Co-adaptations between Ceratocystidaceae ambrosia fungi and the mycangia of their associated ambrosia beetles

by

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DEDICATION

Dedicated to those that came before me, who achieved a sort of immortality through their study of the ambrosia symbiosis, just as the Greek gods of legend derived immortality from their ambrosia. Many of the taxa proposed herein are named in their honor. Also dedicated to those who should follow me, and continue to build upon history.

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ABSTRACT

In a reciprocal, obligate mutualism, ambrosia beetles cultivate gardens of fungi to extract nutrients from sapwood and carve a unique niche in this traditionally nutrient-poor substrate. This mutualism is the most ancient, widespread, and diverse system of insect agriculture. The beetles carry pure cultures of fungal spores to new trees using a variety of organs called mycangia. However, the diversity and evolutionary dynamics of these fungi and of the mycangia that carry them are poorly understood. Beetles with particularly large and specialized mycangia appear to associate with ambrosia fungi in the family Ceratocystidaceae (Microascales). Based on widespread geographic and taxonomic sampling of host beetles, isolation and characterization of their fungi, and molecular phylogenetics of these fungi, the diversity of ambrosia fungi and the evolution of ambrosia symbiosis in the Ceratocystidaceae are related to the development of mycangia in their respective beetle hosts. The mycangia of *Indocryphalus, Remansus, Anisandrus maiche,* and *Corthylus papulans* are newly characterized. Phylogenetic analyses supported six new genera and seventeen new species of fungi. Four unrelated lineages of ambrosia beetles with unique mycangia were found to carry ambrosia fungi in five genera of Ceratocystidaceae. *Ambrosiella* is associated with mesonotal mycangia of the *Xylosandrus* complex (Xyleborini) and the pronotal disk mycangium of *Remansus mutabilis* (Scolytoplatypodini). *Meredithiella* gen. nov. is associated with prothoracic coil mycangia of *Corthylus* (Corthylina). *Phialophoropsis* is associated with prothoracic pleural mycangia of *Trypodendron* (Xyloterini). *Toshionella* gen. nov. is associated with pronotal disk mycangia of Asian *Scolytoplatypus* (Scolytoplatypodini) and the prothoracic pleural mycangia of *Indocryphalus pubipennis* (Xyloterini). *Wolfgangiella*

gen. nov. is associated with pronotal disk mycangia of African *Scolytoplatypus.* Two unrelated symbionts, *Raffaelea* aff. *canadensis* (Ophiostomatales) and *Kaarikia abrahamsonii* gen. et sp. nov. (*incertae sedis*), are associated with oral and prothoracic basin mycangia, respectively, of *Xyloterinus politus* (Xyloterini). Sexual states are characterized for species of *Ambrosiella* and *Wolfgangiella*, the first report of sexual states in mycangial symbionts of ambrosia beetles. Co-adaption between fungal genera and mycangial type was evident, but not species-level co-evolution. It is proposed that there were at least three independant domestication events in the Ceartocystidaceae, the first domestication most likely with the Scolytoplatypodini.

CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

Dissertation Organization

The first chapter of the dissertation is a review of the literature on ambrosia fungi and the mycangia of ambrosia beetles. Chapters two through six are journal-formatted articles with individual methods, results, and discussion sections. Chapter two shows that three genera of ambrosia fungi in the Ceratocystidaceae are each associated with a different type of large mycangium. Chapter three reports the first sexual state in a mycangial ambrosia fungus, the *Ambrosiella* symbiont of the invasive ambrosia beetle *Anisandrus maiche*. Chapter four studies the symbionts of *Corthylus* ambrosia beetles in North, Central, and South America and characterizes mycangium diversity in *Corthylus*. Chapter five is a family-wide phylogenetic study of ambrosia fungi in the Ceratocystidaceae, enabled by the discovery of new ambrosia fungi from the tribe Scolytoplatypodini and a time-calibrated phylogeny comparing the estimated dates of origins of the five ambrosial genera to the origins of their associated mycangia. Chapter six is a study of the varied fungal symbionts and mycangia of ambrosia beetles in tribe Xyloterini. Chapter seven draws general conclusions from the results of the dissertation.

Section 1. Introduction

Ambrosia beetles comprise multiple lineages of wood-dwelling weevils (Curculionidae: Coleoptera: subfamilies Scolytinae and Platypodinae) that share the unique biology of boring tunnels (‘galleries’) into sapwood and cultivating gardens of fungi (‘ambrosia fungi’) (Fig. 1a) that serve as their primary source of sustenance (Baker 1963; Francke-Grosmann 1967; Beaver 1989; Hulcr and Stelinski 2017). Ambrosia fungi form thick layers of nutritious mycelium along the walls of the galleries that both beetles

and larvae feed upon (Francke-Grosmann 1963). The beetles gain a reliable source of food that allows them to extract energy from the nutrient-poor sapwood. In return, the adult beetles carry propagules of their fungal crops in special organs called 'mycangia' and bring their fungi to new trees. The fungi grow in and overflow from the mycangium to inoculate the walls of the galleries (Francke-Grosmann 1967; Schneider 1975; Six 2003). As partners in the most ancient and specialized of insect-fungus symbioses, ambrosia fungi and ambrosia beetles present a fascinating model system for the study of mutualism and co-adaptation.

Many reviews have touched on the ambrosia beetle symbiosis (Baker 1963; Francke-Grosmann 1963, 1967; Beaver 1989; Schneider 1991; Hulcr and Stelinski 2017), on mycangia (Francke-Grosmann 1963, 1967; Six 2003), on bark and ambrosia beetle associations with fungi (Francke-Grosmann 1967; Paine et al. 1997; Harrington 2005), and on ambrosia beetle phylogeny and diversity (Kirkendall et al. 2015; Hulcr et al. 2015). Batra (1985) also published a review on research trends and techniques for working with ambrosia beetles. This literature review will focus on ambrosia beetle mycangia and the ambrosia fungi they carry. Section 2 is a brief summary of the early history of research on ambrosia fungi, a period also reviewed by Baker (1963), Francke-Grosmann (1967), and Beaver (1989); Section 3 details broad aspects of the ambrosia symbiosis, including its ecology and evolution; Section 4 concerns the diversity of ambrosia beetle mycangia; Section 5 is an overview of the currently-known taxonomic groups of primary ambrosia fungi, their associated hosts and mycangia, and their role in the symbiosis; and Section 6 is an overview of gaps in understanding in the ambrosia symbiosis, especially those explored in the dissertation.

Section 2. Early History

The Austrian monk Josef Schmidberger first used “ambrosia” to denote a conspicuous crust he noticed in tunnels bored in wood by *Anisandrus dispar* (Schmidberger 1836). He incorrectly assumed the crust was dried sap processed by the beetles into food. Hartig (1844) realized the crust was fungal and later discovered that the ambrosia was the major diet of the beetles (Hartig 1872a, 1872b), but he incorrectly assumed the fungi spontaneously formed when tree sap mixed with beetle frass. Goethe (1895) provided the first microscopic illustration of an ambrosia fungus (Fig. 1b), and Hubbard (1897) wrote the first comprehensive review on ambrosia beetles and fungi. In addition to various insights on the beetles, Hubbard established several important facts about their fungi: (1) the ambrosia farmed by different ambrosia beetle species varies, but closely-related beetles farm similar fungi; (2) the occurance of ambrosia within the gallery is not accidental, and the fungus is somehow introduced by the mother beetles; (3) the ambrosia is specialized for insect feeding, with nutritious and easily-grazed

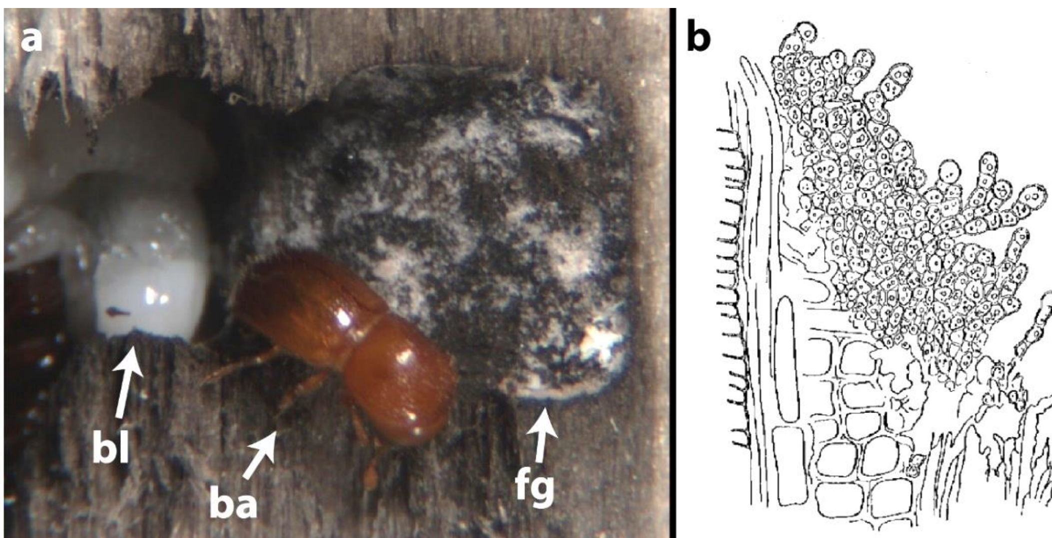

Figure 1. The ambrosia symbiosis. (a) Gallery of *Xylosandrus crassiusculus,* with beetle larvae (bl), adult beetle (ba), and fungal growth of *Ambrosiella roeperi* (fg). (b) The first known illustration of an ambrosia fungus, *Ambrosiella hartigii*. (a) Photograph by C. Mayers. (b) from Goethe (1895).

sporulation; and (4) the ambrosia is maintained in a delicate balance within the galleries by the ambrosia beetles, who keep the ambrosia healthy and pure through significant effort and cooperation. All four of these concepts have held and have been expanded upon in the century-plus since they were proposed.

