fig. 7B, at arrows) indicates that conidiogenesis was holothallic. Although some conidial cells exhibit constriction or contraction of the conidial wall (Fig. 7C, at arrow), these features are not regular, and do not occur in most conidia (Figs. 7B, 7D). 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 (Figs. 7D, 7E, 7F), with individual multiseptate chlamydosporous conidia produced from sparsely branched, 2.5–3.0 μm diam, micronematous conidiophores (Figs. 7E, 7F,

7G), indistinct from mycelial hyphae (Fig. 7F). Conidial secession is rhexolytic (Figs. 7E, 7F), and dispersed conidia may bear remnants of the subtending cell (Fig. 7F, lower arrow).

Type II. This hyphomycetous anamorph exhibits propagation via unequally pigmented, apically dematiaceous chains of amerospores resembling clavate phragmospores; basipetal holoblastic chains are 15–25 µm long and 8 µm diam, with 3–6 transverse septa (Figs. 8A, 8B). Simple septal pores are present between successive cells (Fig. 8B, inset). The conidiogenous cells are 4 µm in diameter and elongated, ranging in length from 5–9 µm; some are ampulliform (Fig. 8A, at left arrow), although most are doliiform. Secession is schizolytic, sometimes occurring at the base of conidiogenous cells, which may remain attached to the dispersed spores (Fig. 8A, 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 (Figs. 8A, 8C). It also appears that multiple conidia were produced from the same conidiogenous cell (Fig. 8C, at arrow), and the conidiogenous locus is therefore indeterminate.

Type III. The 20–25 µm long biseptate, dematiaceous, pyriform phragmospores of this hyphomycetous anamorph (Figs. 8D-G) are characterized by an apical cell which is deeply pigmented when mature (Figs. 8D, compare to Fig. 8F), and markedly inflated, up to 15 µm in diameter. Conidiogenesis is acrogenous and monoblastic. Conidiogenous cells are isodiametric, 5 µm wide, globose (Figs. 8D, 8F), and are retained at the base of the dispersed conidium (Fig. 8E) as a consequence of schizolytic secession from the micronematous conidiophore (Figs. 8D, 8F, at arrows). Some hyphae in close association with spores produce curved branches oriented towards associated assimilative mycelia, the hyphal diameters of which range from 2.5–3 µm (Fig. 8G, 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, as the peel technique is not optimal for recovery of fungal remains (Taylor et al. 2011). Our reinvestigation of *Eorhiza arnoldii* using 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, as many can be attributed to extant lineages (Pirozynski 1976, Pirozynski and Weresub 1979). Therefore, the aim of this study is two-fold: 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 were originally 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. Figs. 7E, 7F), and some also exhibit attachment to distal hyphae (e.g. Fig. 7B, 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 any close affinity with extant genera like *Eriocercosporella* 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 *Scolecosporites* 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 that 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: Jahnulales: Aliquandostipitaceae), a genus of saprotrophic aquatic hyphomycetes that produce thick-walled, dematiaceous, multiseptate chlamydospores (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 chlamydospores of *X. chlamydosporus* 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 chlamydospore 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) chlamydospores, 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 chlamydospores, as conidiogenesis in Type II is holoblastic and the branching conidiogenous cell is terminal upon micronematous conidiophores. Several extant genera produce terminal cylindrical to clavate phragmospores from sympodial conidiogenous cells. It is

possible to exclude *Marielliottia* Shoemaker, as 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 are also apically inflated, 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 amerospores 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 amerospores from obvious phialides (Nag Raj and Kendrick 1975). The dematiaceous conidia, in comparison, are aleuriosporic, and because the cells do not readily undergo schizolytic secession, they often remain attached to hyphae in chains resembling phragmospores (Ellis 1971). Conidiogenesis of aleuriospores from indeterminate loci in *Thielaviopsis* closely resembles the condition seen in some fossil specimens (e.g. Fig 8C). Of the four extant species of *Thielaviopsis* that produce aleurioconidia, the fossils most closely resemble *T. basicola* (Berk. & Br.) Ferr., as 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. 8G) 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 phragmosporic 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 allow us to

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.

Conclusion

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 previous studies (Robison and Person et al. 1973, 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 recently 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 a *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, as several fungal anamorphs are present within most specimens. Additionally, 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* may have been infected by a pathogenic fungus during life, as 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 preferentially produced within intercellular spaces of the cortical aerenchyma, the host tissue was probably 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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Figures

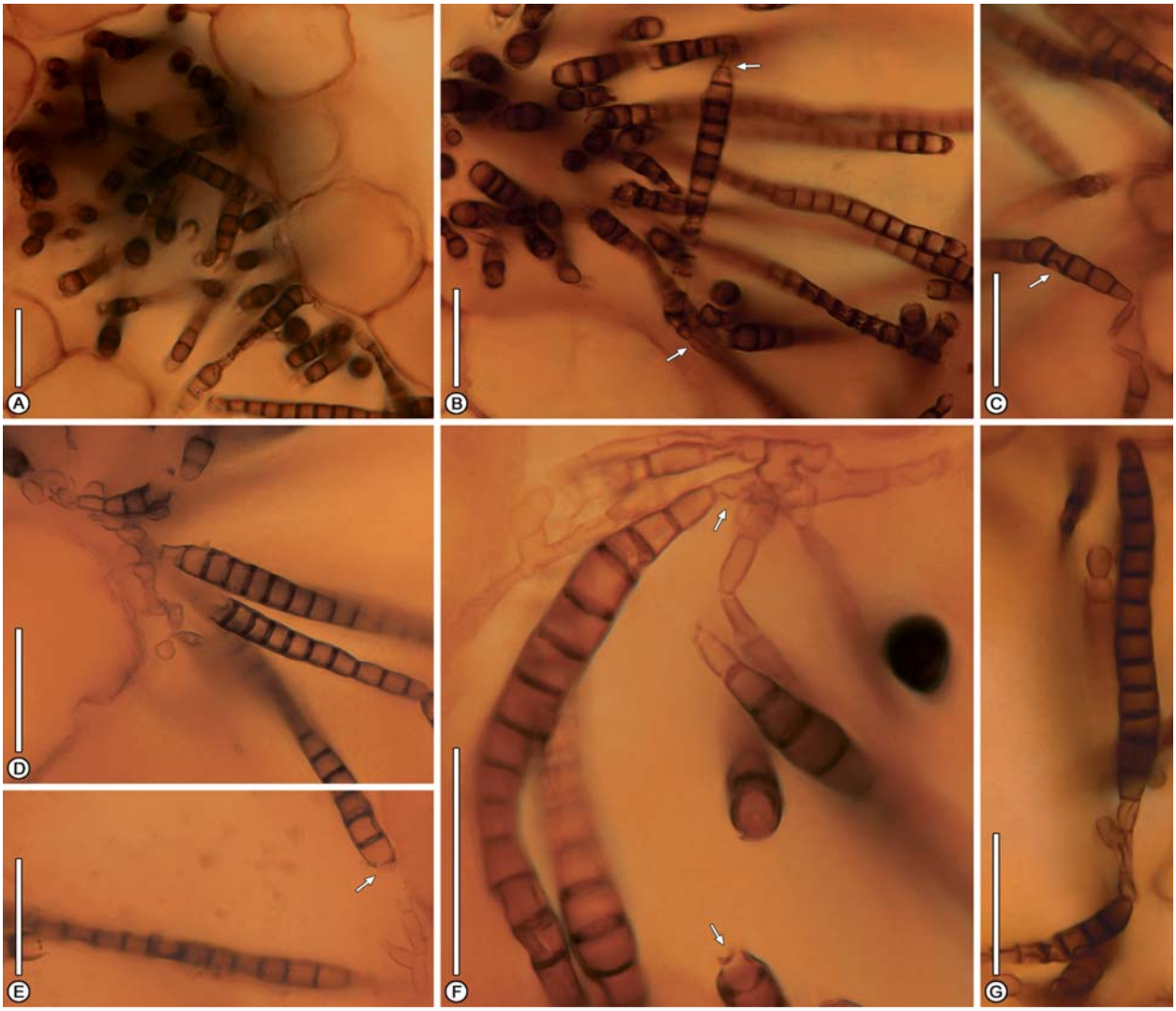

Figure 7: Type I fossil hyphoymycete in *Eorhiza arnoldii*. A,B. Holothallic multiseptate chlamydospores occurring within intercellular spaces in cortical aerenchyma of the middle Eocene vascular plant *Eorhiza arnoldii*. Note subtending hyphae in FIG. 7B (arrows). C. Chlamydospores attached to hyphae; 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 Fbot #001; B, C, D, E, F 17035 Ebot #001; Scale bars = 25 μm.

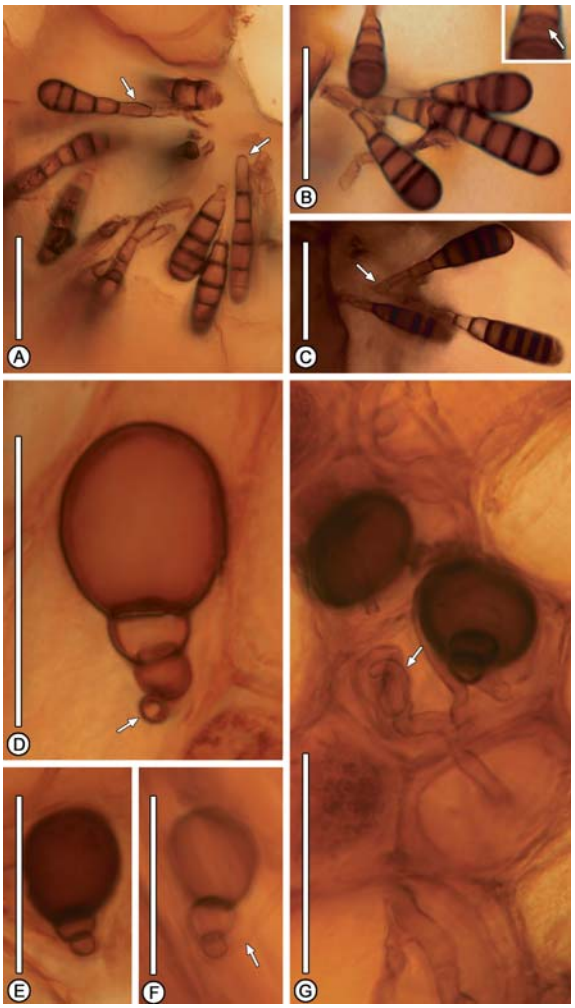

Figure 8: Type II and III fossil hyphomycetes in *Eorhiza arnoldii*. A–C. Type II fossil hyphomycete. Apically pigmented, phragmospore-like aleuriospores with simple pores (B, inset, at arrow), produced from typically doliiform, but occasionally ampulliform (A, at left arrow) conidiogenous cells. Dispersed 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, biseptate phragmosporic chlamydospores 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. Scale bars = 25 μm.

Chapter 4: Dark septate fungi in the aquatic angiosperm *Eorhiza arnoldii* indicate a diverse assemblage of root-colonizing fungi during the Eocene

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Abstract

Tissues of the extinct aquatic or emergent angiosperm, *Eorhiza arnoldii* incertae sedis, were extensively colonized by microfungi, and in this study, we report the presence of several types of sterile mycelia. In addition to inter- and intracellular proliferation of regular septate hyphae, the tissues contain monilioid hyphae with intercalary branching. These filamentous mycelia are spatially associated with two distinct morphotypes of intracellular microsclerotia. These quiescent structures are morphologically similar to loose and cerebriform microsclerotia found within the living tissues of some plants, which have been attributed to an informal assemblage of dematiaceous ascomycetes, the dark septate endophytes. While there are significant challenges to interpreting the ecology of fossilized fungi, these specimens provide evidence for asymptomatic endophytic colonization of the rooting structures of a 48.7 million year old aquatic angiosperm.

Introduction

Fungi are major ecological drivers in extant plant communities, where they play vital roles in decomposition and nutrient mobilization (Cromack and Caldwell 1992, Hoffland et al. 2004), and contribute to niche partitioning and plant species diversity (Gustafson and Casper 2006, Vogelsang et al. 2006). Mutualistic relationships with fungi are thought to have been integral to

the colonization of land by plants (Pirozynski and Mallock 1975, Humphreys et al. 2010, Bidartondo et al. 2011); in the subsequent ~450 million years, intricate associations have evolved, ranging from obligate mutualism through commensalism, parasitism and pathogenicity. A substantial number of vascular plants are also host to internal fungal biota with which they form neither typical mycorrhizal associations, nor produce responses associated with infection (Saikkonen et al. 1998, Jumpponen 2001). There is evidence that relationships between vascular plants and fungal endophytes occur within a continuum: endophytic fungi actively derive carbon from hosts (Barrow 2003), and their presence may inhibit herbivory (Saikkonen et al. 1998) and increase drought tolerance (Rodriguez et al. 2008), but there is also evidence for mutual antagonism between endophytes and hosts (Schulz et al. 1999), and these fungi are known to become weak pathogens or saptrophs with the decline of host plants (Schulz and Boyle 2005). Consequently, the ecological functions of endophytic fungi are of interest, particularly as they are often observed in plants growing in stressed or marginal habitats (Barrow 2003, Newsham 2011), where they may be more common than arbuscular mycorrhizal fungi (Read and Haselwandter 1981, Mandyam and Jumpponen 2005).

Interpreting the ecological role of fungi in the fossil record is a significant challenge. In some instances, there is anatomical or structural evidence that interactions between fossil fungi and host plants were mycorrhizal (Remy et al. 1994), or pathogenic (LePage et al. 1994). Given that a hallmark of an ascomycteous or basidiomycetous endophyte is asymptomatic persistence within a host, there is no proximal method by which to differentiate a fossil endophyte from a saprotroph, particularly as endophytic microfungi can persist as saprotrophs upon the death of their host (Menkis et al. 2005). Ecological interpretations of fossils must therefore take into account secondary lines of evidence, which include the taphonomic profile of host tissue,

systematic affinities of fossils, and associational data. This task is further complicated by the tendency of some fungi, particularly within Ascomycota, to exhibit multiple conidial and mycelial anamorphs (Seifert and Samuels 2000). In this study, we describe several sterile structures systemically distributed within the rhizomes of an aquatic angiosperm, *Eorhiza arnoldii* Robison et Person. We interpret these fungal fossils to as monilioid and regular simple-septate sterile hyphae, which are in spatial association with two types of intracellular microsclerotia. These Eocene fungi are similar to the extant ascomycetes commonly referred to as dark septate endophytes (DSE, Stoyke and Currah 1991), which inhabit the rhizosphere and living tissues of some vascular plants.

Materials and Methods

Fungal body fossils described in this study occur within tissues of the extinct aquatic or emergent angiosperm *Eorhiza arnoldii*, which is known from anatomically preserved vegetative organs (Stockey and Pigg 1994) present in many of the individual bedding planes that constitute the Princeton Chert locality of southern British Columbia, Canada (UTM 10U 678057 5472372; 49°22′40″ N, 120°32′48″ W). This well-known paleobotanical locality comprises 49 layers of silicified peat interbedded with sub-bituminous coal; it has been K-Ar dated to ~48.7 Ma, and is thus latest Ypresian to earliest Lutetian in age (Smith and Stockey 2007, Mustoe 2011, Klymiuk et al. 2013).