Pioneering work on ambrosia fungi was conducted by Neger (e.g. 1908a, 1908b, 1908c, 1909), Beauverie (1910), and Schneider-Orelli (1911, 1913). Hubbard's revelation that these fungi must be introduced to wood by the beetles drove scientists to hypothesize that the fungi were carried superficially (Strohmeyer 1911) or in the gut (Schneider-Orelli 1911, 1913). Beeson (1917) supposed that ambrosia spores might adhere to the secretions produced by pits on the pronotum of *Genyocerus talurae*. However, transmission in the gut remained the commonly-accepted mechanism until Hadorn (1933) reported that guts of female *Trypodendron lineatum* were empty after winter, despite their effective transmission of ambrosia fungi. Nunberg (1951) correctly hypothesized that the pits observed by Beeson, the large organ of *Scolytoplatypus* illustrated by Berger and Cholodkovsy (1916), large paired organs he discovered in *Trypodendron,* and structures in other ambrosia beetles might all serve the common role of storing and transporting fungal propagules. Unaware of Nunberg's work, Francke-Grosmann (1956a), following up on Hadorn's findings, searched for cryptic internal cavities that might carry fungal propagules. In doing so she discovered the large transmission organs of *An. dispar, Xylosandrus germanus,* and *Trypodendron.* In this and follow-up studies (Francke-Grosmann 1956a, 1956b, 1958), she discovered other such organs and definitively proved that they stored masses of fungal propagules. Francke-Grosmann hypothesized that only ambrosia beetles that overwintered needed these organs, but tropical species of ambrosia

beetles were soon found to have them also (Baker 1963). Batra (1963) introduced the term "mycangium" to refer to any of the growing number of described transmission organs, including those described by Schedl (1962). Batra (1967) wrote the first comprehensive treatment on ambrosia fungi, and Francke-Grosmann (1967) produced a seminal review on ambrosia beetles, their fungi, and their mycangia.

Since 1967 there was sporadic study on ambrosia beetles and their fungi, including notable work by Schneider on mycangium dynamics (Schneider and Rudinsky 1969a, 1969b; Schneider 1975) and by Roeper on the diversity of North American ambrosia beetles and their fungi (e.g. Roeper and French 1981; Roeper 1996, 2011). There were two major catalysts responsible for stimulating interest in ambrosia symbiosis in the past two decades. The first was the availability of PCR and DNA sequencing in the 90s that allowed a better recognition of the diversity and classification of ambrosia fungi (Cassar and Blackwell 1996; Blackwell and Jones 1997; Harrington et al. 2010). The second cause of the renewed interest was the outbreak of the devastating laurel wilt, caused by the ambrosia fungus *Raffaelea lauricola* (Fraedrich et al. 2008; Harrington et al. 2008). Thanks to this renewed interest, there is now approximately one research article on ambrosia fungi or ambrosia beetles published per week (Hulcr and Stelinski 2017).

Section 3. The ambrosia symbiosis

Ecology

Insect agriculture

Mueller et al. (2005) defined four requirements for 'agriculture', and they considered only four groups of animals to meet these requirements: fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini: subtribe Attina), fungus-farming termites (Blattodea: Termitidae: Macrotermitinae), ambrosia beetles, and humans. The

four requirements included (1) **planting** their crops with the propagules of previous crops; (2) **cultivating** their crop by removing weeds, fertilizing, and controlling its growth conditions; (3) **harvesting** their crop to eat; and (4) **depending** obligately (or, in humans, facultatively) on the crop for nutrition. Ambrosia beetles are the oldest and the most diverse of these farmers. Fungus-farming originated once in termites at about 31 Ma (Roberts et al. 2016), and they farm a single fungal genus, *Termitomyces* (Agaricales: Lyophyllaceae) (Aanan et al. 2002; Nobre et al. 2011). Fungus-farming also originated only once in ants, at about 55–65 Ma (Branstetter et al. 2017), and they farm one of five lineages of basidiomycetous fungi in three clades (two in Leucocoprineae and one in Pterulaceae) (Schultz and Brady 2008; Branstetter et al. 2017). The single sublineage of fungus-farming ants with fully-domesticated, obligate fungi arose about 25 Ma (Schultz and Brady 2008; Branstetter et al. 2017).

In contrast to the single origins of farming in termites and ants, fungus farming beetles (i.e., ambrosia beetles) evolved from phloem feeding weevils (e.g., bark beetles) in at least 11 separate events (Farrell et al. 2001; Cognato et al. 2011; Jordal and Cognato 2012; Kirkendall et al. 2015; Hulcr and Stelinski 2017). The first origin was in Platypodinae at an estimated 96 Ma, followed by the first of multiple origins in the Scolytinae at an estimated 48–60 Ma (Jordal and Cognato 2012; Jordal 2015; Gohli et al. 2017; Pistone et al. 2017). The numerous evolutionary jumps and the more than 3000 described ambrosia beetle species (Hulcr and Stelinski 2017) attest to the effectiveness of the symbiosis. Central to the ambrosia beetles' success are mycangia, which guarantee cultivar specificity and vertical transmission to a degree that is unmatched in other fungus-farming insects. Fungus-farming ants have infrabuccal pouches to enable verticle

transfer of their fungi, but these pouches are found in all groups of ants (Mueller 2002; 2005). Fungus-farming termites capture their symbionts horizontally from the environment, with two exceptional clades that practice clonal vertical transmission without mycangia (Aanen et al. 2002).

Other insects use mycangia to maintain fungal symbionts, but these insects may or may not not practice true agriculture. Females of ship-timber beetles (Lymexylidae) have mycangia near their ovipositors that inoculate their eggs with *Alloascoidea* symbionts (Saccharomycetales) (Kurtzman and Robnett 2013), which then spread with the larvae as they bore their galleries (Batra and Francke-Grosmann 1961, 1964; Francke-Grosmann 1967). This symbiosis is exceptional within Lymexylidae; fellow lymexylid *Melittomma insulare* does employ a mutualistic relationship with a yeast/bacteria complex in coconut (Brown 1954), but this symbiosis is less specialized (Baker 1963) and may not involve mycangia. Some bark beetles use the ambrosia growth of their ascomycetous and basidiomycetous symbionts to supplement their nutrition, and most of these beetles have mycangia for selective vertical transmission (Francke-Grosmann 1967; Harrington 2005; Hofstetter et al. 2015), but it is not clear if the bark beetles tend to their crops. A recently-suggested fungus farmer, *Doubledaya bucculenta* (Coleoptera: Erotylidae: Languriinae) uses mycangia to maintain a relationship with an apparent yeast cultivar in bamboo (Toki et al. 2012). Certain female wood wasps or horntails (Siricidae and Xiphydriidae) have mycangia and inoculate wood with wood decay fungi during egg-laying, and the larvae eat the decayed wood (Baker 1963; Francke-Grosmann 1963, 1967; Talbot 1977). Larvae of the ambrosia gall midge rely on thick ambrosia growth in their galls, but it is unclear if they introduce the fungus themselves (Kobune et al. 2012). The

leaf-rolling weevil *Euops* (Attelabidae) is the only other curculionid outside of the bark and ambrosia beetles with a well-supported mycangium (Grebennikov and Leschen 2010), but it does not feed on its symbiont (Kobayashi et al. 2008).

Two genera of ambrosia beetles, *Ambrosiodmus* and *Ambrosiophilus* (Xyleborini), tunnel in wood decayed by a basidiomycete, *Flavodon ambrosius* (Kasson et al. 2016; Simmons et al. 2016b; Li et al. 2017). The fungus is found in the beetles' oral mycangia but does not form ambrosia growth in the galleries. Instead, the larvae apparently feed on the decayed wood. The *Flavodon* farmers do not quality for Mueller et al.'s (2005) definition of agriculture, but instead represent an interesting alternative strategy for weevils that is similar to that employed by the mycangial wood wasps/horntails and their decay fungi.

An obligate symbiosis

Ambrosia beetles are obligately dependent on their fungal cultivars for nutrition (Francke-Grosmann 1967; Six 2003; Beaver 1989; Schneider 1991), and the beetles' galleries are usually dominated by primary mycangial symbionts (Batra 1967, 1985; Nakashima et al. 1987; Kinuura et al. 1991; Kinuura 1995; Gebhardt et al. 2004; Harrington et al. 2014). In the period between initial gallery construction and the establishment of the ambrosia, the gallery founder does not eat and will eventually starve if the ambrosia fungus fails to establish (Brader 1964; Francke-Grosmann 1967). Failure of the ambrosia could be due to one of several factors, but perhaps the most important are moisture content and temperature; both have narrow limits for suitable ambrosia growth, outside of which the gallery will fail (Cachan 1957 as cited in Francke-Grosmann 1967; Schneider 1991). Ambrosia beetle larvae either feed exclusively on fungi (mycetophagy) or on both fungi and wood (xylomycetophagy), whereas adults usually are

xylomycetophagous (Francke-Grosmann 1963). The wood ingested by adults (or larvae, while assisting with gallery enlargement) is not necessarily digested, and the larvae of some species are so adapted to fungus feeding that their mandibles are weak and physically unable to macerate wood (Francke-Grosmann 1967; Beaver 1989).

Most ambrosia beetle species practice some form of parental care, at a minimum by tending to the ambrosia as their larvae develop in the gallery, thus helping to maintain a pure fungal culture. The beetles actively remove contaminating fungi from their galleries, either mechanically or chemically (Francke-Grosmann 1967). In some beetle species, multiple generations collaborate in tending the gallery and maintaining the ambrosia (Biedermann and Taborsky 2011; Biedermann et al. 2013), and one species is known to be eusocial (Kent and Simpson 1992). Gallery purity may change during the life cycle of the beetles (Kajimura and Hijii 1992; Yang et al. 2008), and there may be a succession of other fungi (Kinuura et al. 1991; Kinuura 1995), especially once the beetles cease to actively maintain the ambrosia or abandon the gallery (Francke-Grosmann 1967). Other fungi are often found in galleries, even when the ambrosia appears healthy, and these other fungi may contribute to the symbiosis (Haanstad and Norris 1985). Howeve, such fungi are generally also found associated with non-ambrosial bark beetles and should be considered auxiliary fungi. Ubiquitous contaminants, such as *Fusarium* spp., can be carried superficially on the beetles or rarely as interlopers in mycangia (Francke-Grosmann 1967; Bateman et al. 2016), and may be sparse in healthy ambrosia growth. However they can grow aggressively in culture, which can obscure ambrosia fungi in isolations and may lead to inaccurate assessments of associations (Francke-Grosmann 1967). Established ambrosia fungi may be able to exclude weed fungi from

entering the gallery from the surrounding sapwood (Baker 1963; Castrillo et al. 2016), and wood ethanol content may also play a role in controlling gallery fungi (Ranger et al. 2018).

Evolution

Origins of the ambrosia symbiosis

Most ambrosia beetle progenitors were apparently phloem-feeding bark beetles (Jordal 2015; Kirkendall et al. 2015), and early ambrosia beetles in most lineages likely fed on the nutrient-poor wood as well as on phloem- and wood-associated fungi. Delving deeper into the sapwood and farming fungal gardens may have allowed emerging lineages to take advantage of a new ecological niche, away frome competition with bark beetles and other insects in the nutrient-rich inner bark (Harrington 2005; Six 2012). There are two theories on the origin of ambrosia beetle-fungus symbioses (Six 2012). The first, the "transmission first model," sees the fungi first adapt (perhaps using sticky spore drops) to being transmitted by the beetles. The beetles then adapt to the food source they have been contaminated with, and eventually become dependent on it (Mueller et al. 2005). The second, the "consumption first model," hypothesizes that beetles began feeding on fungal contaminants in their galleries, became dependent on them, and in the meantime the fungi adapted transmission strategies that increased both their hosts' and their own fitness.

Ambrosia fungi were likely domesticated from ancestors such as *Ophiostoma sensu lato* (Ophiostomatales) and *Ceratocystis sensu lato* (Ceratocystidaceae), many of which are known to be intimately but not obligately associated with bark beetles (Harrington 2005). These taxa have fruiting structures and spores specifically adapted to stick to the exterior cuticle of bark beetles in order to be transmitted to new trees, and

these fungi were likely present when the ambrosia beetle ancestors first began to domesticate fungal symbionts. Fungi with large, nutritious spores (Harrington 2005) and dimorphic growth (a yeast-like phase for growth in mycangia) would have selective advantage as ambrosia beetle associates. The most grazed-upon species may have been eventually domesticated and gained traits that would make them better food for the beetles. However, these traits may have been at the cost of a loss of the ability to live and disperse freely. The specialized fungi may have then been traded among ambrosia beetles in other genera or tribes. This pattern appears to hold true for the large and diverse genus *Raffaelea* (Vanderpool et al. 2017), and similar patterns may be seen in the small clade of *Fusarium* associated with ambrosia beetles (Kasson et al. 2013; O'Donnell et al. 2015).

The conidia and conidiophores of ambrosia fungi may be large and specialized for beetle grazing, and they may contain abundant lipid bodies, as in the conidiophores and aleurioconidia of *Ambrosiella* (Harrington 2005; Harrington et al. 2010, 2014). Ambrosia growth may provide special nutrients, such as sterols or lipids, that are necessary for insect development (Norris et al. 1969; Kok 1979; Six 2003). Studies have also examined the role of the fungi in concentrating nitrogen, as the nitrogen content of wood would likely be limiting for the beetles (Abrahamson and Norris 1970; Roeper and French 1981; Six 2003). Many ambrosia fungi also produce secondary metabolites, including aromatic fruity esters and deeply pigmented liquids (Francke-Grosmann 1967), and ambrosia beetles may be attracted to the scents of these fungi (Hulcr et al. 2011).

In order to fill and emerge from mycangia, the mycangial symbionts must be able to grow either in a yeast-like phase (Francke-Grosmann 1956a, 1958; Lhoste and Roche 1959; Batra 1967; Harrington et al. 2008, 2010) or as thallic-arthric hyphal fragments

(Batra and Michie 1963; Harrington et al. 2014; Bateman et al. 2017; Li et al. 2017). The mycangia are filled by glandular secretions that presumably select for the growth of the symbionts (Schneider 1975). Francke-Grosmann (1975) presented an exception in *Raffaelea sulphurea*, which is transmitted in the gut of *Xyleborinus saxeseni*. But most ambrosia fungi are dimorphic (Batra and Michie 1963; Francke-Grosmann 1967; Beaver 1989), which allows them to grow in a yeast-like stage inside the mycangium and transition to a filamentous form in the gallery to penetrate the wood, extract nutrients, and eventually form ambrosia.

Young adult beetles fill their mycangia with the fungal propagules lining their natal galleries (Beaver 1989), so their mycangium contents should be derived from the same fungi brought into the gallery by their parents. Ambrosia fungi have been assumed to be asexual, clonal lineages (Farrel et al. 2001; Normark et al. 2003; Harrington 2005; Harrington et al. 2010; van de Peppel et al. 2018), which limits their ability to escape the symbiosis. This links the ancestry of ambrosia beetle farmers and their fungal cultivars through vertical inheritance, which allows for progressive domestication of the fungi by the beetles over many generations. Under these conditions, one would expect the symbiosis to move towards species-specificity (Mueller et al 2005; van de Peppel 2017). This has been questioned, however (Batra 1966; Francke-Grosmann 1967; Bateman et al. 2015; Li et al. 2017). Generally, *Raffaelea* species appear to often associate with multiple ambrosia beetle species in unrelated tribes, whereas *Ambrosiella* may have more specific associations (Harrington et al. 2010, 2014; Kostovcik et al. 2015).

Defining ambrosia fungi

The term ‘ambrosia fungus’ has not been used consistently, and by itself is an ambiguous and imprecise designation. The term ‘fungal symbiont’ is also inadequate

because symbiosis can include any fungus associated with the beetles, as discussed by Skelton et al. (2018). Batra (1985) introduced two terms: 'primary ambrosia fungus' for co-adapted mycangial fungi that dominate tunnels of the beetle during peak growth of the brood and are fed upon by larvae; and 'auxiliary ambrosia fungus' for fungi present facultatively in the galleries that are accidentally associated with ambrosia beetles and carried superficially. Batra's concept of 'primary ambrosia fungus' was sound, except for his argument that primary ambrosia fungi are always species-specific, which has been questioned for both *Raffaelea* (e.g. Gebhardt et al. 2004; Harrington et al. 2010) and *Ambrosiella* (Lin et al. 2017). Instead of 'auxiliary ambrosia fungus', it may be more appropriate to simply use 'auxiliary fungus' or 'weed fungus'. The expansion of 'ambrosia fungus' to include all fungi that may be fed upon and transmitted in mycangia of bark beetles and the use of 'ambrosial mutualist' as the specific term for closely-adapted symbionts (e.g. Hulcr and Stelinski 2017) is confusing and unnecessary, as already discussed by Francke-Grosmann (1963). For the purposes of this dissertation, 'ambrosia fungus' is the equivalent of 'primary ambrosia fungus' and 'mycangial symbiont', and is restricted to those fungi that (1) are associated with and fed upon by ambrosia beetles; (2) are primary, co-adapted mycangial symbionts of their associated beetles; (3) dominate ambrosia growth in galleries of the beetle during brood development; and (4) are not found as free-living species.

The majority of studied ambrosia fungi are placed in the genera *Raffaelea* (Ophiostomatales) or *Ambrosiella* (Microascales: Ceratocystidaceae), though there is evidence for a recent clade of ambrosia fungi in the genus *Fusarium* (Hypocreales: Nectriaceae); all three groups are discussed in Section 5. Species of *Geosmithia* with

large conidia were found dominating ambrosia beetle galleries of *Eupagiocerus dentipes, Cnesinus lecontei,* and *Microcorthylus* sp. (Kolařík and Kirkendall 2010; Kolařík et al. 2015), and these may represent true ambrosia fungi, but their associations with mycangia have not been confirmed.

Yeasts are often associated with bark beetles and their galleries (Davis 2015), as well as ambrosia beetles (e.g. Batra 1963, 1967; Francke-Grosmann 1963; Baker and Kreger-van Rij 1964; Batra and Francke-Grosmann 1964; Giese 1967; Baker and Norris 1968; van der Walt 1972; Endoh et al. 2008; Six et al 2009; Suh and Zhou 2010; Ninomiya et al. 2013; James et al. 2014). However, yeasts are unlikely to be primary ambrosia fungi because they do not form dense ambrosial growth (see Chapter 4 for further discussion). Many other fungi are associated superficially or antagonistically with both ambrosia beetles and bark beetles (Harrington 2005) but are outside of the scope of this study. The so-called ambrosia fungi of ship-timber beetles (Batra and Francke-Grosmann 1961, 1964; Francke-Grosmann 1967), ambrosia midges (Francke-Grosmann 1967; Kobune et al. 2012), and others are also outside of the scope of this dissertation.

Section 4. Mycangia

Each lineage of ambrosia beetles has at least one type of mycangium, which serves several purposes (Francke-Grosmann 1967; Schneider 1975). They protect the valuable but vulnerable ambrosia propagules from desiccation (Batra 1963; Francke-Grosmann 1963), allow ambrosia beetles to retain their symbionts while overwintering (Francke-Grosmann 1963, 1967), and perhaps most importantly, allow ambrosia beetles to bring their specialized fungal cultivars with them to new trees (Batra 1963; Francke-Grosmann 1963; Beaver 1989). Mycangia vary dramatically in size, shape, and where they are located on the beetles' bodies (Francke-Grosmann 1963, 1967; Schneider 1991;

Hulcr et al. 2015). Each separate evolution of an ambrosia beetle lineage was apparently coincidental with the development of a novel mycangium (Hulcr and Stelinski 2017).

The fungal inoculum inside mycangia is typically a pure culture of ambrosia fungi (Gebhardt et al. 2004; Harrington et al. 2010; Harrington et al. 2014). However, as in the galleries, fungal purity and conditions inside the mycangium changes seasonally and over the course of the beetles' life cycle (Kajimura and Hijii 1992; Schneider and Rudinsky 1969a, 1969b; Schneider 1991), and there can be interlopers (Batra 1963; Kinuura 2002; Bateman et al. 2016; Lynch et al. 2016).

Propagules of the primary ambrosia symbiont divide and multiply in the mycangium, with the fungus fed by nearby glandular secretions that are often referred to as 'oily' or 'waxy' (Francke-Grosmann 1956a; Batra 1963; Francke-Grosmann 1963; Schneider 1975; Beaver 1989). Some mycangia may have developed in locations where glandular cells were already present, such as locations of joint or boring lubrication (Francke-Grosmann 1967). Mycangium contents may include fatty acids, phospholipids, free sterols, sterol esters, and triglycerides, as well as an abundance of amino acids such as alanine, valine, and especially proline (Abrahamson 1969; Norris 1979). However, the specific chemicals produced and secreted by the gland cells are unknown, though Francke-Grosmann (1956a) found that the glandular secretions were a clear, slightly acidic, oily liquid. Gland cells were described in detail for *Anisandrus dispar* (Happ et al. 1976), *Platypus cylindrus* (Cassier et al. 1996), *Gnathotrichus* spp. (Schneider and Rudinsky 1969b), and *Cnestus mutilatus* (Stone et al. 2007). Similar glands have been described in bark beetle mycangia, e.g. those of *Dendroctonus* (Six 2003).

Schneider (1975) carefully dissected different sections of different types of mycangia at different times and found that the fungal growth within mycangia follows a similar pattern. First, a few fungal cells enter the opening of the mycangium; next, the cells produce hyphae that disarticulate into individual propagules; finally, mycangium-associated gland cells activate and begin to secrete, prompting the ambrosia propagules to propagate and fill the organ. The size and activity of the gland cells increase when activated, with a corresponding change in the morphology of the mycangial propagules (Schneider and Rudinsky 1969a, 1969b). The gathering of spores into the mycangium may be nonselective, but only the ambrosia fungus is able to grow and proliferate in the mycangium (Beaver 1989). Schedl (1962) and Batra and Batra (1967) reported that immature adults of many species perform distinct rocking movements in order to force spores into their mycangia, and Stone et al. (2007) reported similar movements in *Cnestus mutilatus*. Kaneko (1967) observed that when *Xylosandrus germanus* performed such movements, the pocket-like mycangium turned inside-out, collected spores from the gallery walls, and was then reverted with spores attached. Kent (2008) proposed an interesting method in *Austroplatypus incompertus* in which setae adorning each of the many mycangial pits guide single spores into the adjacent pit when the beetle's body scrapes the gallery walls. Kent supposed that this mechanism may be analogous in other platypodines with similar pit setae.