Slabs of chert containing *E. arnoldii* rhizomes were selectively sectioned into 3–5 cm^2 samples, and mounted on glass slides using Hillquist Two Part mounting medium (Hillquist, USA). Serial thin sections ranging in thickness from 50–200 μm were cut with a Buehler Petrothin®. Serial photomicrographs, taken at different focal planes, were captured directly from the rock surface under oil immersion, using a Leica DC500 CCD attached to a Leica

DM5000B transmitted-light compound microscope. Photomicrographs were compiled as composite focal-stacked images, optimizing visualization of specimens in z-space (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, KS), under specimen accession numbers 17030 B_{bot} #001, 17030 C_{bot} #001, 17035 E_{top} #002, 17035 E_{bot} #002, 17035 F_{bot} #001, 17037 F_{bot} #001, and 17040 B_{bot} #001.

Results

Monilioid hyphae. Chains of dematiaceous monilioid cells, 12–14 μm long by 7–8 μm diam, are produced from acutely branched, melanised regularly septate hyphae, 2–5 μm diam, which exhibit septation ~10 μm below the branching point (Fig. 9A). Smaller monilioid cells occasionally occur at hyphal apices (Fig. 9B, at arrow) which may indicate blastic yeast-like proliferation, but in many monilioid chains the individual hyphal elements do not show as much constriction at septa (Fig. 9C, at lower arrow), which suggests formation by isodiametric enlargement of subdividing cells. A few monilioid cells also exhibit what appear to be inconspicuous lateral scars (Fig. 9C, at upper arrow), but could be taphonomic artifacts. Intercalary branching within monilioid hyphae is occurs frequently, and there is obvious septal constriction of cells at branching loci (Figs. 9D, E, H).

The regularly septate hyphae from which monilioid cells are initially produced may remain micronematous (Figs. 9A, D), or hyphal elements may be somewhat inflated, up to 7–8 μm diam (Figs. 9C, G). Within the pith of some *E. arnoldii* specimens, regular hyphae are absent or rare, and proliferation of monilioid hyphae is extensive (Figs. 9E, F). This is in contrast to the cortex, where regular hyphae are frequently associated with monilioid growth (Figs. 9A–D, G–I),

and also contrasts with the distribution of other fungal remains previously observed within these plants (Klymiuk et al., 2012), which are likewise restricted to cortical tissues.

Loose microsclerotia. Monilioid hyphae are in close spatial association with clusters of monilioid cells that are constrained to host parenchyma cells (Figs. 9H, I). In this manner, aggregations of monilioid cells form loose microsclerotia, up to 65 μm long by 25 μm wide, that show no evidence of differentiation into rind or medullary zones (Fig. 9I). Microsclerotial initiation occurs via the production of monilioid cells constrained to the host parenchyma cell (Fig. 9H), and proceeds until the host cell is filled. Initiation occurs from normal hyphae (Fig. 1H, at arrow), but closely associated inflated hyphal elements (Fig. 9I, at arrow) suggest that microsclerotia may also develop concurrent with growth phases in which monilioid hyphae predominate.

Cerebriform microsclerotia. Densely interwoven hyphal strands form cerebriform microsclerotia ranging from 20–45 μm in diameter (Fig. 10). They are differentiated into medullary and rind zones; the rind is typically composed of a single layer of melanised hyphae, which are narrower in diameter than the medullary hyphae (Fig. 10A). External surfaces of these microsclerotia are undulating or ridged (Figs. 10B, C). Microsclerotia are associated with branching septate hyphae (Figs. 10A, C), and may be connected to one another by septate hyphal stolons (Fig. 10D, at arrow).

Other sterile mycelia in host tissue. In addition to catenulate monilioid hyphae and two microsclerotial morphologies, the cortical tissues of *Eorhiza arnoldii* exhibit extensive intracellular proliferation of assimilative mycelia (Fig. 11A). Hyphae, 2–5 μm in diameter, pass through cell walls as microhyphal strands, 0.25–0.5 μm wide (Fig. 11B, at arrow), without eliciting any obvious host response. In addition to dense intracellular assimilative networks, in

some specimens hyphal growth appears to respond to the architecture of host tissue, in that hyaline to slightly pigmented hyphae are entirely constrained to intercellular spaces (Fig. 11C). Finally, larger 8–10 μm wide septate hyphae may form loose coils which fill the lumen of host cells (Figs. 11D, E). Short, 6 μm by 8 μm, distinctively lobed or invaginated fungal propagules (Fig. 11F) also occur within several outer cortex cells of a single *E. arnoldii* specimen, and are not in close proximity to hyphae. In addition to knobby lobes, these cells are also characterized by the presence of a medial, light-coloured, peg or dot-like structure (Fig. 11F, at arrow), interpreted here as penetration pegs.

Discussion

Monilioid hyphae. In an early review of known Princeton Chert fungi, LePage et al. (1994) observed several monilioid cells within cortical tissues of *Eorhiza arnoldii*, and suggested that they bore a strong resemblance to monilioid hyphae of *Rhizoctonia* DC. To date, relatively few examples of monilioid hyphae have been recognized as such within the fossil record, with the notable exception of the Permian palynomorphs *Reduviasporonites*, which have been interpreted as *Rhizoctonia*-like sclerotia (Visscher et al. 2011). I t is probable that this paucity within the fossil record results from conflation of monilioid hyphae with conidiogenesis.

Palynological maceration techniques may disarticulate chains of cells, and unicellular fungal propagules are typically regarded as amerospores by palynologists (Kalgutkar and Jansonius 2000). For example, the palynological form genus *Haplographites* Felix is used for moniliform chains of ellipsoidal unicells, and despite the absence of diagnostic features of conidiogenesis, these cells are considered amerospores (Kalgutkar and Jansonius 2000, O'Keefe et al. 2011). Similarly, Krings et al. (2009) interpret short chains of spherical to ovoid cells as amerosporic conidia, but supposed conidiogenous loci are undifferentiated (micronematous),

hyphae are not uniformly present in proximity to clusters of cells, neither ramoconidia or connectives are present, and the cells are irregularly arranged in three dimensions within plant cells.

An interpretation of the monilioid cells preserved within *Eorhiza arnoldii* as hyphomycetous conidia can be confidently dismissed. Among extant fungi, amerosporic microconidia of aspergilloid or penicilloid fungi are typically produced from fixed, phialidic conidiogenous loci, whereas the fossil cells do not arise from obvious conidiogenous cells or conidiophores. While catenulate macroconidia are produced from micronematous conidiogenous loci by some species of *Monilia* Bonord., *Phaeomonilia* R.F. Castañeda, Heredia & R.M. Arias, *Seifertia* Partr. & Morgan-Jones, and *Sorocybe* Fr., these genera tend to have conidiomata that are sporodochial, or formed of distinctive, macronematous hyphae (Seifert et al. 2011). Although catenulate conidia of *Cladosporium* Link and *Toxicocladosporium* Crous & U. Braun do resemble the monilioid cells observed in this study, members of these genera also produce numerous septate ramoconidia (Sivanesan 1984, Crous et al. 2007, Seifert et al. 2011). In the fossils, intercalary branching does not result in the production of septate ramoconidia.

We thus concur with LePage et al (1994), in that these chains of fungal cells are monilioid hyphae. However, as *Rhizoctonia* was originally erected as a form genus for soil-borne pathogens and endophytes, and is now understood as a polyphyletic assemblage (Moore 1987, Anderson and Stalpers 1994, García et al. 2006) the taxonomic affinities of these fossil fungi bear reassessment. By examining *E. arnoldii* tissue in palaeontological thin section, we have been able to observe numerous examples of these monilioid hyphae, and it is now apparent that their proliferation through host tissue is frequently extensive. Furthermore, we now know that the monilioid hyphae are produced from simple-septate, acutely branching regular hyphae,

and also occur as loose microsclerotia. These new data suggest that these fossils are unlikely to represent *Rhizoctonia* s.s. or other basidiomycetous species previously classified within the morphotaxon: the regular hyphae associated with the monilioid hyphae tend to be smaller in diameter (<5 μm) than those of *Rhizoctonia* s.l.; they lack clamp connections and dolipore septa; there is no evidence for orthogonal branching in any of the assimilative mycelia observed; and finally, the infection process in *Rhizoctonia* s.l. involves the production of profusely branching masses of hyphae (Parmeter and Whitney 1970, García et al. 2006), whereas only hyphopodium-like cells (FIG. 3F) have been observed in association with the fossils. On the basis of these morphological characters, it is unlikely that the fossils share an affinity with the basidiomycetous *Rhizoctonia*-like soil pathogens, but hyphal features alone do not permit us to more precisely identify them, especially as monilioid growth is common to many fungi that colonize vascular plants, including both plant pathogens and endophytes (Parmeter and Whitney 1970, Melin 1923, Currah et al. 1988). Unambiguous identification of sterile mycelia in living fungi depends upon observing their association with conidial or sexual phases, characterising substrate utilization, or molecular taxonomy (Addy et al. 2005, García et al. 2006).

Microsclerotia. Survival anamorphs, which include aleuriospores, chlamydospores, and sclerotia, represent dormant or quiescent stages of fungal life cycles; they are produced by microfungi in response to changing environmental conditions, and function in long-term survival and dispersal of microfungi (Chet and Hennis 1975, Willets and Bullock 1992, Willets 1996, Siefert and Samuels 2000). Sclerotia in particular have been extensively studied, and are known to be produced in response to accumulation of metabolic staling products, changing temperature and light regimes, and mechanical trauma to the vegetative mycelium (Chet and Hennis 1975). The two types of microsclerotia found within *Eorhiza arnoldii* are consistent in size and

morphology with true, anatomically differentiated sclerotia (Fig. 10) and with the undifferentiated monilioid sclerotia (Figs. 9F-G) produced by non-clavicipitaceous endophytic fungi (Chet and Hennis 1975, Willets 1997, Rodriguez et al. 2009).

The development of true sclerotia is typified by three stages: the formation of sclerotial initials from interwoven hyphae is followed by an increase in size and septation of hyphal initials to form the medulla; as the developing sclerotium matures, the pseudoparenchymatous exterior surface, or rind, thickens and becomes melanised (Chet and Hennis 1975, Willets and Bullock 1992, Erental et al. 2008). The cerebriform microsclerotia present within some rhizomes of *Eorhiza* are fully mature, as the tightly adpressed hyphae forming the rind are deeply pigmented in comparison to vegetative hyphae with which the microsclerotia are associated (Fig. 10). Coiled and interwoven strands of hyphae that occur within some host cells (Figs. 11D, E) may represent initial stages in sclerotial development, but they do not occur in close proximity to mature sclerotia, and intermediate forms have not been observed.

Cerebriform microsclerotia have not been extensively reported in the literature. This sclerotial morphology is best known in association with slow-growing colonial ascomycetes called 'meristematic fungi', which are predominantly found on rock, including marble buildings and monuments. They usually proliferate by short hyphal stolons, although yeast-like phases have been observed (Sterflinger et al. 1999, Sterflinger 2006). Phylogenetically, these fungi are members of orders that contain saprotrophic and plant pathogenic black yeasts (Ruibal et al. 2009). Cerebriform microsclerotia, however, are reported only rarely within plants, but are probably common as they have been observed within a broad taxonomic range of hosts (Hambleton et al. 2003, Ahlic and Sieber 2006, Fernandez et al. 2008).

Unlike the fossil cerebriform microsclerotia, which are invariably born from regular hyphae, the dense aggregations of monilioid cells that completely fill the lumens of host plant parenchyma are associated with both regular and monilioid hyphae. Although some true (differentiated) sclerotia may initiate in this fashion (Townsend and Willets 1954), there is no evidence that these fossil microsclerotia ever became further differentiated into rind or cortex, which is a development that is normally attendant with maturation of a true sclerotium (Willets and Bullock 1992). Willets (1997) considers these structures 'multihyphal reproductive anamorphs', but in most literature they are simply regarded as microsclerotia, and thought to function in the same capacity as other sclerotia (Anderson 1996, Currah et al. 1988, Jumpponen and Trappe 1998). Loose, monilioid microsclerotia similar to the fossils are produced by a number of root-colonizing fungi (Currah et al. 1988, Ahlic and Sieber 2006), and the affinities of these fossil fungi likely lie within the informal assemblage commonly referred to as dark septate endophytes (DSE).

Similarities to extant dark septate endophytes. DSE, which have also been termed DS fungi (DSF) and *Mycelium radicis atrovirens* (MRA), comprise a heterogenous assemblage of predominantly ascomycetous fungi that have been isolated from more than 600 species of vascular plants, and can grow asymptomatically within the living tissue of their hosts (Jumpponen and Trappe 1998, Jumpponen 2001, Rodriguez et al. 2009). In contrast to plant shoots, endophytic colonization of roots is often extensive, with both inter- and intra-cellular proliferation (Schulz and Boyle 2005) of dematiaceous septate hyphae, monilioid hyphae, and yeast-like arthroconidia (Melin 1923, Currah et al. 1988, Dalpé et al. 1989). Unlike arbuscular mycorrhizal fungi, DSE do not form obvious assimilative structures at their interface with host tissues. Instead, there is evidence that DSE are intimately associated with host sieve elements via

mucilaginous hyphae that form integrated networks between the host's vascular system, and the hyphae present within the cortical tissue (Barrow 2003). Intracellular microsclerotia occur in the outer cortex, developing in response to stress or host senescence (Fernando and Currah 1996, Jumpponen and Trappe 1998, Barrow 2003).

Distinctive microsclerotia that co-occur with monilioid and regular hypha growth in the cortical tissues of *Eorhiza arnoldii* are morphologically similar to known DSE (Currah et al. 1988, Ahlich and Sieber 2006, Fernandez et al. 2008). Because the host-fungus interface of DSE involves a network of non-chitinous mucilaginous hyphae (Barrow 2003), direct evidence by which to discriminate an asymptomatic endophyte from a saprotrophic root colonizer is unlikely to be observed in the fossil record, although additional investigations may yield associated conidia. Conidiogenesis can be diagnostic for a number of root endophytes (Fernando and Currah 1995, Addy et al. 2005), but is often rare, frequently occurring only after a period of vernalization (Wilson et al. 2004, Addy et al. 2005). As the sterile mycelia of most DSE are morphologically similar, we are currently unable to more precisely delimit the systematic affinities of these fossils, although the occurrence of two types of survival anamorphs indicates that more than one species of root colonizing fungi may have been present.

As previously mentioned, several lobed or invaginated cells (Fig. 11F) also occur within cortical tissue that hosts monilioid hyphae. Similar cells, co-called 'germlings', have been observed in association with microthyriaceous epiphyllous fungi (Dilcher 1965), but this morphology, particularly with respect to the presence of penetration pegs, is consistent with hyphopodia of the cereal pathogen *Gaeumannomyces graminis* (Sacc.) Arx & D.L. Olivier (van Geel et al. 2011). A hyphopodial growth phase has also been observed in the DSE *Phialocephala fortinii* Wang & Wilcox (Ahlich and Sieber 2006), and may represent the mode of

primary infection for other endophytic fungi. The fossil hyphopodia may represent infection propagules of either of the two DSE-type anamorphs, or a hitherto unknown pathogen of the aquatic host plant, *Eorhiza arnoldii*.