Generally, only one sex has mycangia, and usually this is the female (Francke-Grosmann 1967). In the Corthyline genera *Corthylus* and *Gnathotrichus,* the male has the mycangium (Francke-Grosmann 1967). Typically it is the sex that initiates gallery construction that has the mycangia, though there are exceptions in the Scolytinae and in

much of the Platypodinae (Francke-Grosmann 1967). In *Xyloterinus politus* both sexes have oral mycangia in addition to the female's prothoracic mycangia (Abrahamson and Norris 1966, 1969), and many platypodids have mycangia in both sexes or have more than one mycangium type (Nakashima 1971). There has been an apparent reduction in mycangia in multiple multiple ambrosia beetle genera that have become mycokleptics and bore their galleries directly adjacent to ambrosial galleries so that the ambrosia fungus grows through the wood and along their walls of the kleptic (Hulcr and Cognato 2010). Mycangium reduction has also been reported in a lineage within *Camptocerus* (Scolytini) associated with a possible reversion to phloem feeding (Smith 2013), and some species of *Scolytoplatypus* (Scolytoplatypodini) apparently lack mycangia (Beaver and Gebhardt 2006).

When Batra (1963) first used the term "mycangium" it was defined as sac- or cup-shaped cavities on the exterior of ambrosia beetles for the purpose of holding growing fungal propagules. Batra (1963) and Francke-Grosmann (1963) categorized mycangia by their location on the beetle, and Francke-Grosmann (1963, 1967) reviewed the different types in detail. Subsequent authors broadened the term to include a wider range of structures (e.g. Farris and Funk 1965; Livingston and Berryman 1972; Nakashima 1975), and Furniss et al. (1987) broadened the definition further to "…any repository of the insect cuticle that is adapted for the transport of fungus." Six (2003) proposed a hierarchical classification of mycangia that first separated them into 'pit', 'sac', or 'setal' mycangia, next into glandular or non-glandular, and then into their location on the body. Hulcr et al. (2015) restricted 'mycangium' to refer only to glandular sac mycangia. Some bark beetles utilize mycangia with glands (Francke-Grosmann 1967; Six 2003;

Harrington et al. 2005), but the use of 'mycangium' in this dissertation is generally restricted to the glandular mycangia of ambrosia beetles.

Non-glandular mycangia

Non-glandular mycangia are smaller and are often merely crevices, shallow pits, or setal tufts (Six 2003). However, neither the presence of an exoskeleton cavity in a mycophagous beetle nor the circumstantial presence of fungal spores in such cavities is adequate evidence for its utility as a mycangium (Grebennikov and Leschen 2010). *Scolytodes unipunctatus* has shallow, non-glandular pits, was associated with *Raffaelea scolytodis* and other fungi, and has no other known mycangium (Hulcr et al. 2007b; Kolařík and Hulcr 2009). *Scolytodes unipunctatus* is the only ambrosial species in its genus, so its biology needs further study (Hulcr and Stelinski 2017). *Cnestus mutilatus* has non-glandular scutellum pits (Stone et al. 2007), but it also has a large mesonotal mycangium (Stone et al. 2007) that carries an *Ambrosiella* symbiont (Six et al. 2009). If fungi are carried in the *C. mutilatus* scutellum pits, the pits only serve a secondary role. Certain species in the tribe Bostrosternini (*Bosthrosternus*, *Eupagiocerus*, and *Cnesinus*) have setose patches on the proepisternum that are implicated as mycangia (Hulcr and Stelinski 2017). However, none of the three genera of Bostrosternini are solely composed of ambrosia beetles, and the ambrosial status of certain species remains ambiguous (Wood 2007). *Phloeoborus* (Hylesinini) has similarly-described mycangia and unknown symbionts (Hulcr and Stelinski 2017).

Glandular mycangia

Pronotal pit mycangia

Ambrosia beetles in the Platypodinae have a variety of mycangium types (Nakashima 1975), the most well-characterized of which are pits in the cuticle of the

pronotum associated with active secretory glands (Beeson 1917; Roche and Lhoste 1960; Farris and Funk 1965; Nakashima 1972, 1975; Cassier et al. 1996; Moon et al. 2008, 2012). Gland cells are found on the underside of the cuticle and feed secretions through small tubules to each pit (Cassier et al. 1996; Kent 2008), or there may be several gland cells directly associated with the walls of larger pits, each with a separate secretory channel to the pit (Lhoste and Roche 1961). The number, size, and arrangement of pits varies considerably between genera, within genera, and even within species of Platypodinae (Francke-Grosmann 1967; Nakashima 1972, 1975; Wood 1993; Belhoucine et al. 2013). For example, *Diacavus philippinensis* has only two large pits (Nakashima 1975), whereas in *Platypus koryoensis* there are six large pits that hold large masses of fungal propagules flanked by many smaller pits (Moon et al. 2008, 2012). In *Genyocerus talurae* there are one to two dozen pits that each hold masses of fungal propagules (Beeson 1917). In *Austroplatypus incompertus* there are many pits (in some cases more than 80) in a concentrated patch on the pronotum, and these pits are much smaller, each only large enough to fit a single fungal spore (Kent 2008). The mycangial pits are present in only the female in some Platypodinae, but the pits are sometimes present in both sexes or are reduced in size or number in the male (Farris and Funk 1965; Kent 2008; Moon et al. 2008; Belhoucine et al. 2013). Often, each pit is accompanied by a seta that hold the fungal propagules in place (Kent 2008), and some of these setae are quite elaborate, such as the fan-like pit setae of *Diapus quinquespintus* (Nakashima 1975).

Oral mycangia

Perhaps the most commonly evolved type of mycangia (Beaver 1989; Hulcr et al. 2015; Hulcr and Stelinski 2017) are oral/preoral pouch mycangia, which are small pockets in or near the mouth. Paired bilateral preoral mycangia are found in several

genera in the Xyleborini (Fernando 1959; Francke-Grosmann 1963; Schedl 1962, 1963; Schneider 1987; Hulcr and Stelinski 2017), both sexes of the xyloterine *Xyloterinus politus* (Abrahamson and Norris 1966), and in *Premnobius cavipennis* (Ipini) (Schedl 1962; Bateman et al. 2017). Schedl (1962) illustrated an unpaired, medial oral pouch mycangium is *Pterocyclon bicallosum* (now *Monarthrum bicallosum,* Corthylina; Wood 2007). A mycangium opens into the oral cavity of the platypodine *Crossotarsus niponicus* (Nakashima 1971, 1975); the mycangium consists of a thin membranous cavity wrapped around a conspicuous spherical body (Nakashima 1979).

Elytral mycangia

Elytral mycangia are small, sclerotized cavities on the anterior aspect of the elytra whose openings are fenced with setae. So far, elytral mycangia have been characterized only in *Xyleborinus gracilis* and *X. saxeseni* (Francke-Grosmann 1956a, 1956b, 1967; Schedl 1962; Hulcr et al. 2007a; Biedermann et al. 2012), but they may be present in other Xyleborini (Hulcr et al. 2007a). These mycangia are surrounded by gland cells (Schedl 1962). Though found in a somewhat similar location to mesonotal pouch mycangia, elytral mycangia are posteriad rather than anteriad to the scutellum.

Mesonotal pouch mycangia

A monophyletic group in tribe Xyleborini comprising the genera *Anisandrus, Cnestus, Diuncus, Eccoptopterus, Hadrodemius,* and *Xylosandrus* (hereafter the '*Xylosandrus* complex') have similar pouch-like mesonotal mycangia (Hulcr et al. 2007a; Hulcr and Stelinski 2017). The mycangium is formed by invaginations of the integumental membrane that connects the dorsal side of the pronotum to the mesonotum, that is, the anterior edge of the scutellum (Francke-Grosmann 1956a, 1956b, 1967; Schedl 1962; Stone et al. 2007; Li et al. 2018). The general morphology of these

mycangia is similar among genera of the *Xylosandrus* complex, and their mycangia presumably share a single evolutionary origin. The mycangia differ in the absence or presence of dual lobes and whether the pouch extends forward into the pronotum or underneath the scutellum (Francke-Grosmann 1963). In *Xylosandrus* the mycangium pouch is comprised of two large lobes in the prothorax anterior to the scutellum that are connected to each other and the mycangium entrance via a central bridge, and only a portion of the bridge is under the scutellum (Francke-Grosmann 1956a, 1956b, 1958; Lhoste and Roche 1959; Schedl 1962; Kaneko 1967; Li et al. 2018). The mycangium enlarges as the beetles mature and as it fills with growing fungal propagules (Li et al. 2018). In *Anisandrus,* the mycangium is unlobed and is much smaller, and it wraps under the bottom of the scutellum to form a cavity that lines nearly the entirety of the bottom of its ventral surface (Francke-Grosmann 1956a, 1956b, 1958, 1967). In *Eccoptopterus* the scutellum and mycangium bridge spiral extensively (Francke-Grosmann 1958, 1963, 1967). The mycangium of *Cnestus* is similar to that of *Xylosandrus*, but the mycangium bridge travels further back under the scutellum and spirals slightly, though not as extensively as in *Eccoptopterus*, and the mycangium wall is reticulated (Stone et al. 2007). The mycangia of *Diuncus* and *Hadrodemius* have not been characterized, though *Diuncus* may have reduced or absent mycangia as a result of specialization to mycokleptism/fungus-stealing (Cognato and Hulcr 2010). Gland cells are found on the portions of the scutellum inside the mycangium lumen (Francke-Grosmann 1963, 1967), and in *Cnestus mutilatus* the gland cells are interspersed across the entire reticulated wall of the organ (Stone et al. 2007).

Subcoxal mycangia

Fungal propagules are carried in enlargements of the prosternal subcoxa in the corthyline genera *Gnathotrichus* (Farris 1963; Francke-Grosmann 1963, 1967; Schneider and Rudinsky 1969a, 1969b) and *Monarthrum* (Schedl 1962; Lowe et al. 1967). In both genera, the cavities are lined with gland cells (Schedl 1962; Schneider and Rudinsky 1969a, 1969b), and in *Gnathotrichus* the glands feed secretions into the cavities via small channels (Schneider and Rudinsky 1969b). Similar mycangia are also found in the Platypodinae (Nakashima 1972, 1975).