Ecological interpretations. In the absence of defining features of conidiogenesis that would permit clear attribution to extant DSE lineages, it is impossible to conclusively identify the ecological role of these fossil fungi. Hyphae associated with the putative DSE do, however, appear to have interacted with the cell wall structure of the host plant: penetration across cell walls is via microhyphal strands (a feature which to our knowledge has not previously been demonstrated for fossil fungi), and in some specimens, hyphal growth has been constrained to intercellular spaces of host tissue. Finally, the *Eorhiza* tissue contains several loose intracellular coils of hyphae, which are similar to ericoid mycorrhizae, and to 'peloton-like' DSE structures observed in some boreal orchids that have been interpreted as functioning as ectendomycorrhizae (Currah et al. 1988, Petersen et al. 2004). We favor the latter interpretation, as the structures are isolated and rare, and the host plant is thought to represent an extinct family of basal angiosperms perhaps most closely affiliated with Nympheales (Stockey and Pigg 1991, 1994). Currah et al. (1993) noted, however, that the peloton-like, coiled, branching hyphae associated with some DSE can also occur in moribund tissues, and are therefore not necessarily indicative of a biotrophic relationship.

Conclusion

Previous assessments of fungal diversity within *E. arnoldii* have revealed the presence of several microfungi, some of which are known to be saprotrophic (LePage et al. 1994, Klymiuk et al. 2012), providing indication that the host tissue was moribund at the time of fossilization. As chitin is a highly resistant biopolymer (Briggs 1999), it is likely that the fungi preserved within

the plants of the Princeton Chert are a palimpsest of fungal succession: endophytes colonized living tissue, which senesced, died, and was incorporated into the organic substrate of a peat-forming mire, where it became subject to biodegradation by saprotrophs. We hypothesize that *E. arnoldii* was colonized by dark septate endophytes which persisted commensally within the cortex during the plant's life; during this period, regular hyphal growth was likely restricted to intercellular spaces, with monilioid growth occurring predominantly in association with sclerotial development, which occurred within the confines of host cells. Subsequent to the death of the host, and upon incorporation into the inundated substrate, the fungi persisted as saprotrophs, with assimilative mycelia proliferating through the degrading host tissue.

Our suggestion that the relationship between the *E. arnoldii* plant and the dark septate endophytes was one of commensalism should be understood only as a parsimonious hypothesis, especially as the ecology of living endophytic microfungi remains largely unknown. It has been experimentally demonstrated that DSE can function as both pathogens and saprotrophs (Wilcox and Wang 1987, Menkis et al. 2004, 2005). Additionally, they display little host specificity (Ahlich and Sieber 2006, Walker et al. 2011), and are known to colonize species which simultaneously host AMF or ectomycorrhizal fungi (Wagg et al. 2008, Ghanta et al. 2012). Nevertheless, they are ubiquitous in alpine, boreal, arctic, and arid environments (Gardes and Dahlberg 1996, Barrow 2003, Schmidt et al. 2008), and there is some indication that DSE can form mutualistic mycorrhizal-like associations with plants that lack typical mycorrhizae (Petersen et al. 2008). It has been hypothesized that they can positively contribute to plant growth through nutrient solubilisation, or by water retention (Mandyam and Jumpponen 2005). That some heliotealean DSE have also been shown to enhance nitrogen uptake in graminoids and

eriocoids (Zijlstra et al. 2005, Newsham 2011) is of particular interest when considering plants growing in inundated peat-forming mires, as these environments are generally nitrogen-poor.

Many of the plants preserved in the Princeton Chert, including *E. arnoldii*, have structural adaptations to an aquatic habitat, and obviously grew within or near the periphery of the Eocene mire that has been preserved as a succession of silicified peats (Cevallos-Ferriz et al. 1991). Exquisite preservation of botanical remains has allowed insight into the microbial constituents of this environment, which in turn provide new information about the ecology of this renowned paleobotanical locality. The sterile mycelia described here provide an important new fossil record for plant-fungal interactions, and simultaneously expand our understanding of the diversity of root-colonizing fungi within the Chert. In addition to arbuscular mycorrhizae and ectomycorrhizae (LePage et al. 1997, Stockey et al. 2001), there is now evidence for the presence of dark septate endophytes. We anticipate that continued research into the distribution and prevalence of these enigmatic fungi will better enable us to draw ecological parallels between modern temperate mires and the fossil biota of these Eocene peats.

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Figures

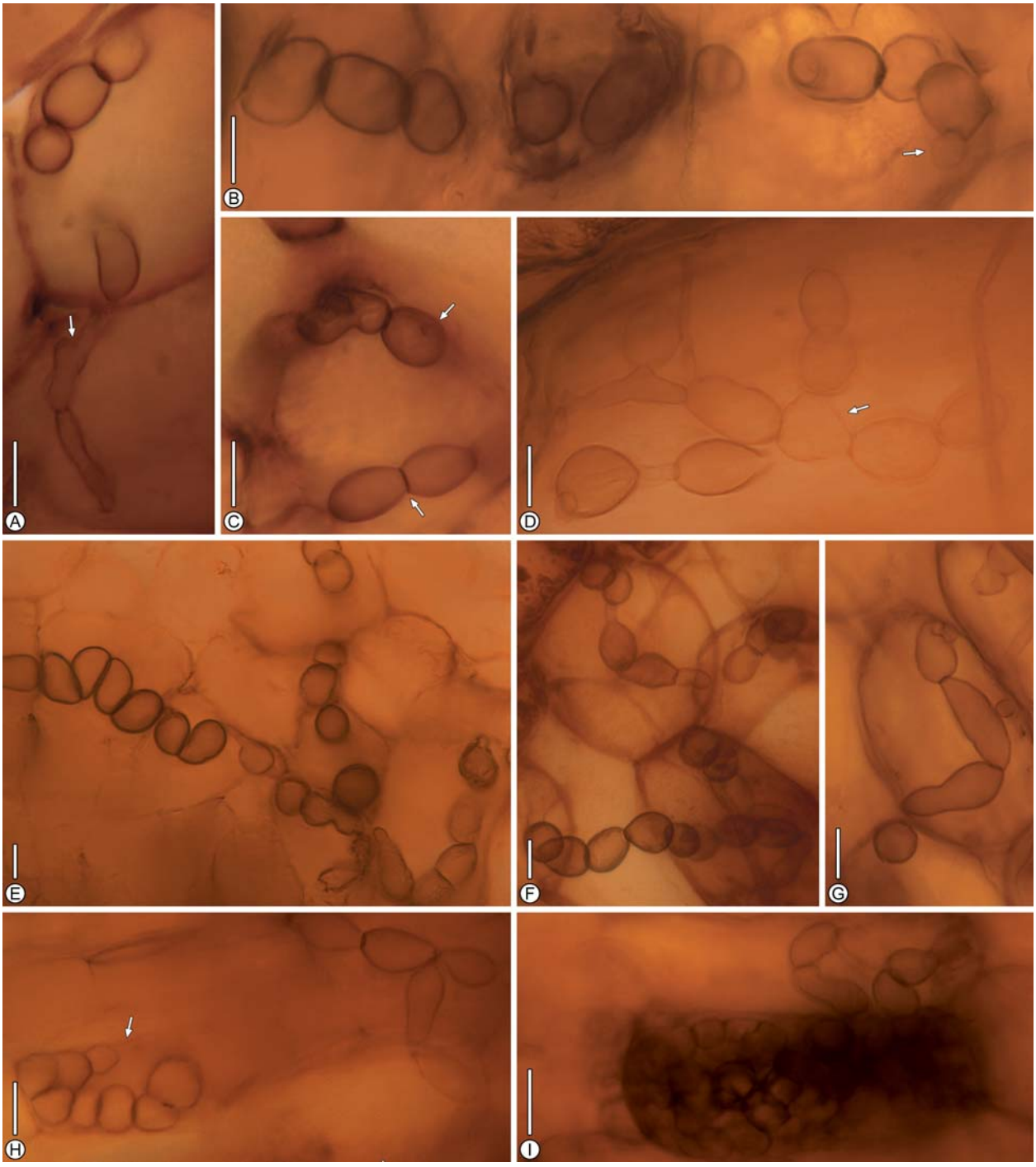

Figure 9: Dematiaceous monilioid hyphae. A. Chains of monilioid cells in association with septate hyphae; note acute hyphal branching at arrow. B. Newly-produced cells occur at the terminus of monilioid hypha (arrow). C. Branching hyphae may be inflated. Note relatively unconstricted septa between some cells (lower arrow), and presence of putative lateral bud scar (upper arrow). D. Intercalary branching (arrow). E-F. Extensive proliferation of monilioid hyphae through host tissue. G. Short, inflated hyphal segments frequently associated with isodiametric monilioid cells. H-I. Loose microsclerotia formed of monilioid hyphae that fill lumen of host cells. Monilioid

hyphae are produced from regular simple-septate hyphae (H, arrow). Scale bars = 10 μm. A-F, H-I: 17037 Fbot #001 G: 17035 Fbot #001.

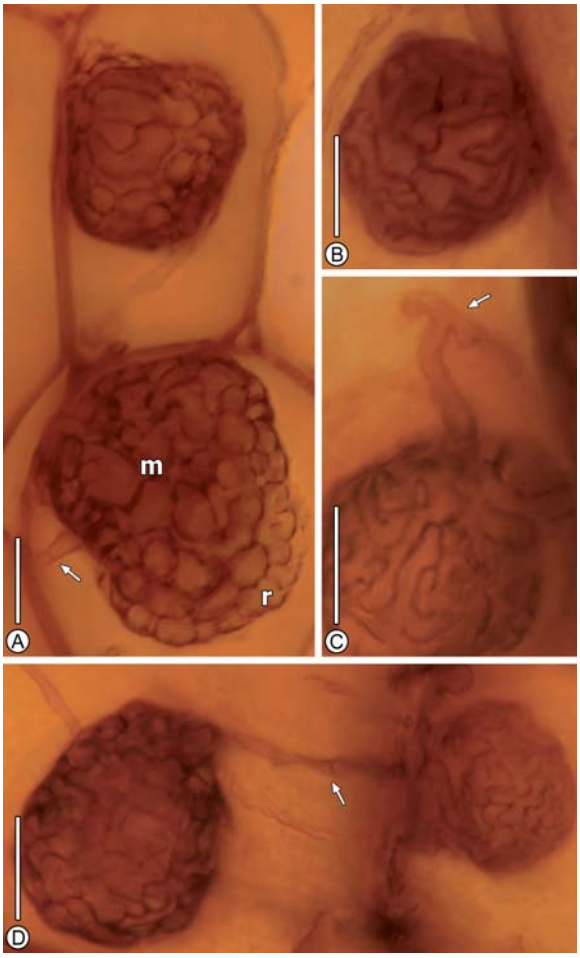

Figure 10: Cerebriform microsclerotia. A. Microsclerotia in transverse section, exhibiting differentiation into rind (r) and medulla (m), and attachment to septate hyphae (arrow). B-C. Microsclerotia in plan or surficial view; hyphae that form the rind are tightly adpressed. Note attachment to branching hyphae (C, at arrow). D. Multiple microsclerotia may be attached by hyphal stolons (arrow). Scale bars = 10 μm. A, B, D: 17030 Bbot #001; C: 17037 Fbot #001.

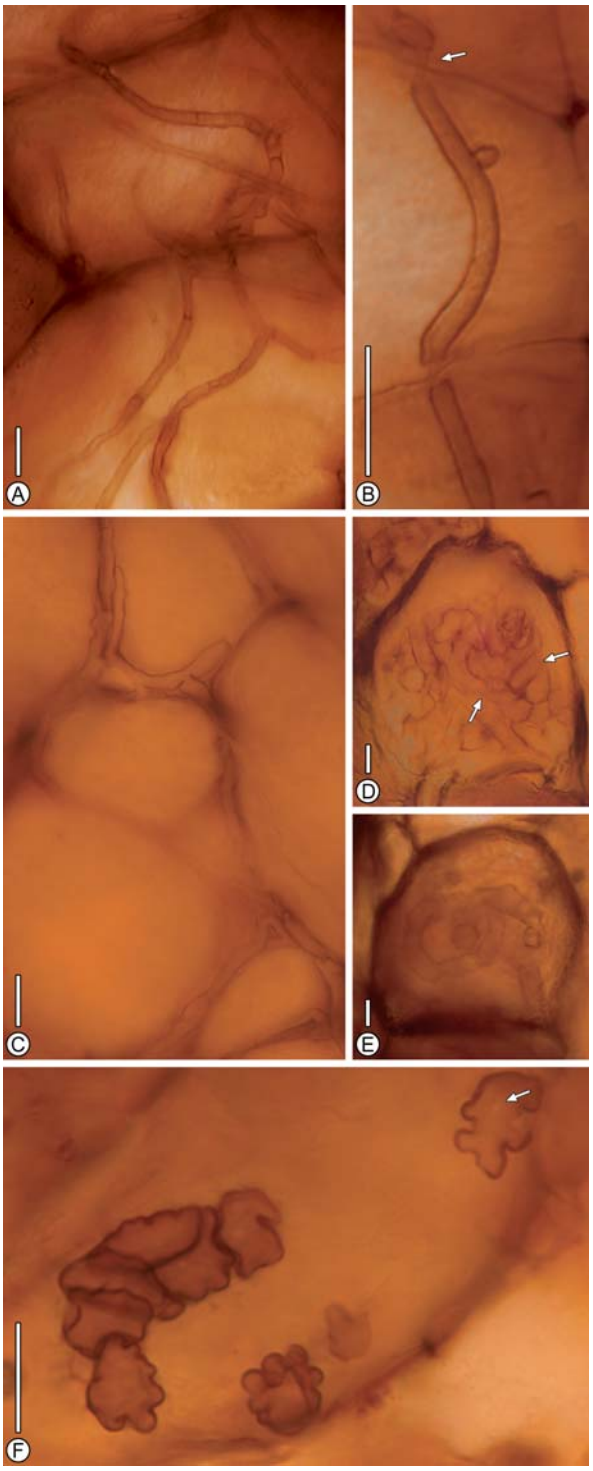

Figure 11: Variation in mycelial growth through tissue of host plant. A-B. Extensive intracellular hyphal proliferation with microhyphal cell wall penetration (B, arrow). C. Restriction of hyphae to intercellular spaces. D-E. Loose coils of large diameter (10 µm) hyphae within host cell lumens; septa visible at arrows. F. Vegetative hyphal elements with irregularly lobed or invaginated morphology; note medial cellular structures interpreted as penetration pegs, all cells and indicated at arrow. Scale bars = 10 µm. A, C: 17035 Ebot #002; B: 17030 Cbot #001; D, E: 17035 Etop #002; F: 17040 Bbot #001.

Chapter 5: Dictyosporic microfungi, *Monodictysporites princetonensis* gen. et sp. nov., associated with decayed rhizomes of an Eocene semi-aquatic fern.

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Abstract

This study builds on previous investigations of paleomycological diversity within permineralized plants of a significant Eocene paleobotanical locality, the Princeton Chert. The fungal body fossils described here occur in decayed rhizomes of the extinct semi-aquatic fern *Dennstaedtiopsis aerenchymata*. Fungi include vegetative hyphae throughout the plant tissue, as well as a dense assemblage of >100 dematiaceous spores. The spores occur in a discrete zone surrounding two extraneous rootlets of other plants, which penetrated the fern tissue post-mortem. Spores are obovoid and muriform, composed of 8–12 cells with constricted septa, and produced from hyaline or slightly pigmented hyphae. The spores are morphologically similar to both asexual reproductive dictyospores of phylogenetically disparate microfungi attributed to the morphogenus *Monodictys*, and perennating dictyochlamydospores that occur in the anamorph genus *Phoma*. In addition to expanding the early Eocene fossil record for Ascomycota, these specimens also provide new insight into the rapidity of initial phases of the fossilization process in this important paleobotanical locality.