Prothoracic coil mycangia

Two long, winding or spiraling, hose-like tubes inside the prothorax exit into the procoxal cavities of male *Corthylus* (Schedl 1962; Finnegan 1963; Giese 1967; Nord 1972; Orañegui and Atkinson 1984) and *Microcorthylus* species (Schedl 1962). The mycangium wall is intricately reticulated (Finnegan 1963) and presumably holds gland cells, but these have not been identified. Presumably this mycangium type is derived from relatives in the Corthylini with smaller, subcoxal mycangia in males such as *Monarthrum,* to which *Corthylus* is closely related (Gohli et al. 2017).

Prothoracic pleural mycangia

Females of *Trypodendron* in tribe Xyloterini have two large, inverted U-shaped chambers on the inside of the prothorax, and these chambers empty through seta-lined slot openings on the sides of the prothorax (i.e. the pleura) (Nunberg 1951; Francke-Grosmann 1956a, 1956b, 1958, 1959; Abrahamson et al. 1967; Schneider and Rudinsky 1969a). These cavities are very similar across *Trypodendron* spp. and mostly vary in the size of the ascending portion of the cavity or in how extensively the terminus curls (Francke-Grosmann 1956a). The opening of the mycangium opens and closes via the leg

muscles (Francke-Grosmann 1963). The walls of the mycangium are sclerotized and reticulated (Francke-Grosmann 1956a; Abrahamson et al. 1967) and are lined with gland cells (Schneider and Rudinsky 1969a). The contents of the ascending and descending portions of the mycangia can differ from each other in the off season, and various debris can fill the mycangia at that time (Schneider and Rudinsky 1969a). However, during dispersal and gallery initiation the mycangia are packed with homogenous propagules of yeast-like propagules (Francke-Grosmann 1956a, 1958; Abrahamson et al. 1967; Schneider and Rudinsky 1969a). Another genus in tribe Xyloterini, *Indocryphalus* (=*Dendrotrypum*), has what appear to be mycangium openings on the sides of the prothorax in a location similar to the openings of *Trypodendron* mycangia (Wood 1957; Beaver 2000; Cognato et al. 2015). However, the internal morphology of the *Indocryphalus* mycangium is unknown. *Indocryphalus* may have split early from *Trypodendron* and *Xyloterinus politus* (Gohli et al. 2017; Pistone et al. 2017), the second of which has different mycangia as discussed below.

Prothoracic basin mycangia

The monotypic *Xyloterinus* (Xyloterini) appears to have simpler prothoracic mycangia that are shallow basins rimmed with setae (Francke-Grosmann 1963, 1967; Abrahamson and Norris 1966; MacLean and Giese 1968). The protoracic mycangium of X. *politus* could be a reduced form of the *Trypodendron* mycangia (Francke-Grosmann 1963), but the two mycangial types are located on different parts of the prothorax. The prothoracic mycangia of X. *politus* carry an unknown fungus with large spherical conidia (Abrahamson and Norris 1969). *Xyloterinus politus* is unique among Scolytids in that it also has oral mycangia in both sexes (Abrahamson and Noris 1966).

Pronotal disk mycangia

Beetles in the genus *Scolytoplatypus* (Scolytoplatypodini) have a single pore on the anterior pronotum that leads to a large saucer- or disc-shaped mycangium just under the cuticle. The mycangium is surrounded by large masses of gland cells, and the mycangium walls are lined with setae that feed towards and through the pore, presumably helping guide spores to exit the organ (Berger and Cholodkovsky 1916; Schedl 1962; Beaver and Gebhardt 2006). There are African and Asian sublineages of *Scolytoplatypus* (Jordal 2013). The mycangium wall setae are seated on reticulated cones in the Asian species (Berger and Cholodkovsky 1916; Schedl 1962; Beaver and Gebhardt 2006), but the African species have simpler setae attached directly to the wall of the mycangium (Schedl 1962). The only other genus in the tribe, *Remansus,* has a pronotal pore that leads to a presumably similar but unstudied mycangium (Jordal 2013).

Mycangia with unconfirmed glandular nature

Several types of mycangia have been characterized that remain understudied. Three of these mycangia were discovered in the underappreciated work of Nunberg (1951). The platypodid *Diapus pusillimus* has a crevice-type mycangim on the posterior side of the pronotum (Nunberg 1951). Similarly, *D. quinquespinatus,* which also has glandular pit mycangia, has a pair of elongated, transverse, crevice-type mycangia that are covered by fences of protective setae on the posterior side of the pronotum (Nakashima 1975). In another platypodid, *Periommatus excisus,* Lhoste and Roche (1961) found small notches on the prothorax that held fungal spores but did not believe the notches to be associated with glands.

Camptocerus (Scolytini) has paired circular depressions on the prothorax (Smith 2013). *Sueus* (Hyorrhynchini) is a genus of ambrosia beetles with an obvious fungus-

farming habit (Beaver 1984, as cited in Hulcr and Stelinski 2017), but its mycangium and symbiont are unknown (Hulcr and Stelinski 2017). Mycangia appear to be present in two scolytids whose ambrosial habits are unclear (Hulcr and Stelinski 2017). *Dactylpalpus transversus* (Hylesinini) has a broad, central-transverse, slot-like mycangium on the anterior pronotum (Nunberg 1951). *Phloeoborus rudis* (Ipini) has paired mycangia that are circular cups ringed with protective setae on the sides of the pronotum, with pores at the bottom of the cups that may be associated with gland cells (Nunberg 1951).

Section 5. Ambrosia fungi

Baker (1963) and Francke-Grosmann (1967) give reviews of the convoluted taxonomy of ambrosia fungi prior to 1967, which was confused by misunderstandings of primary symbionts, morphological convergence toward easily-grazed conidia, lack of observed sexual states, and the difficulty of isolating ambrosia fungi in pure culture. The major ambrosia fungus genera *Raffaelea* and *Ambrosiella* were described in 1965 (von Arx and Hennebert 1965). Batra (1967) published a taxonomic revision and review of ambrosia fungi, in which he placed most ambrosia beetle symbionts into one of the two genera. The advent of PCR and DNA sequencing gave the first indications that *Raffaelea* and *Ambrosiella* were distantly related (Cassar and Blackwell 1996; Blackwell and Jones 1997; Rollins et al. 2001; Paulin-Mahady et al. 2002; Harrington 2009) and that each genus contained species that should have been treated in the other. Harrington et al. (2010) revised the genera so that *Raffaelea* accommodated symbionts in the Ophiostomatales and *Ambrosiella* accommodated symbionts in the Microascales.

Raffaelea

Raffaelea is related to genera such as *Leptographium*, *Ophiostoma*, and *Sporothrix* in the Ophiostomataceae (Ascomycota: Sordariomycetes: Ophiostomatales)

(Harrington et al. 2010; de Beer and Wingfield 2013; Simmons et al. 2016a; Vanderpool et al. 2017). *Ophiostoma* includes some important plant pathogens, such as the causative agent of Dutch elm disease *Ophiostoma novo-ulmi*, but many *Ophiostoma* spp. are saprophytes carried on the exoskeleton of bark beetles (Harrington 2005). *Sporothrix* is the only genus in the family that contains human pathogens (de Beer et al. 2016). Within the Ophiostomataceae, *Raffaelea* is currently polyphyletic and contains two or three clades. *Raffaelea sensu stricto* (which contains the type species *Raffaelea ambrosiae*) is clearly separate from the *Raffaelea sulphurea* complex, which is placed inside *Leptographium sensu lato* (Dreaden et al. 2014; Simmons et al. 2016a; Vanderpool et al. 2017). *Raffaelea sensu lato* currently includes at least 27 species (Simmons et al. 2016a) with evidence for additional, cryptic species. *Raffaelea* spp. produce asexual conidia holoblastically on the end of long, thin, branched conidiophores (Gebhardt and Oberwinkler 2005; Harrington et al. 2010), in contrast to the larger phialidic conidiophores of *Ambrosiella* discussed below. Sexual states for some species placed in *Raffaelea* were recently reported (Musvuugwa et al. 2015), though the *Raffaelea* species were not clearly associated with ambrosia beetles.

Raffaelea species have been associated with a wide variety of mycangium types, do not appear to be limited to specific ambrosia beetle species or tribes, and a single adult beetle can carry multiple *Raffaelea* spp. in its mycangia (Harrington et al. 2010; Harrington and Fraedrich 2010; Kostovcik et al. 2015; Vanderpool et al. 2017). The studied Platypodinae have *Raffaelea* symbionts, regardless of mycangium type (Harrington et al. 2010; Vanderpool et al. 2017). Most identified symbionts of oral mycangia in the Platypodinae and Scolytinae are *Raffaelea* spp. (Harrington et al. 2010).

Subcoxal mycangia in *Monarthrum* and *Gnathotrichus* also harbor *Raffaelea* symbionts (Batra 1967; Funk 1970), as do elytral mycangia of *Xyleborinus* (Verral 1943; Harrington et al. 2010; Biedermann et al. 2013; Gharabigloozare 2015).

Ambrosiella

Ambrosiella as defined by Harrington et al. (2010) is in the family Ceratocystidaceae (Ascomycota: Sordariomycetes: Microascales) (de Beer et al. 2014). Many relatives in the family have insect vectors or are dispersed in frass of ambrosia beetles, and many are tree pathogens (Harrington 2009; Harrington 2013). *Ambrosiella* consists of six species: *A. xylebori* (Brader 1964), *A. hartigii* (Batra 1967), *A. beaveri* (Six et al. 2009), and *A. roeperi* (Harrington et al. 2014) associated with the mesonotal pouch mycangia of the *Xylosandrus* complex; and *A. ferruginea* and *A. trypodendri* associated with the prothoracic pleural mycangia of *Trypodendon* spp. (Mathiesen-Käärik 1953; Batra 1967; Harrington et al. 2010). Unlike *Raffaelea* spp., *Ambrosiella* spp. produce phialidic conidiophores (Gebhardt and Oberwinkler 2005; Harrington et al. 2010). *Ambrosiella* conidiophores are often monillioid and produce distinctive asexual conidia termed aleurioconidia (Harrington et al. 2014). The sexual state of *Ambrosiella* is unknown, and its species show limited genetic variation (van de Peppel et al. 2018).