Introduction

The Princeton Chert, a renowned paleobotanical succession in southern British Columbia, Canada, is emerging as an important resource for the study of fossil microfungi, owing to high-fidelity preservation of plant tissues — and the fungi they contain — by cryptocrystalline quartz

(SiO_2) in three dimensions at a cellular level of detail. The succession contains temperate and subtropical (Greenwood et al. 2005) vascular plants that grew in association with a peat-forming mire. Host plant tissues contain numerous pathogenic, saprotrophic, and endophytic fungi (LePage et al 1994, Stockey et al. 2001, Klymiuk et al. 2013a, 2013b). Previous reports of plant-associated fossil fungi have emphasized that many are morphologically comparable to living genera, particularly as features pertaining to conidiogenesis may be inferred owing to their exceptional preservation (Klymiuk et al. 2013a, 2013b).

The new microfungi described in this report occur within anatomically preserved rhizomes of the extinct polypodialean fern (Dennstaedtiaceae), *Dennsteadtiopsis aerenchymata* Arnold et Daugherty (1964). Tissues of these ferns have not previously been examined for microbial remains. *D. aerenchymata* occurs in several bedding planes of the Princeton Chert, as well as within the Clarno Formation of Oregon, from which it was originally described (Arnold and Daugherty 1964, Cevallos-Ferriz et al. 1991). As compared to samples from the Clarno Formation, *D. aerenchymata* specimens from Princeton exhibit substantially higher levels of kerogen, or geochemically altered cell wall components (Czaja et al. 2009). The Princeton samples are therefore likely to yield the most inclusive picture of associated fungal diversity, owing to higher-fidelity preservation.

In addition to expanding the known saprotrophic component of paleoecosystems, the study of fossil fungi can contribute to our understanding of events and processes preceding fossilization. Taphonomy, the paleontological discipline concerned with explicating post-mortem fates of organisms and their potential for recruitment into the fossil record (Efremov 1940), can complement paleoecological investigations of fossil biota by defining biases in preservation (e.g., decay of plant tissues, most often accomplished by saprotrophic fungi) and

clarifying the temporal window in which fossilization occurred. The manner in which these fungi have been preserved reveals that the taphonomic profile of this Eocene mire likely includes phases of very rapid silica deposition. While this mode of preservation is common in sinter-associated cherts, the Princeton Chert is not thought to have been associated with hot-springs; rapid silicification of these specimens is thus difficult to reconcile with current depositional hypotheses for this system (Mustoe 2011). By clarifying the temporal window of fossilization processes, these fungal fossils further increase our appreciation of the taphonomic complexity inherent in this succession of silicified peats

Materials and Methods

Specimen provenance. The Princeton Chert locality (UTM 10U 678057 5472372; 49°22′40″ N, 120°32′48″ W) is a single inclined outcrop composed of ~49 anastomosing layers of silicified peat interbedded with sub-bituminous coal. An ash within the succession has been K-Ar dated to ~48.7 Ma; the locality is thus latest Ypresian to earliest Lutetian in age (Smith and Stockey 2007, Mustoe 2011). Samples of silicified rhizomes of the aquatic fern *Dennstaedtiopsis aerenchymata* were taken from Layer 24, as informally numbered by RA Stockey and colleagues. Small blocks of chert containing rhizomes were mounted on glass slides using Hillquist Two Part mounting medium (Hillquist, USA), and serially thin sectioned (100–200 μm) with a Buehler Petrothin®. Thin sections are deposited in the University of Alberta Paleobotanical Collections (UAPC-ALTA); figured specimens comprise accession numbers P3301 $D_{top(B)}$ #001 and P2954 G_{3top} #002.

Photomicrography. Serial photomicrographs taken through 10-30 focal planes were captured directly from the rock surface under oil immersion, using a Leica DC500 CCD attached to a Leica DM5000B transmitted-light compound microscope. To optimize visualization of

specimens, I compiled composite focal-stacked images with Helicon Focus v5.3.7 (Helicon Soft Ltd, Kharkov, Ukraine) under default pyramid parameters (Method C). Spinning disk confocal microscopy was performed using an Olympus IX71 microscope equipped with a Yokagawa CSU10 spinning disk confocal illumination system. Excitation was performed with a 561 nm Coherent solid-state laser. Emission was collected using a Semrock longpass 586 nm filter, and image capture was performed with a Hamamatsu 1000 × 1000 back-thinned electron multiplying CCD (quantum efficiency, 94%). Additional image processing (contrast and tonal adjustment for all images) was performed with Adobe Photoshop CS5 12.1, and source image data are available upon request.

Results

The fossil fungi described here occur in anatomically-preserved rhizomes of the semi-aquatic fern *Dennstaedtiopsis aerenchymata* which are entrained within a layer of silicified sapric-textured (sensu Boelter 1969, Henderson 1981), or highly-decayed, peat. Rhizomes exhibit substantial degradation of the epidermis and cortex (Fig. 12A). Two cortical layers, composed of thin-walled, isodiametric parenchyma cells, are present: the outer cortex, ~400 μm wide, surrounds the aerenchymatous inner cortex, wherein chains of parenchyma cells separate lacunae. In several places, the cortex contains 250 μm diam rootlets of another plant (Fig. 12A, arrows). Although assimilative hyphae are present throughout the fern rhizomes (not figured), fungal spores have only been observed in proximity to two of the intruding rootlets, which do not themselves contain any hyphae. The spores form a dense assemblage around the entire periphery of one of these rootlets (Figs. 12A, box, 12B-F), and occur sporadically, in lower numbers and at less mature developmental stages, around another (Fig. 12G, intruding rootlet cells at bottom).

Fungal remains comprise more than a hundred dematiaceous, obovoid, muriform spores, each typically composed of 8–12 cells (Figs. 12C–G), which appear bulbous and are predominantly transversely and longitudinally septate. Occasional dispersed spores morphologically similar to these occur in isolation in other layers; this is the first observation of these spores in developmental context and organic position. Mature spores are 10–15 μm diam (Figs. 12C, D), while the most immature specimens range from 3 to 5 μm, and are less pigmented than at maturity (Figs. 12E–G). In very immature specimens, septation appears to be primarily longitudinal (Fig. 12G); when spores are viewed from their bases (Fig 12E, spore at arrow), however, it is apparent that cells are irregularly arranged. The appearance of longitudinal septation may be an optical artefact, and further specimens are necessary to confirm this pattern of development. Spores are produced from hyaline to slightly pigmented doliiform to cylindrical cells, 1–3 μm diam (Figs. 12E-G, Fig 13). If the spores are interpreted as conidia, the condiogenous locus appears to be integrated and terminal, and the subtending hyaline hyphae (Figs. 12E-G, Fig. 13A at arrows) may be interpreted as micronematous (undifferentiated) conidiophores. Secession is not apparent.

Spinning disk confocal microscopy (Fig 13A) was employed both to corroborate photomicrographs of the subtending hyaline hyphae, and to investigate the distinctive halos or rimes of chert surrounding the spores (Fig 13B). These rimes consist of cryptocrystalline quartz that is substantially lower in organic carbon content than the fungal tissues themselves, or the dark-coloured, organic-rich chert deposited around the rimes.

Taxonomy

Monodictysporites Klymiuk gen. nov.

Mycobank MB 815858

Typification. — Monodictysporites princetonensis Klymiuk

Etymology. — The genus reflects a resemblance to the spores of living fungi deposited in *Monodictys.* The specific epithet references the paleobotanical locality.

Diagnosis. — Dictyosporic conidia or dictyochlamydospores; spores 8–12 cells, muriform, obovoid, dematiaceous. Subtending hyphae hyaline, cells doliiform to cylindrical where producing spores.

Monodictysporites princetonensis Klymiuk sp. nov., Fig. 12-13.

Mycobank MB 816284

Typification. — Canada: British Columbia. Allenby Formation, Princeton Group; Ypresian–Lutetian (Eocene, ~48.7 Ma.) Specimens occurring within paleontological thin sections cut by AA Klymiuk, from rocks P13301 D_{top} B, slide #001; P2954 $G3_{top}$, slide #002. Specimens are deposited in the University of Alberta Paleobotanical Collection (UAPC-ALTA)

Etymology. — The specific epithet references the paleobotanical locality.

Diagnosis. — Dictyosporic conidia or dictyochlamydospores; spores 8–12 cells, muriform, obovoid, dematiaceous and 10–15 μm diam at maturity. Subtending hyphae hyaline, 1–3 μm diam; cells doliiform to cylindrical where producing spores.

Discussion

Affinities. Although it is a common practice in paleomycology to describe fossil spores as palynological form genera, many Cenozoic fungi are morphologically consistent with extant lineages (Pirozynski 1976, Pirozynski and Weresub 1979). Morphological classification of extant microfungi, however, requires observation of developmental features, particularly those relating to conidiogenesis (Hughes 1953); these data are often unavailable for fossilized microbes (Girard and Adl 2011). The value of exceptional preservation, whether in amber, or by

permineralization of host plants by silica or marine carbonates (LePage et al. 1994, Schmidt et al. 2008, 2010, Sadowski et al. 2012, Bronson et al. 2013, Klymiuk et al. 2013a), lies in the potential to infer developmental features that can aid in systematic classification. Although much of this data is available for the fossils illustrated here, they cannot be unequivocally assigned to an extant lineage, as spores similar to the fossils are produced by many phylogenetically disparate microfungi.

Among described fossil taxa, the new specimens most closely resemble dispersed spores described as *Staphlosporonites conoideus* Sheffy and Dilcher (1971), and *Dictyosporites loculatus* Felix (Kalgutkar and Jansonius 2000). Neither of these described fossils occur in association with hyphae, hence their deposition in *Sporae dispersae* morphogenera. Isolated fungal palynomorphs deposited in these genera were globally distributed in the late Mesozoic and Paleogene (e.g., Sheffy and Dilcher 1971, Parsons and Norris 1999, Kalgutkar and Jansonius 2000, Kalgutkar and Braman 2008, Singh and Chuahan 2008). Species deposited therein, however, exhibit substantial morphological heterogeneity. Given that dictyosporic morphology is common across phylogenetically unrelated taxa, these palynomorph genera are unlikely to reflect monophyletic groups. As the new fossils described here are not dispersed spores, and some developmental data is available, they are more appropriately compared with extant fungi, consistent with the comparison of other Princeton microfungi to living lineages (Klymiuk et al. 2013a, 2013b).

Among living fungi, spores similar to the fossils include both asexual reproductive propagules (hereafter, dictyospores) and propagules functioning in perennation (hereafter, dictyochlamydospores). Differentiating between dictyochlamydospores, some of which are formed through deposition of a secondary ‘cell wall’ inside of the normal, bi-layered hyphal wall

(Campbell and Griffiths 1975), and dictyospores requires a level of ultracellular detail unavailable in these fossils. There is thus no way in which to ascertain whether these structures represent dispersive or perennating propagules, and comparisons with extant taxa needs must accommodate both possibilities.

One group of fungi, the phylogenetically heterogeneous 'black yeasts', produces both dictyosporic conidia and dictyochlamydospores. These include *Coniosporium* Link, *Knufia* L.J. Hutchison & Unter., *Phaeococcomyces* de Hoog and *Phaeotheca* Sigler, Tsuneda, & J.W. Carmich., which also produce multicellular structures called 'meristematic bodies' that are superficially similar to the fossils (de Hoog et al. 1997, Sterflinger et al. 1997, Tsuneda et al. 2008, 2011). These meristematic structures develop from detached conidia or hyphae, and subsequently function in the production of endoconidia (de Hoog et al. 1997, Tsuneda et al. 2008, 2011). The fossils do not contain endoconidia, and they co-occur with normally-branching, uninflated hyphae – indeed, there are no monilioid hyphae or yeast-like cellular proliferations in the specimens at all, as would be expected for any of the 'black yeast' taxa.

Among taxa that produce abundant dictyochlamydospores, those of the genus *Pochonia* resemble the fossils in size and shape (e.g., Gams and Zare 2001, Zare et al. 2001, Nonaka et al. 2013). The fossils, however, are deeply pigmented, whereas chlamydospores of *Pochonia* are hyaline. If the fossils represent dictyochlamydospores, better candidates for their affinities can be found among members of Didymellaceae consistent with the anamorph morphogenus *Phoma* (Davey and Currah 2009, de Gruyter et al. 2009, Zhang et al. 2009, Aveskamp et al. 2010), which frequently produce dictyochlamydospores (Boerema et al. 2004; Aveskamp et al. 2009). Although a *Phoma*-like fungus is known to have infested seeds of the Princeton Chert angiosperm *Princetonia allenbyensis* Stockey & Pigg, dictyochlamydospores were not reported

(LePage et al. 1994). Because *P. allenbyensis* does co-occur with *D. aerenchymata* in several layers of the Chert, it is possible that the new spores represent chlamydospores of the *Princetonia* seed parasite. The fossils, however, are morphologically inconsistent with most dictyochlamydosporous '*Phoma*' anamorphs: They are ellipsoidal to only weakly alternarioid (c.f. Fig. 1B, C), in contrast with species like *P. pomorum* (Boerema et al. 2004) and *P schachtii* Aveskamp, Gruyter & Verkley (Aveskamp et al. 2009); moreover, none are catenate (i.e, borne in chains) as in *P. glomerata* Corda) Wollenw. & Hochapfel, *P. pimprina* P.N. Mathur, S.K. Menon & Thirum., and *P. subglomerata* Boerema, Gruyter & Noordel. (Boerema et al. 2004). Nor do the fossils bear a close resemblance to many botryoid *Phoma* dictyochlamydospores, as their shape is generally more regular: They exhibit less variability in terms of size and cell number than dictyochlamydospores of *P. omnivirens* Aveskamp, Gruyter & Verkley (Aveskamp et al. 2009), *P. zeae-maydis* Punith. *P. sorghina* (Sacc.) Boerema, Dorenb. & Kesteren, *P. narcissi* (Aderh.) Boerema, Gruyter & Noordel. and *P. zantedeschiae* Dippen. (Punithalingam 1990, Boerema 1993, Boerema et al. 2004, Aveskamp et al. 2009). When they are immature, dictyochlamydospores of the alternarioid species *P. sancta* Aveskamp, Gruyter & Verkley (Aveskamp et al. 2009) and *P. jolyana* Piroz. & Morgan-Jones (Pirozynski and Morgan-Jones 1968) most closely resemble the fossils. As fossilization of the spores was rapid, and several exhibit immature morphology, it is possible that their affinities do lie within Didymellaceae.

The sheer abundance of the fossil spores, and the small space to which their production was constrained (as opposed to being associated with the hyphae proliferating throughout the rhizomes), are lines of evidence against their being dictyochlamydospores. Among the dictyospore-producing hyphomycetes, many, such as *Annellophorella* Subram, *Parapithomyces* Thaung, *Thyrostroma* Höhn., and *Thyrostromella* Höhn., may be dismissed as these taxa are leaf

pathogens (Subramanian 1962, Ellis 1971, Thaung 1976, Alcorn 1992, Seifert et al. 2011), a habit inconsistent with the fossils. The lack of both ornamentation and distinctive pigmentation can also be used to exclude affinities with some extant hyphomycetes. Although faint surface features may be occluded by taphonomic effects (Klymiuk et al. 2013a), palynological records (Kalgutkar and Jansonius, 2000) indicate that distinctive ornamentation, like that of *Paradictyoarthrinium* Matsushima (1996) or the *Phoma* anamorph *Epicoccum* (Aveskamp et al. 2010), are amenable to fossilization. Diagnostic patterns of conidial pigmentation, such as the darkened basal cell of *Acrodictyopsis* P.M. Kirk, darkened apex of *Junewangia* W.A. Baker & Morgan-Jones, or pigmented central cells of the bulbil-like conidia of *Papulospora* Preuss (Kirk 1983, Baker et al. 2002a, Seifert et al. 2011) are also expected to be evident in well-preserved fossils. Such gradations in dematiaceous pigmentation have been observed in other microfungi reported from the Princeton Chert (Klymiuk et al. 2013 a, b); by comparison, it is apparent that the fossil spores are uniformly pigmented, and unornamented. As robust or macronematous conidiophores would be expected to fossilize, and confocal scanning laser microscopy (Fig. 13) reveals only narrow, hyaline hyphae subtending the spores, I infer that — if the spores *are* conidia, and not dictyochlamydospores — the conidiogenous locus is integrated, with conidia produced from micronematous or undifferentiated conidiophores.

Given these considerations, the fossils accord best, among hyphomycetes, with *Monodictys* S. Hughes, of which more than 50 species have been described. The fossils resemble *M. putredinis* (see Hosoya and Hutinen 2002), but a specific diagnosis cannot be made at this time, as a thorough revision of the genus is necessary. For instance, Mouzouras and Jones (1985) suggested that *M. pelagica* (T. Johnson) E.B.G. Jones is the anamorph of *Nereiospora cristata* (Kohlm.) E.B.G. Jones, R.G. Johnson & S.T. Moss (Sordariomycetes: Microascales:

Halosphaeriaceae,), Day and Currah (2006) place *M. arctica* M.J. Day & Currah within Leptosphaeriaceae (Dothideomycetes: Pleosporales), and Hosoya and Hutinen (2002) suggest that *Hyaloscypha albohyalina* (Leotiomycetes: Heliotales: Hyaloscyphaceae) has a *M. putredinis* anamorph. Moreover, it is probable that some spores described as *Monodictys* are, in fact, chlamydospores. As previously discussed, it is not possible to conclude that the fossils necessarily represent conidia either. As they cannot be unequivocally assigned to any extant lineage, and would be inappropriately deposited within a 'sporae dispersae' palynological form genus, they are consequently described as a new fossil taxon, *Monodictysporites princetonensis*.

Insights into taphonomic complexity of the Princeton Chert. Hot-spring sinter deposits, like the Devonian Rhynie Chert (Channing and Edwards 2003, 2009), are well-recognized as sources of exceptional preservation within rapid time intervals (e.g., discharge of spermatozoa from an antheridium; Kerp et al. 2003). The cherts at Princeton, however, are depauperate with respect to heavy metals indicative of geothermal origins (Mustoe 2011). The silicified coal-forming peats that comprise the locality are thus unlikely to have originated as shallow wetlands near sinter-depositing hot-springs. Although the locality has been considered geologically unique (Mustoe, 2011), similar chert layers or lenses have been described in at least four other silicifed peat-coal deposits (Schopf 1970, Ting 1972, Taylor et al. 1989, Sykes and Lindqvist 1993, Umeda 2003, Slater et al. 2015); most have plant remains with preservational fidelity comparable to Princeton plants (e.g., Sykes and Lindqvist 1993, Plate 3; Umeda 2003, fig. 5).

Despite the fact that peat-associated cherts are geologically common, there remain some difficulties in understanding how fossilization proceeds in such assemblages. For instance, silica has exceedingly low solubility in peat water (Siever 1962), and is more soluble in circumneutral

water than that which has a low pH (Bennett 1991). Stoichiometric shifts may explain silicification at depth in the peat-forming depositional systems: even acidic peat bogs become circumneutral deep in their profiles (Siegel and Glaser 1987), and both the rate of dissolution and solubility of silica increase in the presence of organic acids (Bennett 1991, Bennett and Casey 1994), with dissolved silica in peats increasing at depth (Bennett et al. 1991). This *does* accommodate fossilization of plants buried deeply within peats, as in the silica-rich groundwater model posited by Mustoe (2011). However, hydraulic impedance is an intrinsic property of peats (Boulter 1969, Rycroft et al. 1975, Ivanov 1981), and this model is more problematic when considering specimens like these decayed *Dennsteadtiopsis* rhizomes and the fungi they contain. Although a number of phylogenetically disparate ascomycetes may remain metabolically active for some time under the type of anaerobic conditions occurring deep in the peat column (Kurakov et al. 2008), mycelial proliferation is greatest in the acrotelm, the 'active' layer above the resident water table (Golovchenko et al. 2002, 2013, Lin et al. 2012), where aerobically respiring fungi are the principal agents of decay (Thormann and Rice 2007). The combination of both intruding rootlets, indicative of actively-growing plants in proximity to the decayed rhizomes, as well as extensive fungal growth, suggests that these specimens were entrained within the acrotelm when they were fossilized.

A full understanding of the sequence of events leading to the preservation of these fossils remains intractable, but the fungal spores do provide important new data with respect to the temporal window in which the earliest stages of silicification occurred. While fungal remains are common in fossil plants (Taylor et al. 2014), it is usually difficult to infer the amount of time between fungal proliferation and fossilization. Given that hyphae and spore walls contain chitin, a molecule highly resistant to degradation and known to be readily preserved in the fossil record

(Briggs 1999, Flannery et al. 2001), this temporal window could be significant, as surrounding plant cell walls provide protection against disarticulation. The fungal body fossils illustrated here are thus a rarity: The spores are preserved in multiple, co-occurring developmental stages, indicating that initial silicification occurred during sporulation, and was rapid – on the order of days – in these particular specimens. If the fern rhizomes *were* entrained within acrotelm peats, as seems likely, this provides important insight into the rapidity of localized silicification in peat normally above the resident water table. Additionally, the rime of low-organic quartz surrounding the specimens suggests that this initial phase may have occurred under hydrologic conditions differing from later stages of fossilization (Fig 2A). Whether rapid silicification produced other layers of the Princeton Chert, and the extent to which individual layers are themselves palimpsests of successive silicification, is unknown at this time.

Conclusion

As paleomycological investigations of the Princeton Chert continue, it is becoming increasingly apparent that ascomycetous microfungi comprise much of the microbial diversity. The presence of numerous *Monodictys*- or *Phoma*-like spores provides a first record of fungi within tissues of the semi-aquatic fossil fern *Dennsteadtiopsis aerenchymata*, and expand our knowledge of the saprotrophic component of the Princeton mire, which includes *Alternaria*-, *Ascochyta*-, *Thielaviopsis*-, and *Xylomyces*-like fossils (LePage et al. 1994, Klymiuk et al. 2013a). Taphonomic features of these new fossils also suggest the need for a more nuanced understanding of the silicification processes that produced this important paleobotanical locality.

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Figures

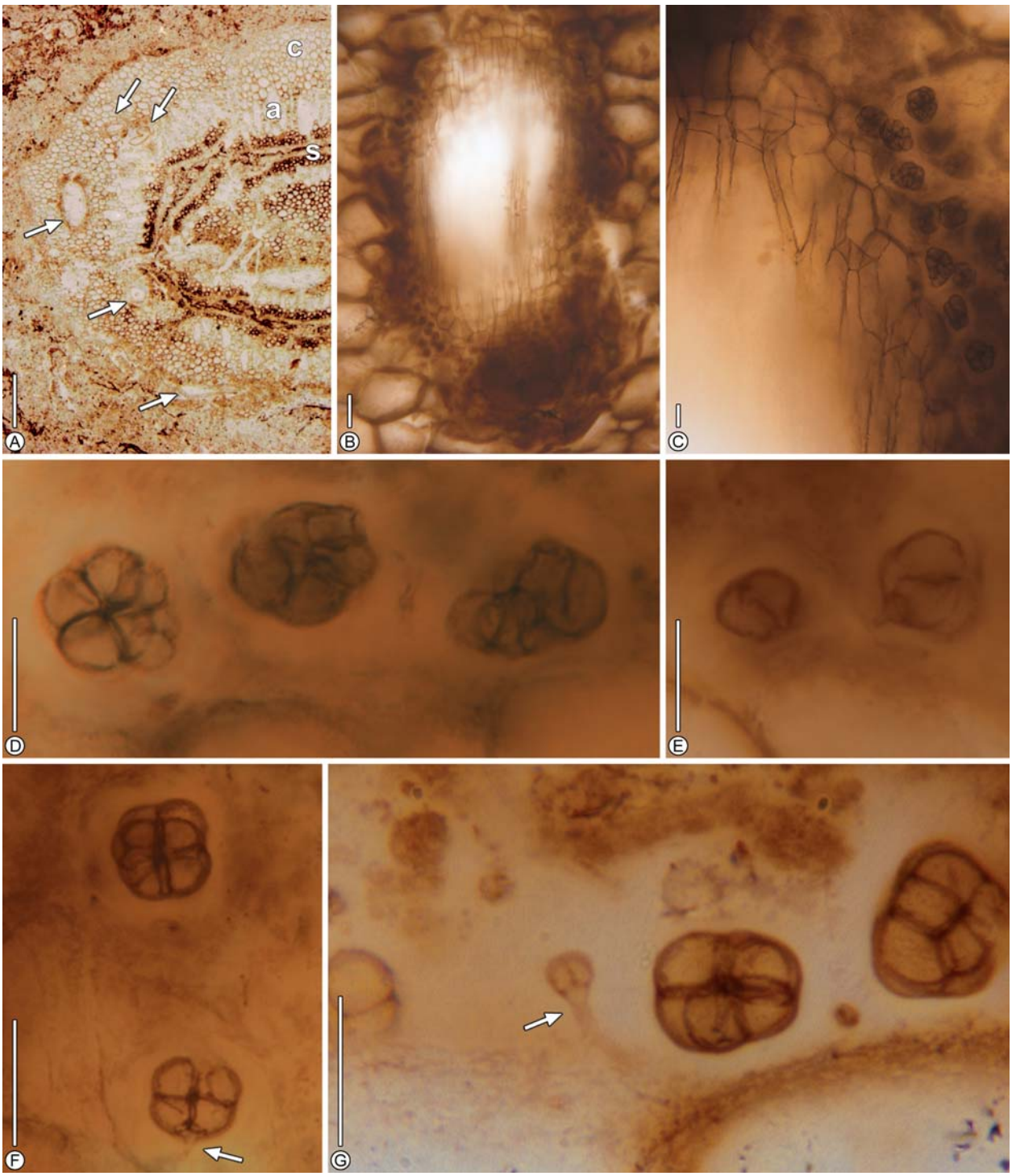

Figure 12: Spores in *Dennsteadtiopsis aerenchymata*. A. Transverse section through fern rhizome. Note penetrating rootlets at arrows, c = outer cortex, s = stele (vascular tissue), a = aerenchyma, box = portion magnified in 12B. B. Oblique transverse section of penetrating rootlet with fungal fossils around circumference, box = portion magnified in 12C. C. Spores in degraded zone between disrupted fern tissue, right, and rootlet, left. D-G Spores. Note subtending hyphal fragments at arrows. A-F: P13301 Dtop B 001, G: P2954 G3top 002.

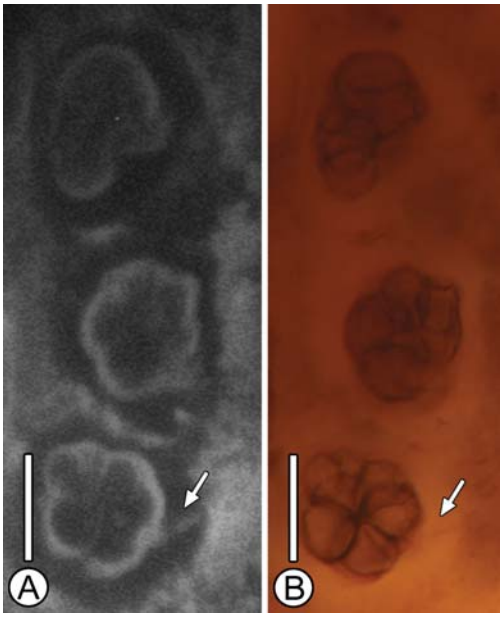

Figure 13: Mineralization of fossil spores, c.f. 12D. A. Spinning disk confocal micrograph; lighter regions have higher organic content. Note hypha at arrow. B. Light micrograph, c.f. 2A. Hypha at arrow. P13301 Dtop B 001.

Chapter 6: Suppression of root-endogenous fungi in persistently inundated *Typha* roots

Abstract

Owing to the anoxic and reducing conditions that predominate in wetland soils, these environments impose biogeochemically hostile conditions on plant roots, and the fungal communities endogenous to them. While the effects of inundation on mycorrhizal fungi have been subject to investigation, few studies have explored how the incidence or diversity of other root endogenous fungi changes in response to prolonged inundation. The cosmopolitan wetland plant *Typha* L. is highly efficient at mitigating root-zone anoxia, ergo roots of these plants may constitute fungal habitats similar to roots in subaerially-exposed soils, or fungi may be in competition with plant cells for diminishing oxygen, particularly at the deepest limits of growth. We hypothesized that extrinsic environmental factors would affect the internal root environments, and predicted that incidence of fungal hyphae would be negatively correlated with depth of inundation, that spore production (a common stress response) would increase commensurate with depth, and that community composition would differ between roots that were deeply inundated versus those growing in subaerially-exposed soils. To assess our hypotheses, we sampled roots of *Typha* plants (n = 108) from three transects at each of three constructed water catchment reservoirs in the state of Kansas. Along each transect, we collected roots for three plants at the deepest and highest extent of their growth along the local inundation gradient, as well as at the measured median of each transect. For each plant, roots were surface-sterilized and a) aseptically plated so as to culture root-endogenous fungi, b) cleared and stained for microscopic examination of fungal structures. Contrary to our expectations that hyphal incidence would be negatively correlated with depth, we found that the defining difference between the incidence of aseptate, hyaline, and dematiaceous hyphae in roots was whether the

roots were taken from subaerially-exposed soils, or from those that were persistently inundated. Spore production did not vary across transects, nor did the incidence of chytrids, which are facultative aerobes. Similarly, sampling points did not vary with respect to community composition of culturable fungi; we recovered 83 morphologically distinct types of fungi but found these communities did not significantly vary by inundation depth, or between reservoirs, a finding consistent with earlier metagenomic assays. This suggests that the suppression of hyphae which we observed in root samples did not result from changes in community composition. Instead, we consider it likely that low hyphal incidence in inundated *Typha* roots reflects germinal inhibition or unsuccessful initial colonization, owing to plant-mediated redox dynamism in the surrounding soil.