All studied species with mesonotal mycangia in the *Xylosandrus* complex have been associated with *Ambrosiella* symbionts (Batra 1967; Francke-Grosmann et al. 1967; Roeper 1996, 2011; Yang et al. 2008; Harrington et al. 2010, 2014; Bateman et al. 2015, 2016; Ito and Kajimura 2017; Lin et al. 2017; van de Peppel et al. 2018). Multiple *Trypodendron* spp. have been associated with *A. ferruginea* (=*Monilia ferruginea*), and *T. scabricollis* is associated with *A. trypodendri* (Funk 1965; Batra 1967; Francke-Grosmann 1967; Harrington et al. 2010; Roeper 1996). However, *Ambrosiella* may be

polyphyletic (Harrington et al. 2010, 2014). The mesonotal mycangium symbionts *A. beaveri, A. hartigii, A. roeperi,* and *A. xylebori* appear to be closely related to *Ceratocystis adiposa,* whereas *Ambrosiella ferruginea* is more closely-related to the oak wilt fungus *Bretziella fagacearum* (de Beer et al. 2017) than to other *Ambrosiella* (Harrington et al. 2014). *Ambrosiella*-like symbionts have also been implicated as associates of the prothoracic coil mycangia of *Corthylus punctatissimus* (Batra 1967; Nord 1972; Roeper 1996) and the pronotal disk mycangia of Asian *Scolytoplatypus* spp. (Nakashima et al. 1987; Nakashima 1989; Kinuura et al. 1991). *Ambrosiella* may be associated with mycangia that are more developed when compared with the mycangia that carry *Raffaelea* symbionts (Roeper 2011).

Other ambrosia fungi

The recently-described *Afroraffaelea* is monotypic and phylogenetically placed within the Ophiostomatales, perhaps near *Raffaelea*, and it is an oral mycangium symbiont of *Premnobius cavipennis* (Ipini) (Bateman et al. 2017). The prothoracic basin mycangia of *Xyloterinus politus* carry an unknown fungus that forms large, dark, spherical propagules (Abrahamson and Norris 1966, 1969a; MacLean and Giese 1968). Some *Euwallacea* spp. (Xyleborini) have been associated with an ambrosial clade of *Fusarium*, though *Euwallacea* spp. also carry *Raffaelea* symbionts (Eskalen et al. 2012; Kasson et al. 2013; O’Donnel et al. 2015).

Section 6. Gaps in understanding

Although there has been recent progress on understanding the numerous lineages of ambrosia beetles and mycangial fungi, there is still only a basic understanding of the dynamics of this symbiosis. Comprehensive and in-depth studies of coevolution between the different lineages of beetles and fungi, as has been done in fungus-farming ants

(Branstetter et al. 2017) and fungus-farming termites (Aanen et al. 2002), is lacking in ambrosia beetles. There has been little attention given to the evolution of mycangia and the fungal lineages adapted to the most advanced mycangia. The study by Farrell et al. (2001) was limited by an incomplete understanding of the fungi, and a recent study on the coevolution of *Raffaelea* and its ambrosia beetle hosts (Vanderpool et al. 2017) did not accommodate the symbiotic patterns of Ceratocystidaceae symbionts.

Only about half of the known ambrosia beetle lineages have clearly associated fungal symbionts (Harrington et al. 2010; Hulcr et al. 2015; Kirkendall et al. 2015; Hulcr and Stelinski 2017), and only a small fraction of ambrosia beetle species have been studied. Sampling has been hindered by the cryptic nature of ambrosia beetles and the limited geographic distribution of some groups. Further, ambrosia fungi are notoriously difficult to isolate and grow, and sometimes it is only possible to get barcode DNA sequences from mycangia or galleries with no associated live culture. Studies using next-generation sequencing to elucidate the diversity of ambrosia fungi present in mycangia and galleries can be done, but in the absence of microscopic observation or isolation of cultures, it is difficult to identify the important members of the symbiosis.

This dissertation study targets the groups of ambrosia beetles with relatively large and complex mycangia and those said to carry symbionts in the Ceratocystidaceae, i.e. *Ambrosiella.* Preliminary evidence suggested that cryptic diversity existed in the *Ambrosiella* symbionts of the *Xylosandrus* complex and of *Trypodendron,* and in the unstudied symbionts of *Corthylus* and *Scolytoplatypus*. Additionally, we sought to characterize the understudied mycangia of *Corthylus, Scolytoplatypus*, and *Indocryphalus*, and to attempt to relate these mycangia to apparent specificity in

symbionts. We suspected that the four beetle groups with large mycangia might harbor four distinct groups of *Ambrosiella* symbionts. We also wanted to confirm that these mycangia are not associated with the otherwise-ubiquitous *Raffaelea* and have only *Ambrosiella* symbionts.

CHAPTER 2. THREE LINEAGES IN THE *CERATOCYSTIDACEAE* ARE THE RESPECTIVE SYMBIONTS OF THREE INDEPENDENT LINEAGES OF AMBROSIA BEETLES WITH LARGE, COMPLEX MYCANGIA

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Abstract

The genus *Ambrosiella* accommodates species of *Ceratocystidaceae* (*Microascales*) that are obligate, mutualistic symbionts of ambrosia beetles, but the genus appears to be polyphyletic and more diverse than previously recognized. In addition to *A. xylebori, A. hartigii, A. beaveri,* and *A. roeperi,* three new species of *Ambrosiella* are described from the ambrosia beetle tribe *Xyleborini*: *A. nakashimae* sp. nov. from *Xylosandrus amputatus*, *A. batrae* sp. nov. from *Anisandrus sayi*, and *A. grosmanniae* sp. nov. from *X. germanus*. The genus *Meredithiella* gen. nov. is created for symbionts of the tribe *Corthylini*, based on *M. norrisii* sp. nov. from *Corthylus punctatissimus*. The genus

Phialophoropsis is resurrected to accommodate associates of the *Xyloterini*, including *P. trypodendri* from *Trypodendron scabricollis* and *P. ferruginea* comb. nov. from *T. lineatum*. Each of the 10 named species was distinguished by ITS rDNA barcoding and morphology, and the ITS rDNA sequences of four other putative species were obtained with *Ceratocystis*-specific primers and template DNA extracted from beetles or galleries. These results support the hypothesis that each ambrosia beetle species with large, complex mycangia carries its own fungal symbiont. Conidiophore morphology and phylogenetic analyses using 18S (SSU) rDNA and TEF1α DNA sequences suggest that these three fungal genera within the *Ceratocystidaceae* independently adapted to symbiosis with the three respective beetle tribes. In turn, the beetle genera with large, complex mycangia appear to have evolved from other genera in their respective tribes that have smaller, less selective mycangia and are associated with *Raffaelea* spp. (*Ophiostomatales*).

Introduction

The six recognized species of *Ambrosiella* Brader ex Arx & Hennebert (1965) (*Sordariomycetes*: *Microascales*: *Ceratocystidaceae*) are obligate, mutualistic symbionts of ambrosia beetles. Ambrosia beetles are an ecological group of more than 3400 species of mycophagous sapwood-boring beetles in the subfamilies Platypodinae and Scolytinae (Coleoptera: Curculionidae). Whereas their bark beetle relatives generally feed on the nutritious inner bark (secondary phloem) of trees, ambrosia beetles tunnel in the nutrient-poor sapwood and depend on mutualistic fungi for their nutrition (Harrington 2005). The beetles generally do not eat wood while boring (Beaver 1989), though the larvae of some ambrosia beetles ingest fungus-colonized wood (xylomycetophagy) (De Fine Licht and Biedermann 2012, Roeper 1995). The major food of both larvae and adults is ambrosial

growth of fungi within the sapwood tunnels. A diverse fungal flora grows in ambrosia beetle galleries, but the dominant fungi are obligate symbionts of ambrosia beetles and have not been found as free-living species (Harrington et al. 2010).

The fungal symbionts rely on the beetles for dispersal and are primarily carried by adult beetles in special sacs called mycangia, in which the fungi grow in a budding yeast-like or arthrospore-like phase (Fraedrich et al. 2008, Harrington et al. 2014). In the studied cases of ambrosia beetle mycangia, gland cells secrete material into or near the mycangium to support growth of the fungal symbionts (Schneider and Rudinsky 1969a, 1969b), and the overflow of spores from the mycangium inoculates the galleries during construction. Of the two weevil subfamilies, the *Platypodinae* consist entirely of ambrosia beetles and have relatively small and simple mycangia (Cassier et al. 1996, Marvaldi et al. 2002, Nakashima 1975). In contrast, ambrosia beetles arose at least 10 separate times from bark beetle lineages within the *Scolytinae* (Farrell et al. 2001, Jordal and Cognato 2012, Kirkendall et al. 2015), each event apparently marked by the development of novel mycangia. Generally, the *Scolytinae* mycangia are relatively small and harbor one or more species of *Raffaelea* Arx & Hennebert (1965) (*Ophiostomatales*) (Cassar and Blackwell 1996, Harrington and Fraedrich 2010, Harrington et al. 2010, 2014). These small mycangia include oral pouches, pronotal pits, elytral pouches, and coxal enlargements, each of which are simple modifications of the adult beetle's exoskeleton, with secreting gland cells near the opening of the mycangium (Beaver 1989, Francke-Grosmann 1967). In contrast, certain genera of *Scolytinae* exhibit markedly larger and more elaborate mycangia that are set entirely within the body and are composed of a reticular structure punctuated by gland cells secreting directly into the

mycangium (Finnegan 1963, Francke-Grosmann 1956, Schneider and Rudinsky 1969b, Stone et al. 2007). These larger, complex mycangia have specialized channels or tubes that direct the overflow of fungal growth to the outside of the beetle for tunnel inoculation.

Ambrosiella spp. have so far been recovered or reported from five beetle genera (Harrington et al. 2014), and each of these genera appear to have relatively large and complex mycangia with secretions directly through reticulated mycangial walls. Within the tribe *Xyleborini*, species of *Xylosandrus*, *Anisandrus*, and *Cnestus* have large, internal mesonotal mycangia in female adults (Beaver 1989, Cognato et al. 2011a, Francke-Grosmann 1956, 1967, Hulcr and Cognato 2010, Hulcr et al. 2007, Kinuura 1995, Stone et al. 2007). In the *Corthylini*, *Corthylus* spp. have long, folded tubes that open into the procoxae of adult males (Finnegan 1963, Giese 1967). In the *Xyloterini*, female *Trypodendron* spp. have large, tubular, pleural-prothoraic mycangia (Francke-Grosmann 1956, 1967, Schneider and Rudinsky 1969b).

Ambrosiella initially included ambrosia beetle symbionts with percurrent proliferation of conidiogenous cells vs. sympodial proliferation by *Raffaelea* spp. (Batra 1967, Brader 1964, von Arx and Hennebert 1965). Gebhardt et al. (2005) demonstrated that some *Raffaelea* spp. have percurrent and sympodial proliferation, but *Ambrosiella xylebori* Brader ex Arx & Hennebert (1965), *A. hartigii* L.R. Batra (1968), and *A. ferruginea* (Math.-Käärik) L.R. Batra (1968) produced conidia from phialides. Harrington et al. (2010) limited *Ambrosiella* spp. to the phialidic ambrosia fungi within the *Ceratocystidaceae* and *Raffaelea* spp. to symbionts within the *Ophiostomatales*. All

known *Ambrosiella* spp. produce a fruity aroma (Harrington 2009), and these volatiles may play a role in attracting ambrosia beetles within the galleries (Hulcr et al. 2011).