Introduction

Root-endogenous fungi are complex communities of mutualists, commensals, parasites, and pathogens that are endogenous to living vascular plant roots. Their incidence and diversity is a function of complex interactions between biotic and abiotic factors, including host taxonomy (Stevens et al. 2011), soluble carbon availability (Jones et al. 2009), soil nutrient availability (Johnson 1993), geography (Higgins et al. 2007), and climate (Augé 2001, Newsham et al. 2008). Of particular interest are fungi inhabiting the roots of plants subject to extreme levels of abiotic stress – in some cases, endogenous fungi may have beneficial or protective effects upon their hosts, and in other instances, they are deleterious (Saikkonen et al. 1998, Mandyam and Jumpponen 2005, Rodriguez et al. 2009). From a fungal perspective, roots of stressed plants may comprise environments which impose strict limits on fungal growth, or even which species can persist in these habitats, as is the case for root-endogenous fungi of plants growing in saline soils (Carvalho et al. 2001, 2003), highly acidic soils (An et al. 2008), or in the presence of

phytotoxic metals (Entry et al. 2002). By understanding the incidence and diversity of root-endogenous fungi in extreme environments, and how these fungi vary in response to abiotic stressors, we stand to gain considerable insight into their biology.

Wetland soils are among the most biogeochemically challenging environments (Pezeshki and Delaune 2012) that vascular plants and root-endogenous fungi inhabit, because prolonged inundation imposes a suite of interlinked abiotic stressors: Inundated soils are anoxic and therefore reducing chemical environments, which influences availability of limiting nutrients, and causes phytotoxic metals and organic acids to accumulate in the rhizosphere (Ponnamperuma 1984, Pezeshki 2001, Weis and Weis 2004, Reddy and Delaune 2008). Within the root environment, aerobically-respiring fungi compete with host cells for diminishing oxygen; declining oxygen availability causes plant cells to switch from oxidative respiration to fermentation pathways (Vartapetian and Jackson 1997, Gibbs and Greenway 2003, Greenway and Gibbs 2003, Kreuzwieser et al. 2004), thereby also diminishing soluble carbon that might otherwise be available to root-endogenous fungi. Owing to the anoxic and reducing conditions that predominate in most wetland soils (Vepraskas and Faulkner 2001, Reddy and Delaune 2008, Mitsch et al. 2009), root-endogenous fungi have historically been considered minor contributors to wetland soil ecosystems (Khan and Belik, 1995).

Most early work on root-endogenous fungi in both terrestrial and aquatic systems focused on arbuscular mycorrhizal fungi (AMF), but in recent decades it has become obvious that parasites, pathogens, and asymptomatic ‘endophytic’ fungi also regularly occur in plant roots. Arbuscular mycorrhizal fungi comprise a mucoromycotan subphylum (Spatafora et al. 2016), and engage in obligate mutualisms with members of every vascular plant lineage inhabiting subaerially-exposed soils (Feijen et al. 2017). AMF were reported as rare or absent in wetlands

(Stahl 1949, Khan 1974, Currah and Aan Dyk 1986, Thoen 1987), but it has since become apparent that AMF are common, occurring in fully submerged through emergent wetland plants (e.g., Søndergaard and Laegaard, 1997, Read et al. 1976, Turner et al. 2000, Beck-Nielsen and Madsen 2001, Cornwell et al. 2001, Šraj-Kržič et al. 2006, Sudová et al. 2011, Wang et al. 2011, Zhang et al. 2014; comprehensively reviewed by Zhouying et al. 2016). Similarly, dark septate endophytes (DSE), a phylogenetically heterogenous guild of potentially mutualistic or weakly parasitic fungi that inhabit plant tissues without obvious host response (Jumpponen and Trappe 1998, Schulz and Boyle 2005, Mandyam and Jumpponen 2005, Mandyam et al. 2013), are also consistently observed in wetland plant roots (Cook and Lefor 1998, Weishampel and Bedford 2006, Kai and Zhiwei 2006, de Marins et al. 2009, Sudová et al. 2011, Kohout et al. 2012), as are true pathogens (Evans and Reeder 2000). Metagenomic assays corroborate our contemporary understanding of wetland plant roots as environments with highly diverse fungal communities (Kohout et al. 2012, Sandburg et al. 2014).

The ubiquity of fungi in wetland plant roots may be substantially owed to the ability of these plants to mitigate anoxia within their roots. To compensate for these conditions, plants that thrive in wetlands employ a variety of physiological and anatomical adaptations to mitigate anoxia (Armstong 1979, Jackson and Armstrong 1999, Strand 2002, Gibbs and Greenway 2003, Greenway and Gibbs 2003, Evans 2004, Colmer and Voesneck 2009). Many of these strategies involve active or passive aeration of roots (Strand 2002, Evans 2004), and some wetland plants are so effective at oxygenating submersed roots that there is abundant extra-radicle oxygen leakage into surrounding sediments (Flessa 1994, Pezeshki and Delaune 2012). Well-aerated wetland plant roots may therefore closely resemble roots in subaerially-exposed soils, in terms of constituting suitable habitat for root-endogenous fungi. Conversely, the effectiveness of plants'

mitigation strategies is known to decline with depth, accounting for wetland community structure (Spence 1982, Brix et al. 1992, Lemoine et al. 2012) and perhaps causing root-endogenous fungal communities to experience seasonal or persistently hypoxic to anoxic conditions. Such root environments are clearly not paralleled in most soil environments, but comparatively few studies have assessed the extent to which deeply inundated wetland fungal communities resemble those in subaerially-exposed soils.

Here, we explored root-endogenous fungal incidence and community structure in the cosmopolitan wetland plant *Typha* L., and tested the extent to which fungi inhabiting the roots of *Typha* spp. vary across inundation gradients. *Typha*, colloquially known as cattails or reedmace, are cosmopolitan emergent macrophytes that span the entirety of inundation gradients. *Typha* spp. have been used as model plants in inundation studies (Ray and Inoue 2006, Inoue and Tsuchiya 2009). They employ pressurized convective ventilation to mitigate root hypoxia (Brix et al. 1992, Bendix et al. 1994, Tornberg et al. 1994, White and Ganf, 1998, 2001), which is one of the most efficient aeration strategies (Sorrell and Hawes 2009). Nevertheless, oxygen diffuses more readily out of roots in reducing conditions (Kludze and Delaune 1996), most oxygen within roots is consumed by plant respiration (Bedford et al. 1991, Chabbi et al. 2000) and some species even exhibit increased metabolic oxygen demand when inundated (Matsui and Tsuchiya 2006). Actively growing fungi are thus in direct competition with host plant cells for diminishing oxygen. As such, we hypothesized that *Typha* roots in subaerially-exposed soils would be more hospitable to aerobically respiring fungal endophytes than those at depth, which may experience more frequent or longer periods of hypoxia. To determine the effect of inundation (and declining oxygen availability by proxy), we assessed the incidence of microscopically visible fungal structures within roots to determine if their abundance differed across inundation gradients. We

also utilized culture methods to assess whether community structure of culturable root-endogenous fungi differed across gradients. Our study was replicated at three geographically disparate locations to account for potential geographic differences in fungal diversity and basin hydrology.

Our specific hypotheses and associated predictions are: (i) Under increasing inundation, plant roots will become increasingly inhospitable to aerobically respiring fungal endophytes. We predict the incidence of mycelial structures attributable to aerobically-respiring fungi will diminish commensurate with depth; structures associated with stress response, like sclerotia or conidia (asexual spores), should increase. Morphological structures attributable to facultative aerobes or anaerobes will be unaffected. (ii) Inundation will influence the composition of culturable fungal endophyte communities. We predict different morphotaxa will be cultured from roots grown in subaerially exposed soils, versus those at depth. (iii) Some variation in incidence of structures or composition will be explained by geographic proximity alone (i.e. spatial autocorrelation). We predict fungal incidence and community composition of samples within a reservoir will more closely resemble each other than those from other reservoirs.

Materials and Methods

Organism, site selection and transect design. Three species, *Typha angustifolia, T. glauca* (*T. angustifolia* x *latifolia*), and *T. latifolia*, are present in the state of Kansas, and are known to readily hybridize (Kirk et al. 2011). In this study, we inventoried root-endogenous fungi in *Typha* at the peak of flowering (early June, 2014), within three Kansas catchment basins: University of Kansas West Campus (38°56'58"N, 95°15'48.86"W), Cross Reservoir (39° 3'8"N, 95°11'2"W) and Melvern Lake Outlet (38°30'40"N, 95°41'59"W). All three reservoirs are drainage catchment basins constructed in limestone parent rock; they are dammed along their

south aspect, and grassy vegetation atop the dam is mown throughout the growing season. Three transects were established along the dam in each reservoir, and extended from the deepest to highest incidence of *Typha* plants.

Sample collection and processing. Along each transect, we excavated three plants: the most deeply inundated, the most subaerially-exposed, and at the measured midpoint of each transect (Fig. 14). We also compared fungi in *Typha* roots fungi with fungi in grasses growing in immediate proximity at the upper terminus of each transect (Fig. 14). Above-ground growth was removed in the field, and rhizomes with attached roots were sealed in individual sterile bags and transported to the laboratory on ice, where they were washed of sediment, subsequent to surface sterilization by sequential immersion in 95% EtOH (10 seconds), 10% sodium hypochlorite (2 min), and 70% EtOH (2 min). Root clippings were taken from each plant 5 cm below the divergence of stem from rhizome.

Incidence of fungal structures and culturable fungi in roots. For each of three plants taken at every transect point (n = 108), we assessed the incidence of morphological structures by microscopic examination of *Typha* roots, and by culturing endogenous fungi. Surface-sterilized plant root clippings for microscopic examination were stored in 95% EtOH at -20 °C, then cleared with 10% potassium hydroxide (KOH), fuchsin stained, and permanently mounted to glass slides using Eukitt mounting medium (O. Kindler GmbH). Following the root intersection method (McGonigle et al. 1990), roots of each plant were examined across 200 intersections, for the presence of vegetative mycelia (comprising coenocytic/aseptate hyphae, hyaline septate hyphae, and dematiaceous hyphae) and other fungal structures (vesicles, conidiospores, or sporangia attributable to epi- and endobiontic chytrids). To assess community composition of culturable endogenous fungi, we aseptically plated surface-sterilized root clippings from each

plant on potato dextrose agar (PDA) and V8 agar supplemented with ampicillin. Resultant fungi were sequentially isolated on PDA until pure cultures (n = 83) were achieved. Pure cultures were photographed, morphotyped, and archived in ultrapure (PCR-grade) H_2O.

Statistical analyses. Differences in the incidence of microscopic fungal structures were assessed using generalized linear mixed effects models (GLMMs) and generalized linear models (GLM) with negative binomial distributions. GLMs were marginally favoured under AIC, BIC, and Vuong's *z*-stat (Vuong 1989) in goodness of fit tests (Table 1), however both the experimental design and null hypothesis expectations of spatial autocorrelation are better reflected using a random effect term that incorporates the transect point nested by reservoir. Mixed effect multinomial logistic regressions for each type of fungal structure were implemented with the GLMER.NB function, which builds on GLMER, a component of the LME4 1.1-18-1 package (Bates et al. 2015). We performed post-hoc general linear hypothesis testing of fitted models using multiple pairwise (Tukey) comparisons implemented with the GLHT function of the MULTCOMP package (Hothorn et al. 2008) in *R*. Explanatory significance of fixed and random effects was assessed with likelihood ratio tests.

To assess the community structure of 83 culturable root-endogenous fungal moprhotypes present in n = 108 samples, we performed NMS ordinations in PC-ORD 6.08 (McCune and Mefford 2011). Ordination parameters tested 6 axes, and the dataset was sampled in 250 runs. A random number specified starting coordinates, and the stability criterion was set as 0.000001; stability was evaluated with 10 iterations, to a maximum of 500 iterations, stepping down in dimensionality with an initial step length of 0.20. Tie-breaking penalized unequal ordination distances (Kruskal's secondary approach). PerMANOVA tests of variance were also conducted in PC-ORD, comparing experimental data against 10 000 random permutations.

Results

Incidence of microscopic fungal structures is not linearly correlated with inundation. All three types of vegetative mycelia were most prolific in roots taken from subaerially-exposed soils at the highest transect points (Figs. 15A–C). Rather than diminishing commensurate with increasing depth, however, the incidence of all hyphae only differed significantly between plants that were subaerially exposed, and those from inundated soils, regardless of inundation depth (P = <0.05 for all Tukey pairwise comparisons, Figs. 15A–C). Non-hyphal fungal structures did not evidence clear distinctions between roots taken from inundated versus subaerially exposed soils. Vesicles were rare to absent in all *Typha* roots (Fig. 15D), and neither conidiospores (Fig. 15E) nor chytrid fungi (Fig. 15F) varied significantly between samples (P = >0.05 for all Tukey pairwise comparisons, Figs. 15D–F). We tested inundation depth as a predictor of sample variance, and found it was: not predictive of the incidence of chytrids ($\chi^2(3) = 2.6147$, $P = 0.4549$), spores ($\chi^2(3) = 6.3949$, $P = 0.0939$), or aseptate hyphae ($\chi^2(3) = 7.0128$, $P = 0.07149$); marginally significant for hyaline hyphae ($\chi^2(3) = 7.6709$, $P = 0.05333$); and strongly predictive of the incidence of dematiaceous hyphae ($\chi^2(3) = 15.907$, $P = 0.001185$), which declined with depth.

Community composition does not vary with respect to inundation. We cultured 83 morphologically distinct fungi from $n = 108$ plants (Fig. 16); the composition of these communities did not differ with respect to inundation gradients ($F = 0.99920$, $P = 0.475037$). Ordination via nonmetric multidimensional scaling suggested a 4-dimensional solution (final stress= 19.67295, final instability = 0.00030 over 500 iterations), with four principal axes explaining r^2=0.548 of the variance (Axis 1, r^2= 0.1627; Axis 2, r^2= 0.1612; Axis 3, r^2= 0.1317;

Axis 4, r^2=0.0925). Samples from inundated transect points are indistinguishable from those in subaerially-exposed soils (Figs. 16A–B).

Geographic proximity does not structure fungal incidence or community composition. The incidence of fungi within plant roots did not vary significantly between reservoirs (aseptate: $\chi^2(1) = 0$, $P = 0.998995$; hyaline: $\chi^2(1) = 0.295$, $P = 0.7249593$; dematiaceous: $\chi^2(1) = 3.042$, $P = 0.1498147$; vesicles: $\chi^2(1) = 0$, $P = 0.9999984$; spores: $\chi^2(1) = 0.1183$, $P = 0.8367079$; chytrids: $\chi^2(1) = 0$; $P = 0.999524$). Community composition of culturable fungi (Fig. 3C) was also similar between reservoirs ($F = 1.2276$, $P = 0.159060$).

Discussion

Our study presents the first explicit investigation of inundation effects on the broader community of root endogenous fungi in wetland plants; previously, most research has focussed on the ecology of arbuscular mycorrhizal fungi in these biogeochemically stressful environments. Contrary to our predictions that incidence of root-endogenous fungi would be negatively correlated with inundation depth, we found that any degree of inundation diminished the incidence of hyphae compared to roots taken from subaerially-exposed soils; deeply inundated roots contained similar amounts of hyphae as shallowly-inundated specimens. These trends were apparent for all vegetative mycelia we examined, which comprised: simple-septate hyaline hyphae consistent with ascomycete pathogens and/or saprotrophs; dematiaceous septate hyphae attributable to dark septate endophytes; and aseptate or coenocytic hyphae, which may represent arbuscular mycorrhizal fungi, but could be attributed to other mucoralean fungi, as hyphal morphology alone cannot reliably distinguish AMF from other mucoromycotan taxa (Field et al. 2016). AMF are known to form associations with *Typha* (Stenlund and Charvat 1994, Wetzel and van der Valk 1996, Turner et al. 2000, Bauer et al. 2003, Dunham et al. 2003,

Ray and Inouye 2006), but this may be a facultative rather than obligate mutualism (Dunham et al. 2003, Janos 2007), as many studies also report absence of AMF in *Typha* roots (Anderson et al. 1984, Thormann et al. 1999, Cornwell et al. 2001). Because vesicles were rare in our *Typha* samples, but occasionally observed in the grass roots, we hold it more likely that the aseptate hyphae in *Typha* roots represent other mucoromycotan fungi, but cannot rule out the possibility that they are glomalean.

Our study demonstrates that a variety of fungi are suppressed when host roots are inundated, results which are substantially similar to reports of AMF response to inundation. Investigations employing hydrologic gradients (Anderson et al. 1984, Rickerl et al. 1994, Stevens and Peterson 1996, Miller and Bever 1999), or inferring hydrologic effects by sampling different basins with varying depths or soil moisture (Wetzel and van der Valk 1996, Bauer et al. 2003) have typically demonstrated that occurrence and intensity of AM colonization declines with depth and redox potential (Clayton and Bagyaraj 1984, Tanner and Clayton 1985, Khan and Belik 1995, Miller 2000). For AMF, however, factors like plant phenology (Bohrer et al. 2004), and possibly phosphorus availability (Ramirez-Viga et al. 2018) are more important drivers of colonization and diversity. Moreover, if previously established, i.e., during dry seasons, mycorrhizal associations in submerged roots do not appear to be affected by inundation (Miller and Sharitz 2000, Ray and Inouye 2006, but see Ipsilantis and Sylvia 2007). Some plants like *Typha latifolia* L. even exhibit increased AMF incidence following inundation (Ray and Inouye 2006), either due to increased availability of photosynthates (Li et al. 2004) or slower root growth under flooding, resulting in merely an apparent increase in colonization (Miller 2000, Ray and Inouye 2006). It should be noted, however, that arbuscules, which are indicative of active mycorrhizal associations, are rare in persistently inundated roots (Stevens and Peterson

1996, Ray and Inouye 2006). The presence of AM hyphae alone is therefore indicates neither plant-fungal interaction, nor active fungal growth, stipulations which may be true for the broader community of root-endogenous fungi in our study as well.

Previous studies have suggested that much as water depth and sediment anoxia structure aquatic plant communities (Spence 1982, Brix et al. 1992, Mitsch et al. 2009, Lemoine et al. 2012), above-ground zonation in plant tolerance may be mirrored by below-ground zonation of commensal fungi (Anderson et al. 1994, Khan and Belik 1995, Miller and Bever 1999, Choudhury et al. 2010). The apparent suppression of hyphae which we observed could conceivably result from changes in community composition, with exclusion of obligate aerobes and competitive release of facultative aerobic fungi. Our results, however, suggest otherwise: we observed exemplars of all three categories of hyphae in roots taken at all depths; the incidence of chytrids, which are facultative aerobes, did not vary with respect to inundation; and there were no significant differences between communities of cultured fungi. Our culture assays were consistent with previous metagenomic assays, which demonstrated that phylogenetically diverse root endophyte communities do not differ significantly between collection periods, among host plants, among reservoirs, or as a function of depth (Sandberg et al. 2014). Our observations of mycelial incidence in roots, taken in conjunction with culture assays, suggest that reduced mycelial incidence is suppression of root-endogenous fungi, and not simply exclusion of fast-growing obligate aerobes that might be expected to predominate in subaerially-exposed soils.

We hypothesize that low hyphal incidence in inundated *Typha* roots reflects germinal inhibition (Damm et al. 2003) or unsuccessful initial colonization, as has been suggested for AMF (Daniels and Trappe, 1980, Le Tacon et al. 1983, Saif 1981, 1983), where initial

colonization is strongly suppressed by inundation (Miller 2000, Miller and Sharitz 2000). Spores of AMF are abundant in wetland soils, and frequently concentrated in the wettest portions of hydrologic gradients (Khan 1974, Rickerl et al. 1994, Khan and Belik 1995, Miller and Bever 1999, Miller 2000, Miller and Sharitz 2000) where they may remain viable for many years (Wolfe et al. 2007). Rickerl et al. (1994) suggested that high spore numbers represent a stress response, whereas Miller and Bever (1999) maintained that spore number alone is uninformative compared to spore volume, which they found did not vary across inundation gradients. Asexual spores of other fungi are also abundant in wetland soils (Bettucci et al. 2002, Verma et al. 2003, Card and Quideau 2010), and conidiogenesis has also been considered a stress response (Chang et al. 2011). We had anticipated that asexual sporulation would be enhanced owing to environmental stress, but found no evidence that asexual conidiospores varied in response to inundation. Germination of spores entrained in wetland soils may be effected by extra-radicle oxygen leakage; indeed, *Typha* stands have been shown capable of oxidizing the entirety of the rhizosphere (Aldridge and Ganf 2003). High redox potential is, however, a condition subject to diel fluctuation in wetland soils: plants transport oxygen to their roots only while photosynthesizing, and surrounding sediments thus become anoxic and reducing at night (Sorrell and Dromgoole 1989, Caffrey and Kemp 1991), while residual pore-water oxygen is consumed by bacterial metabolisms (Jespersen et al. 1998, Vepraskas and Faulkner 2001, Nikolausz et al. 2008). Fungal spores germinating in the sediments around inundated roots would thus have a narrow temporal window for successful infection of the root environment.

In conclusion, our investigation of fungi endogenous to *Typha* roots illustrates that their abundance is impacted by inundation of the host plants' roots, regardless of depth, but that inundation does not impose a taxonomic filter. Communities of root-endogenous fungi may be

influenced more strongly by external environmental factors, than by the environments that plant roots comprise, as has recently been suggested of foliar endophytes (Whitaker et al. 2018). Future research should investigate whether trends identified here hold for root-endogenous fungi in other wetland plants, across a wider variety of hydrological regimes. Mechanistically, pot experiments which address root colonization with a view to redox conditions would be of great utility in determining whether apparent suppression of root-endogenous fungi results from germinal inhibition, or depressed mycelial proliferation. As we continue to develop a comprehensive understanding of plant-fungal interactions in biologically hostile settings, it is evidently necessary to consider not only the root environments that fungi inhabit, but also the extrinsic factors which may have broad impacts on fungal recruitment and colonization thereof.

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Figures

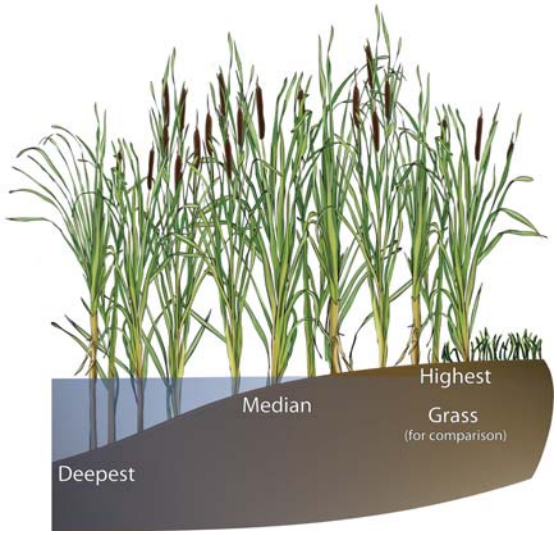

Figure 14: Experimental design. Three transects were established at each reservoir, and rhizomes with attached roots of three plants were taken from every sampling point: deepest- and highest-growing *Typha* plants, the measured median of each transect, and grasses growing adjacent to the highest-growing *Typha* plants (n = 108).

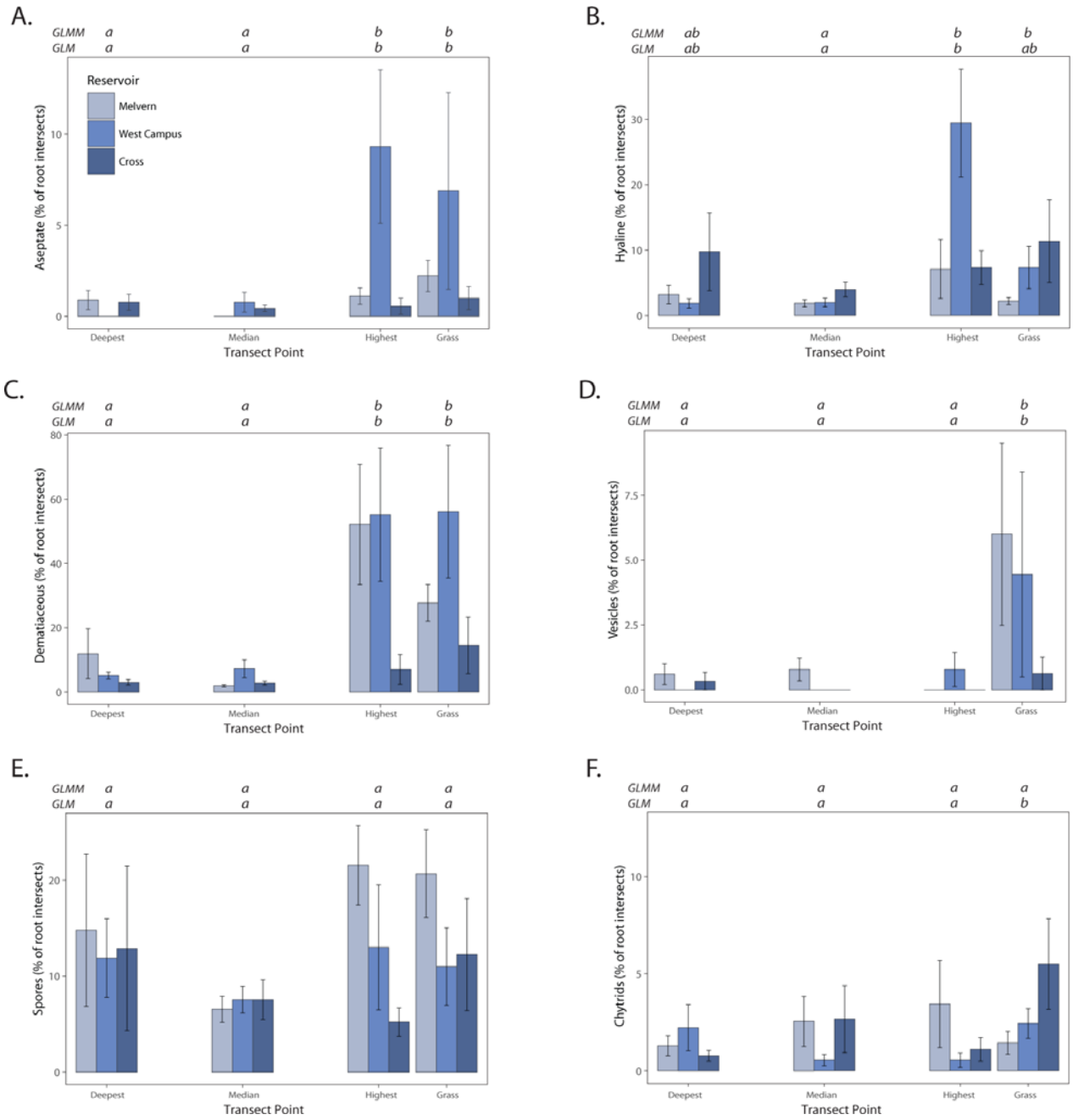

Figure 15: Incidence of fungal structures in plant roots. A. Aseptate hyphae, B. hyaline hyphae, C. dematiaceous hyphae, D. vesicles, E., asexual spores (=conidia/conidiospores), F. epi- and endo-biontic chytrid sporangia. X-axes reflect transect points, as per FIG. 14, lower y-axes express the percent of root intersects (as per McGonigle et al. 1990) that contain fungal structures of interest. Shading of bar graphs reflects reservoir identity, as detailed in FIG 15A. Error bars represent the standard error (SE) of the mean. Upper y-axes convey the results of Tukey post-hoc multiple pairwise comparisons (P = <0.05) for general linear mixed models (GLMM) and generalized linear models (GLM) with negative binomial distributions.

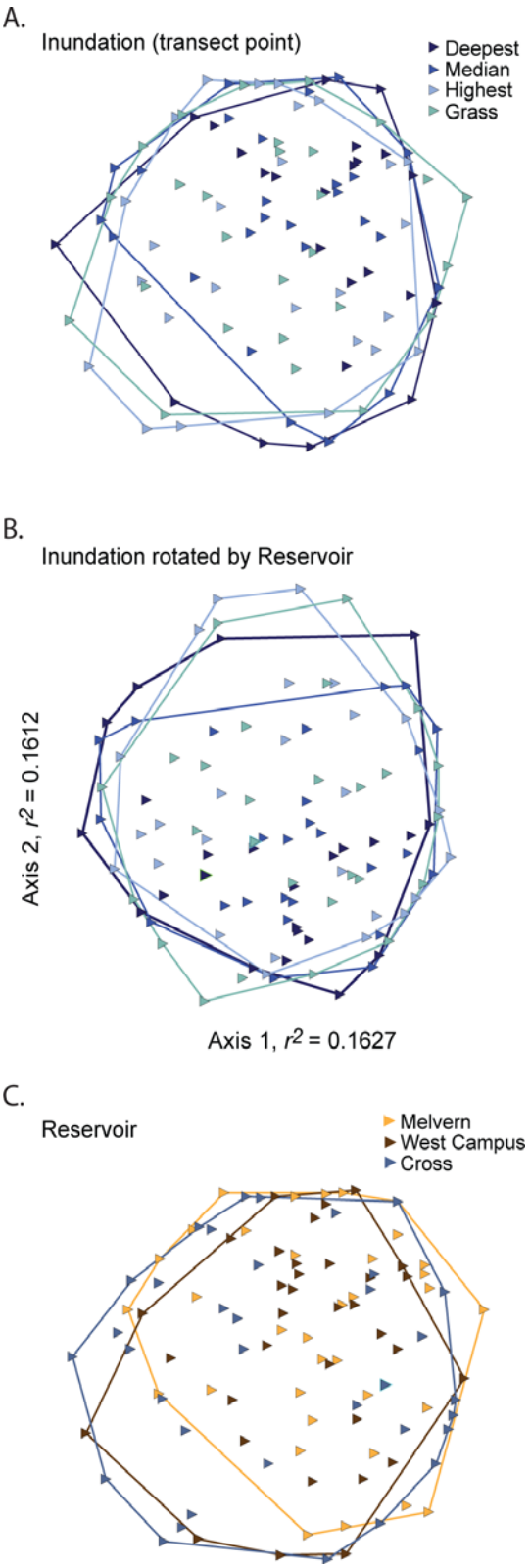

Figure 16: Nonmetric multidimensional scaling plot of n = 108 samples, scaled by n = 83 visibly distinguishable root-endogenous fungi cultured from surface-sterilized *Typha* roots. A., B., samples grouped by inundation, C. samples grouped by reservoir of origin