As now recognized, most *Ambrosiella* spp. produce large, thick-walled, ovoid, terminal aleurioconidia with inconspicuous collarettes and/or basipetal chains of cylindrical to barrel-shaped phialoconidia via ring-wall building (Minter et al. 1983, Nag Raj and Kendrick 1993, Riggs and Mims 2000). The deep-seated phialides of the symbiont of *T. scabricollis* (tribe *Xyloterini*), *A. trypodendri* (L.R. Batra) T.C. Harr. (2010), was used to erect the monotypic genus *Phialophoropsis* L.R. Batra (1968), but Harrington et al. (2010) placed *Phialophoropsis* in synonomy with *Ambrosiella*. However, phylogenetic analyses have generally suggested that the two genera are distinct (Alamouti et al. 2009, de Beer et al. 2014, Harrington 2009, Harrington et al. 2010, Six et al. 2009).

At present, there are six named species of *Ambrosiella*: *A. trypodendri* (Harrington et al. 2010) from *T. scabricollis* (Batra 1967); *A. ferruginea* from *T. lineatum* (Batra 1967); *A. xylebori* from *Xylosandrus compactus* (Brader 1964); *A. hartigii* from *Anisandrus dispar* (Batra 1967); *A. beaveri* Six, de Beer & W.D. Stone (2009) from *Cnestus mutilatus* (Six et al. 2009); and *A. roeperi* T.C. Harr. & McNew (2014) from *X. crassiusculus* (Harrington et al 2014). In addition to *X. compactus, A. xylebori* has been reported from *Corthylus columbianus* (Batra 1967, Nord 1972) and *C. punctatissimus* (Roeper 1995). Besides *An. dispar, A. hartigii* has been reported from *An. sayi* and *An. obesus* (Hazen and Roeper 1980, Roeper and French 1981), as well as *X. germanus* (Roeper 1996, Weber and McPherson 1984). *A. ferruginea* has been reported from several *Trypodendron* spp., including *T. lineatum, T. domesticum*, *T. retusum*, *T.*

rufitarsis, and *T. betulae* (Batra 1967, French and Roeper, 1972, Roeper 1981, Roeper 1996), and Nakashima et al. (1992) illustrated a fungus from *T. signatus* with similar conidiophore morphology.

Most of the above identifications of *Ambrosiella* spp. were based on morphological characters only, and more detailed phylogenetic analyses may reveal cryptic species and genera among the fungal symbionts. Preliminary DNA sequence analyses and observations of cultures from beetles with large mycangia suggested that there was more species diversity within *Ambrosiella* than previously recognized, and each studied ambrosia beetle with large, complex mycangia appeared to be associated with a single, unique species, either within *Ambrosiella* or a closely related genus in the *Ceratocystidaceae*.

We studied fungal isolates, beetle galleries and insect specimens of 14 ambrosia beetle species with large, complex mycangia to determine the identity of their fungal symbionts and infer an evolutionary history of the fungi. Our hypothesis was that each beetle would yield a unique fungal species, and that all species recovered from beetles with large, complex mycangia would form a monophyletic genus (*Ambrosiella*) within the *Ceratocystidaceae*, stemming from a single evolutionary jump to ambrosia beetle symbiosis.

Materials and Methods

Beetle collection and fungal isolation

Most of the adult beetles were caught in flight using Lindgren traps with water or polyethylene glycol in collection cups, while the cups of other traps were dry and had No Pest insecticide strips (vapona, Spectrum Brands, Middleton, Wisconsin). The traps were baited with either ethanol lures or lineatin flexlure (Contech Enterprises, Victoria, British

Columbia) in the case of *T. lineatum* and *T. scabricollis*. Some adult beetles were caught in-flight with rotary net traps. Other mature adults were taken directly from fresh galleries by splitting infested wood sections.

Most fungal isolates were obtained by grinding beetles and dilution plating (Harringon and Fraedrich 2010, Harrington et al. 2011) or by placing whole beetles or parts of beetles containing mycangia on plates of SMA (1% malt extract, Difco; 1.5% agar, Sigma-Aldritch; and 100 ppm streptomycin sulfate added after autoclaving). Isolations were also attempted directly from ambrosia growth in beetle galleries by scraping with a sterile needle and transferring to SMA or MYEA (2% malt extract, 0.2% Difco yeast extract, 1.5% agar).

For mycangial examination, adult female *Xyleborini* were dissected in 20% lactic acid on a deep well slide using fine forceps and a scalpel. An incision was made just posterior to the scutellum to expose the interior of the beetle without damaging the mycangium, which sits directly beneath the mesonotum and is attached to the scutellum. Fine forceps were then used to gently tease out the mycangium and scutellum. The mycangium/scutellum was either transferred to a drop of Cotton blue on a slide and covered with a cover slip for microscopic examination, or the spore mass separated and used for isolation of the fungal symbiont or for DNA extraction with PrepMan® Ultra (Applied Biosystems, Foster City, CA).

DNA extraction and sequencing

Isolates were grown at room temperature on MYEA, and DNA was extracted using one of two methods: either the cultures were grown 2-7 days and DNA was extracted using PrepMan® Ultra, or isolates were grown 4-14 days and extracted using the ProMega Wizard® Genomic DNA Purification Kit (Promega, Madison, WI).

PrepMan® Ultra was used to extract DNA from scraped fungal material in beetle galleries or beetle mycangia. In some cases, the extracted DNA was concentrated using Amicon® Ultra-0.5 Centrifugal Filter Devices (EMD Millipore, Billerica, CA). Whole beetles preserved in ethanol were ground with a tissue grinder and Prepman® Ultra extraction buffer, and the resulting mix was transferred to a microcentrifuge tube for DNA extraction.

ITS barcoding

Sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA for initial identification of unknown cultures utilized the general fungal primer ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) and the PCR conditions of Paulin-Mahady et al. (2002) and Harrington et al. (2000). When using extracted DNA from gallery material, mycangial masses, or ground beetles, *Ceratocystis*-specific primers were used to amplify the ITS region in two parts: primer pairs Cerato1F (5' GCGGAGGGATCATTACTGAG 3') and ITSCer3.7R (5' GTGAAATGACGCTCGGACAG 3') for ITS1 and primer pair ITSCer3.1 (5' CAACGGATCTCTTGGCTCTA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') for ITS2 (Harrington et al. 2014).

All ITS sequences generated from cultures, beetles and galleries were compared with the ITS sequences of representative *Ceratocystidaceae* in a manually aligned ITS rDNA dataset. There were regions of ambiguously aligned characters in both ITS1 and ITS2 due to numerous areas of insertions and deletions (indels), and the indel regions had limited reliable phylogenetic signal. Nonetheless, the aligned dataset was analyzed by UPGMA in PAUP 4.0b10 (Swofford 2002) using uncorrected ("p") distance, and gaps were treated as missing data.

Phylogenetic analysis

Sequences of the small subunit rDNA (SSU, 18S rDNA) and translation elongation factor 1-alpha (TEF1α) were used for phylogenetic placement of the ambrosia beetle symbionts. Taxa selected (Table 1) included a representative of each symbiont and other representatives of the newly recognized genera in the *Ceratocystidaceae*, which were previously treated as *Ceratocystis* spp. or *Thielaviopsis* spp. (de Beer et al. 2014). The representative taxa are well characterized, except for the members of the *C. moniliformis* complex, now treated as *Huntiella*. Our *H. moniliformis* isolate C792 from a *Populus* sp. in Minnesota is probably an undescribed species, while *H. moniliformis* isolate C1007 (CBS 204.90, CMW 11046) from India has the ITS sequence of *H. omanensis* (DQ074739). Other taxa in the *Microascales* included *Pseudallescheria* spp. (mixed species; *P. ellipsoidea* for 18S rDNA (U43911) and *P. angusta* for TEF1α) and *Gondwanamyces capensis* (18S rDNA, FJ176834,). Outgroup taxa were *Plectosphaerella cucumerina* (18S rDNA, AF176951) and *Neurospora crassa* (18S rDNA, X04971).

The SSU sequences were amplified and sequenced using a variety of primers (Vilgalys 2005, White et al. 1990), typically using the overlapping sequences from NS-1/NS-6 and SR-9R/NS-8, but sometimes overlapping sequences were obtained with NS-1/NS-4, NS-3/NS-6, and NS-5/NS-8. These overlapping sequences yielded an aligned sequence of approximately 1700 bp.

Amplification of TEF1α used the forward amplification primer EFCF1a (5' AGTGCGGTGGTATCGACAAGCG 3') or EFCF1.5 (5' GCYGAGCTCGGTAAGGGYTC 3') and the reverse primer EFCF6 (5' CATGTCACGGACGGCGAAAC 3') following the protocol of Oliveira et al. (2015). Sequencing was generally performed with the PCR primers as well as the internal

primers EFCF2 (5' TGCTCAACGGGTCTGGCCAT 3') and EFCF3 (5' ATGGCCAGACCCGTGAGCA 3'). The aligned sequences were approximately 1200 bp.

A combined SSU and TEF1α dataset (TreeBase URL: http://purl.org/phylo/treebase/phylows/study/TB2:S17680) of 2781 characters was used for phylogenetic analysis. Model testing using ModelTest 2.1.7 v20141120 (Darriba et al. 2012) on both the combined dataset and the separate datasets for each gene showed the GTR+I+G model to be most appropriate. MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003) was used for Bayesian analysis with this GTR+I+G model. A single MCMC run with four chains (one cold, three heated) ran for 600,000 generations, which was sufficient to bring the convergence diagnostics below 0.01; a burn-in of 25% was applied before creating a majority rule consensus tree with the function "sumt". The tree was visualized with FigTree.

A full heuristic, maximum parsimony (MP), 10,000-replicate bootstrap analysis and 50% majority rule consensus tree was created with PAUP to add bootstrap support values to the Bayesian inference tree. All characters had equal weights, and the heuristic search was performed with simple stepwise addition. The MP analysis used the same combined dataset but treated gaps as a new state (5th base).

Species descriptions

For growth rate studies, selected isolates were grown on MYEA plates. Plugs from the leading edge of growth taken with a sterile #1 cork borer were placed upside down on three new MYEA plates per isolate, and the plates were incubated at 25°C for 4-6 days. Color descriptions of cultures followed Rayner (1970.)