Tables

Table 1: Model Fitting

Model		*df*	AIC	BIC	Vuong *z*
Aseptate Hyphae					
GLM binomial	*Aseptate_hyp*	4	722.6285	733.3570	
GLM poisson	*Aseptate _hyp.p*	4	742.9651	753.6936	
GLM negative binomial	*Aseptate _hyp.nb*	5	340.8709	354.2816	z = 1.910, P = 0.028048
GLM zero-inflated neg binomial	*Aseptate _hyp.zinbn*	6	342.8709	NA	
GLMM binomial	*Aseptate _hyp.mixbn*	6	587.0708	603.1635	
GLMM poisson	*Aseptate _hyp.mixpois*	6	604.5271	620.6199	
GLMM negative binomial	*Aseptate _hyp.mixnegbn*	7	339.0815	357.8565	
Hyaline Hyphae					
GLM binomial	*Hyaline_hyp*	4	1481.7494	1492.4779	
GLM poisson	*Hyaline_hyp.p*	4	1559.3067	1570.0352	
GLM negative binomial	*Hyaline_hyp.nb*	5	621.5095	634.9202	z = 2.797, P = 0.0025763
GLM zero-inflated neg binomial	*Hyaline_hyp.zinbn*	6	623.5095	NA	
GLMM binomial	*Hyaline_hyp.mixbn*	6	1231.7489	1247.8416	
GLMM poisson	*Hyaline_hyp.mixpois*	6	1292.2353	1308.3281	
GLMM negative binomial	*Hyaline_hyp.mixnegbn*	7	621.4969	640.2718	
Dematiaceous Hyphae					
GLM binomial	*Dematiaceous_hyp*	4	2962.8254	2973.5539	

GLM poisson	*Dematiaceous_hyp.p*	4	3443.7569	3454.4854	
GLM negative binomial	*Dematiaceous_hyp.nb*	5	792.1705	805.5812	z = 3.163, P = 0.0007804
GLM Zero-inflated neg binomial	*Dematiaceous_hyp.zinbn*	6	794.1705	NA	
GLMM binomial	*Dematiaceous_hyp.mixbn*	6	2336.9054	2352.9982	
GLMM poisson	*Dematiaceous_hyp.mixpois*	6	2727.1929	2743.2857	
GLMM negative binomial	*Dematiaceous_hyp.mixnegbn*	7	786.7182	805.4931	

Vesicles

GLM binomial	*Vesicles_dist*	4	498.9037	509.6322	
GLM poisson	*Vesicles_dist.p*	4	510.0956	520.8241	
GLM negative binomial	*Vesicles_dist.nb*	5	188.3554	201.7660	z = -0.195, P = 0.42266701
GLM zero-inflated neg binomial	*Vesicles_dist.zinbn*	6	190.2396	NA	
GLMM binomial	*Vesicles_dist.mixbn*	6	458.1098	474.2025	
GLMM poisson	*Vesicles_dist.mixpois*	6	468.6953	484.7881	
GLMM negative binomial	*Vesicles_dist.mixnegbn*	7	192.3554	211.1303	

Spores

GLM binomial	*Spores_dist*	4	1672.2366	1682.9652	
GLM poisson	*Spores_dist.p*	4	1782.6289	1793.3574	
GLM negative binomial	*Spores_dist.nb*	5	766.2424	779.6531	z = 7.373, P = 0.23046
GLM zero-inflated neg binomial	*Spores_dist.zinbn*	6	768.2424	NA	
GLMM binomial	*Spores_dist.mixbn*	6	1591.2613	1607.3541	
GLMM poisson	*Spores_dist.mixpois*	6	1694.2588	1710.3516	
GLMM negative binomial	*Spores_dist.mixnegbn*	7	770.1241	788.8990	

Chytrids

GLM binomial	*Chytrids_dist*	4	613.6413	624.3699	
GLM poisson	*Chytrids_dist.p*	4	621.5244	632.2529	

GLM negative binomial	*Chytrids_dist.nb*	5	402.8511	416.2618	$z = 7.891$, $P = 0.21504$
GLM zero-inflated neg binomial	*Chytrids_dist.zinbn*	6	404.8511	NA	
GLMM binomial	*Chytrids_dist.mixbn*	6	581.3324	597.4252	
GLMM poisson	*Chytrids_dist.mixpois*	6	588.6327	604.7255	
GLMM negative binomial	*Chytrids_dist.mixnegbn*	7	406.8426	425.6175	

Chapter 7: Conclusion

In the course of this dissertation, I have: presented a re-investigation of putative bacterial fossils in the decayed petiole of a Carboniferous fern; described multiple saprotrophic and putatively endophytic fossil fungi from a silicified Eocene mire; and performed an investigation of fungal incidence and community structure within living aquatic plants, which data aids in interpreting the distribution and ecology of fossil fungi in wetland successions. These research projects are applicable to taphonomic questions, investigating microbes as the agents of decay, their roles in preservation, and using microbial fossils to establish temporal limits around the processes of fossilization. My ecological research also suggests that plant-microbe interactions during key periods of Earth's history must be assessed in light of the biogeochemical processes at work in wetland soils, which are not paralleled in most subaerially-exposed soils systems.

My re-investigation of Carboniferous structures originally described as actinomycete bacteria (Smoot and Taylor 1983) utilized a combination of investigative imaging technologies and comparisons with morphology of living actinobacteria to demonstrate that these structures are biomimetic carbonate minerals. Authigenic biomimetic carbonates may be precipitated abiogenically (Reitner 2004, Butler et al. 2008, Chen et al. 2009, Zhang et al. 2009, Yang and Xu 2011, Roberts et al. 2013), or in association with anaerobic sulfate reducing bacteria (Vasconcelos et al. 1995, Wright and Wacey 2005). Scanning electron microscopy with energy-dispersive spectroscopy suggests that the biomimetic structures are disordered ferrous dolomites, an interpretation supported by the dumbbell-shaped morphology of some crystals, which can be indicative of bacteriogenic dolomite (e.g., Warthmann et al. 2000, Van Lith et al. 2003). Pyrite also occurs within the inter-cellular spaces of the decayed fern petiole containing biomimetic structures, which provides further evidence that stages of permineralization were anoxic.

Chytrid zoosporangia that also occur in the plant tissue are covered in fine mineral precipitates, which are less prevalent near their discharge pores, suggesting that local mineralization began before the operculum was removed by zoospore discharge, and that some stages of mineralization were synchronous with saprotroph proliferation. Biomimetic structures are likely ubiquitous in permineralized plants, as similar structures have been described in other carbonate-permineralized plants (e.g., Andrews and Lenz 1943, Andrews 1945, Stewart 1951, Delevoryas and Morgan 1954, Rothwell and Taylor 1972, Rothwell 1980). I hypothesize that dolomitic replacement of plant kerogen is common in carbonaceous concretions, and that cellular permineralization occurs through multiple stages, the earliest of which may be bacteriogenically mediated.

My work with the fossil fungi of the Eocene (Ypresian, 48.7 Ma) Princeton Chert provides insight into the biostratinomy of this succession. In cataloguing an array of microfungi associated with *in-situ* aquatic or marginal plants of the Princeton mire, I have demonstrated that these plants likely reflect palimpsests of plant-fungal interactions. The enigmatic dicot, *Eorhiza arnoldii* Robison et Person (1973) is ubiquitous in most chert layers of the Princeton succession, and many *Eorhiza* fossils were permineralized in growth position (Pigg and Stockey 1996). Rhizomes of *Eorhiza* contain basipetal phragmospore-like chains of amerospores similar to *Thielaviopsis basicola* (Chapter 3), suggesting that this plant may have been prone to infection by root pathogens (e.g., Paulin-Mahady et al. 2002) during life. I also described microsclerotia and hyphae attributable to dark septate endophytes, the first fossil record for this heterogenous assemblage of plant commensals (Chapter 4). The putative endophytes may have persisted as part of the saprotrophic assemblage, which includes several facultative-aquatic hyphomycetes. Multiseptate, holothallic, chlamydospore-like phragmoconidia were observed in previous studies

(Robison and Person et al. 1973, LePage et al. 1994), and on the basis of new ontogenetic information, I consider it similar to extant *Xylomyces giganteus* (Chapter 3). I have also described two new hyphomycetes: The first are characterized by biseptate, chlamydosporic phragmoconidia with darkly melanized, inflated apical cells, which I deem morphologically similar to *Brachysporiella rhizoidea* or *Culcitalna achraspora* (Chapter 3). The second occurs in rhizomes of the semi-aquatic fern *Dennstaedtiopsis aerenchymata* Arnold et Daugherty (1964), and consists of vegetative hyaline or slightly pigmented hyphae producing a dense assemblage of >100 dematiaceous obovoid and muriform spores, which I have deposited within a new fossil taxon, *Monodictysporites princetonensis* (Chapter 5). These saprotrophic hyphomycetes are comparable to genera that are facultative aquatic taxa and consistently occur on submerged substrates (Rao and de Hoog 1986, Goh and Hyde 1996, Shearer et al. 2007, Buesing et al. 2009).

To establish appropriate ecological hypotheses for fossil fungi like those of the Princeton Chert, many of which are attributable to extant lineages or even species, it is necessary to complement their identification with an understanding of extant fungi in contemporary environments. As noted previously (Chapter 1), some of our most valuable insights about plant-fungal interactions in the fossil record come from localities that represent wetland communities, like the Rhynie and Windyfield Cherts of Scotland (Remy et al. 1994, Kenrick and Strullu-Derrien 2014, Selosse et al. 2015, Strullu-Derrien et al. 2017, Brundrett et al. 2018). Yet, little work has been done on understanding fossil microbes with respect to extant wetland soils, which are biogeochemically appropriate analogues. Because anoxic and reducing conditions predominate in wetland soils (Ponnamperuma 1984, Reddy and Delaune 2008), these environments impose biogeochemically hostile conditions on plant roots, and the fungal

communities endemic to them. Extant wetland plants mitigate hypoxia within their roots through a variety of physiological and anatomical mechanisms (Strand 2002, Gibbs and Greenway 2003, Sorrell and Hawes 2009, Pezeshki and Delaune 2012). Some strategies, like aerenchyma tissue formation, are common in the fossil record, as in the aquatically-adapted plants of the Princeton Chert (Cevallos-Ferriz et al. 1991), or Carboniferous lycopsids with extensive lacunar systems of aerenchyma (Rothwell 1984, Rothwell and Erwin 1985, Hetherington 2016).

My investigations of fungal incidence and diversity in living reedmace, *Typha* L. (Chapter 6), suggests that even pressurized convective ventilation, which is highly efficient at oxygenating roots and even the surrounding rhizosphere (Ray and Inoue 2006, Sorrell and Hawes 2009, Lemoine et al. 2012, Pezeshki and DeLaune 2012), does not support the same kind of fungal proliferation that is apparent in plant roots growing in subaerially-exposed soils. Whether this reflects germinal inhibition of spores in the surrounding inundated sediments (Le Tacon et al. 1983), or competition with plant cells for limited oxygen (Bedford et al. 1991, Chabbi et al. 2000, Matsui and Tsuchiya 2006), it is clear that hyphal incidence in roots is affected by inundation. Interestingly, the community composition of culturable root-endogenous fungi does not appear to be affected by inundation, at least in living roots. When addressing the fossil record, and the paleoecology of plants and microbes therein, the taxonomic identity of fossil microbes may be less informative to paleoenvironmental reconstruction than the incidence of body fossils. Studies of extant plant-fungal interaction under inundation should be expanded to additional taxa, including those used as modern analogues for Rhynie Chert plants, and investigation of fungal proliferation in moribund tissue under different redox conditions should also be undertaken to provide insight into biostratinomic parameters.

My work with the fossil fungi of the Eocene Princeton Chert succession also provides insight into early-diagenesis mineralization of plant tissues preserved by silicification. In characterizing the fungi that inhabited living and moribund tissues of these wetland plants, I have suggested that aspects of their development and distribution garner insight into timing of silicification of the Princeton plants. Some fossil spores were preferentially produced within intercellular spaces of cortical aerenchyma; ergo the host tissue was probably colonized quickly, before becoming so degraded as to become waterlogged. Although a number of phylogenetically disparate ascomycetes may remain metabolically active for some time in anoxic soils below the resident water table (Kurakov et al. 2008), mycelial proliferation is greatest in aerated wetland soils and peats (Golovchenko et al. 2002, 2013, Lin et al. 2012), where aerobically respiring fungi are the principal agents of decay (Thormann 2006, Thormann and Rice 2007). I consider it probable that most of the saprotrophic fungi observed thus far in the Princeton Chert colonized plant tissues either very soon after they died, or that colonization occurred in necromass entrained above the resident water table. Given that hyphae and spore walls contain chitin, a molecule highly resistant to degradation and known to be readily preserved in the fossil record (Briggs 1999, Flannery et al. 2001), the temporal window between fungal growth and permineralization could be significant, as surrounding plant cell walls provide protection against disarticulation; it is rare to be able to infer the timing between fungal proliferation and fossilization. As such, fossils like *Monodictysporites princetonensis* provide valuable taphonomic information: Because they are preserved in multiple, co-occurring developmental stages, we know that silicification in these specimens began during sporulation, and that initial stages were rapid, probably occurring within several days.

In several respects, the Princeton Chert assemblage presents a geological and taphonomic conundrum. Unlike silicified paleobotanical assemblages that originated as shallow wetlands near sinter-depositing hot-springs (e.g., the Devonian Rhynie Chert; Channing and Edwards 2003, 2009), which are well-recognized as sources of exceptional preservation within rapid time intervals (Kerp et al. 2003, Massini et al. 2016), cherts at Princeton are depauperate of heavy metals indicative of geothermal origins (Mustoe 2011). Although the succession of silicified coal-forming peats that comprise the Princeton Chert locality have been considered geologically unique (Mustoe, 2011), chert layers or lenses have been described in several other peat/coal deposits (Schopf 1970, Ting 1972, Sykes and Lindqvist 1993, Umeda 2003, Slater et al. 2014). Permian and Triassic silicified peats in the Beardmore Glacier region of Antarctica have long been recognized (Schopf 1970, Taylor et al. 1989), and a chert bed with abundant anatomically preserved plant and fungal fossils forms the uppermost unit of a Permian coal seam in East Antarctia (Slater et al. 2014). Thin bands and lenses of chert have been described from a Palaeocene lignite in North Dakota (Ting 1972), and from three lignitic through semi-anthracitic coalfields in New Zealand (Sykes and Lindqvist 1993). A Miocene succession in Fukui Prefecture, Japan, contains chert beds with anatomically preserved plants that alternate with unlithified peat and finely laminated mudstone (Umeda 2003). Silicified peats are therefore *not* rare, and occur in diverse palaeoenvironmental and stratigraphic contexts, suggesting this mode of plant preservation likely results from geochemical dynamics that are intrinsic to peat-forming depositional systems. Investigating microbes preserved within other silicified peats may elucidate whether rapid silicification is also a widespread feature of this mode of fossilization.

In conclusion, my dissertation research demonstrates that investigating plant tissues and the microbes inhabiting them expands our understanding of the biostratinomy and

early-diagenesis mineralization which contributes to fossilization of plants at cellular level of detail. I have catalogued components of the saprotrophic assemblage of the Princeton mire, some of which may have inhabited the living rhizomes of wetland-adapted plants that grew there. Through ecological investigation of fungi in contemporary wetlands, I have demonstrated that root-endogenous fungi of living plants are consistent across inundation gradients, but that their incidence in roots reflects whether those roots are inundated. This has clear implications for reconstructing the growth habit of exinct plants like *Eorhiza arnoldii*. I have also provided evidence that phases of both permineralization pathways, via silicification or carbonate precipitation, not only occurred rapidly, but that the earliest stages of permineraliztion were synchronous with microbial proliferation in these tissues. This research builds paths forward in better understanding the processes of information loss and preservation in the paleobotanical record.

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