Results

Using ITS rDNA sequences as a barcode to delineate putative species, we were able to associate an Ambrosiella sp. with each of 14 studied species of ambrosia beetles with large, complex mycangia: eight species from tribe Xyleborini (Cnestus mutilatus, Xylosandrus amputatus, X. germanus, X. crassiusculus, X. compactus, Anisandrus dispar, An. sayi, and Eccoptopterus spinosus), two from tribe Corthylini (Corthylus punctatissimus and C. consimilis), and four from tribe Xyloterini (Trypodendron lineatum, T. domesticum, T. scabricollis, and T. retusum.) The ITS sequences were obtained from pure cultures or from DNA extracted from dissected mycangial spore masses, whole beetles, or from sporulation in beetle galleries. Gallery sporulation and isolates on MYEA showed fungi with macro- and microscopic characteristics expected of *Ambrosiella* spp. (Figs. 1, 2), including a fruity aroma when grown on MYEA. Each of the beetle species yielded a different *Ambrosiella* sp. based on unique ITS sequences (Fig. 3) and morphology (conidiphores and/or culture characteristics). No species of ambrosia fungus was found associated with more than one beetle species.

Beetle associations

Xyleborini

Two *Cnestus mutilatus* females caught in a trap in Barrow County, Georgia in September 2013 were ground and plated on SMA, and the recovered isolates had both aleurioconidia and phialoconidia typical of *A. beaveri* (Six et al. 2009.) The ITS sequence of these isolates was identical to a culture (CBS 121750) from the holotype of *A. beaveri* from *C. mutilatus* in Mississippi (Six et al. 2009).

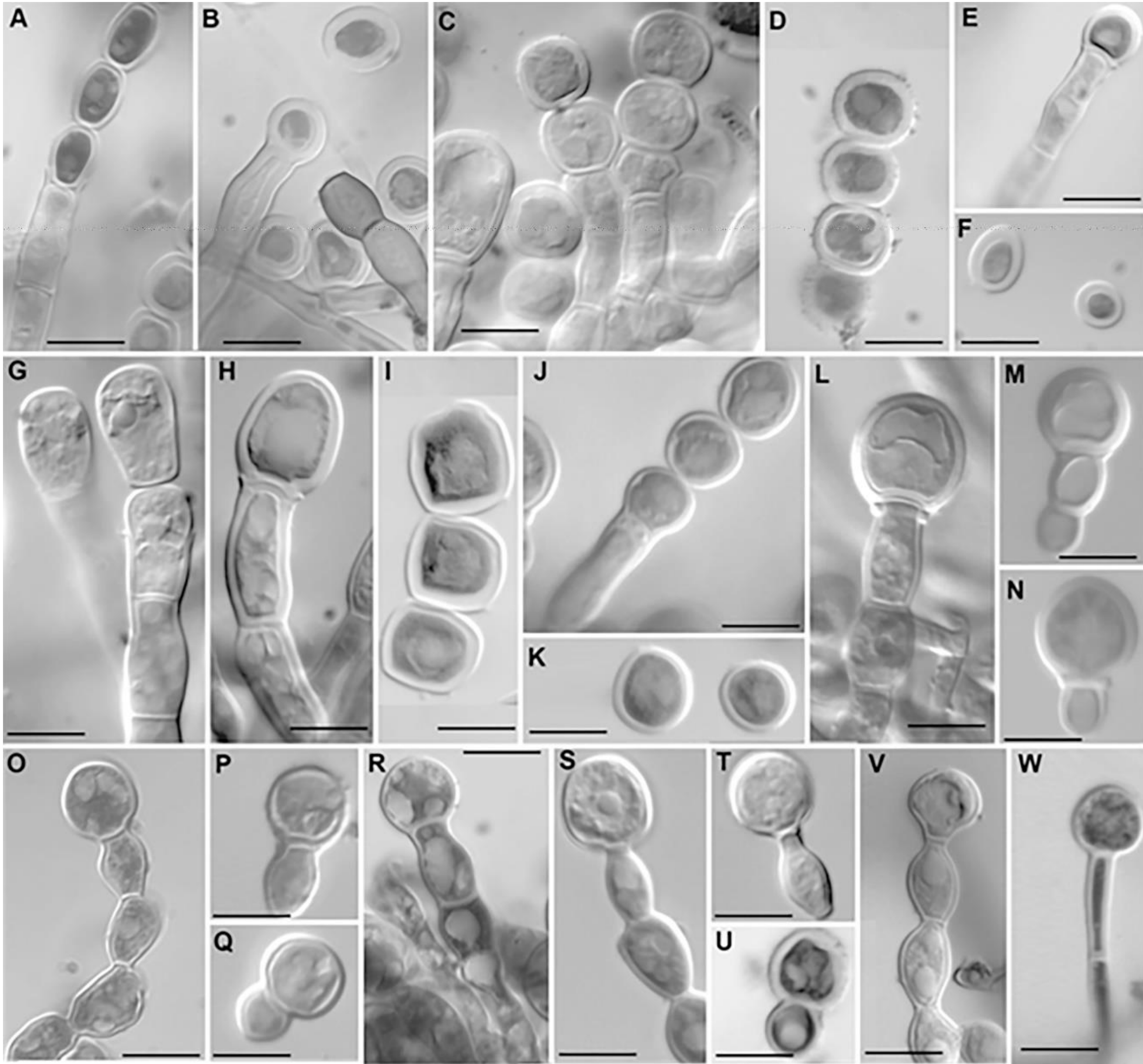

Figure 1. Conidiophores and conidia of *Ambrosiella* spp. A, B. *A. beaveri* isolate C2749 (CBS 121750,ex para-type). A. phialoconidiophore. B. aleurioconidiophore. C-F. *A. nakashimae.* C, D. in gallery, specimen BPI 893134 (holotype). E, F. Isolate C3445 (CBS 139739, ex-type). G-I. *A hartigii* isolate C3450 (CBS 139746). G. phialoconidiophore. H, I. aleurioconidiophore and detached aleurioconidia. J-L. *A. batrae* isolate C3130 (CBS 139735) and M, N. isolate C3045 (CBS 139736). O-R. *A. grosmanniae*. O-Q in gallery, specimen BPI 893133. R. Isolate C3125 (CBS 137357). S, T. *A. roeperi* isolate C2448 (CBS 135864, ex-type). U-W. *A. xylebori* isolate C2455. All photos by Nomarski interference microscopy of material stained with cotton blue. Bar = 10 µm.

Galleries of *Xylosandrus amputatus* in two stems of *Cinnamomum camphora* in Lowndes County, Georgia in August 2014 were lined with a thick, grey-white mycelium with aleurioconidiophores, and protoperithecia were scattered in the mycelium (Fig. 2A-D). Isolations from the fungal growth in five beetle galleries yielded an *Ambrosiella* sp.

whose ITS sequence differed from that of *A. beaveri* by having an extra T in a string of Ts at the end of ITS2 (Fig. 3).

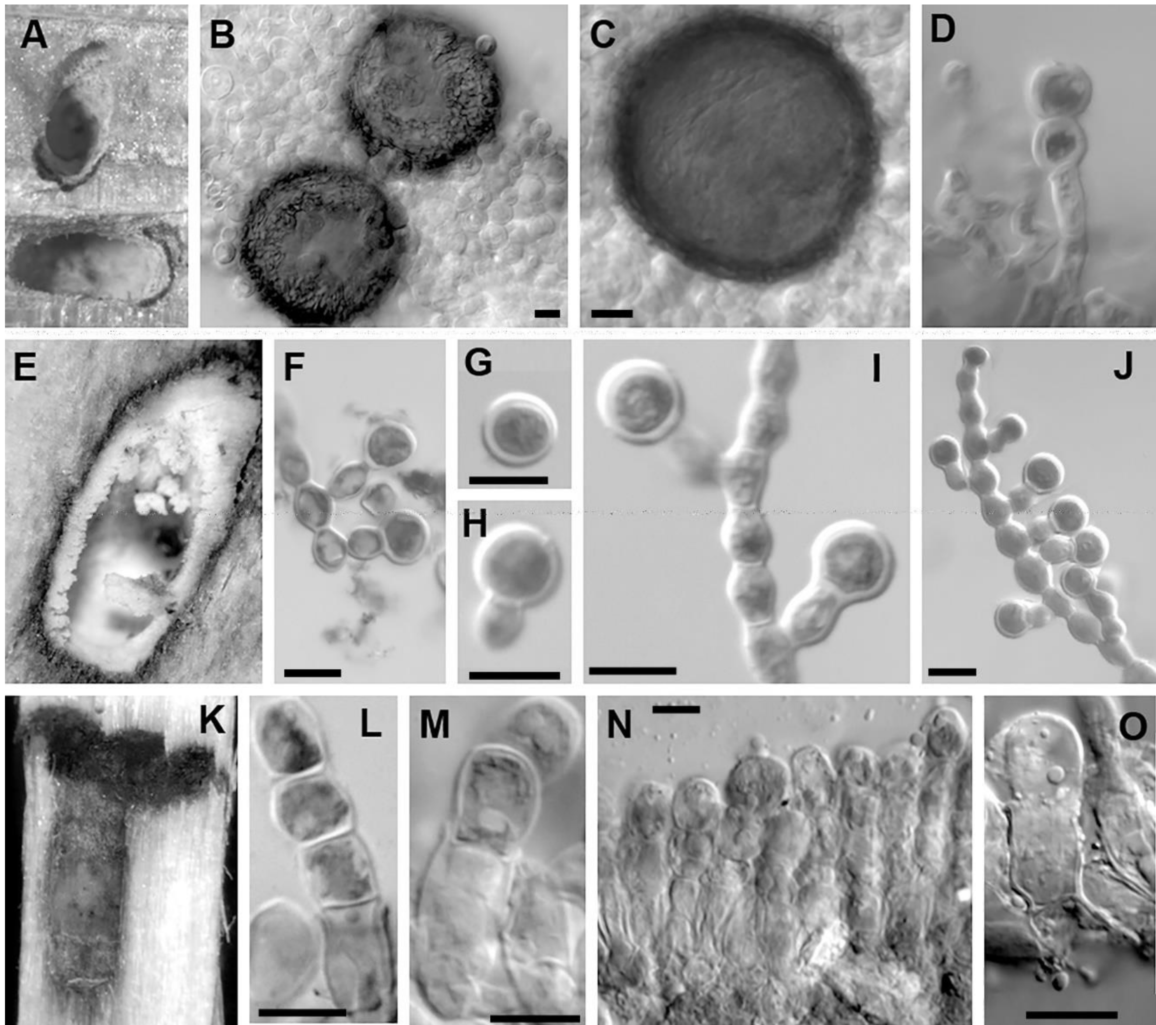

Figure 2. Growth and sporulation of ambrosia fungi in galleries of ambrosia beetles from three beetle tribes. A-D. *Ambrosiella nakashimae* in galleries (BPI 893134) of *Xylosandrus amputatus.* A. growth in tunnels. B, C. protoperithecia and aleurioconidia. D. chained aleurioconidia on aleurioconidiophore. E, F. *Meredithiella norrisii* in galleries of *Corthylus punctatissimus*. E. Growth in gallery (BPI 893135). F. Branched aleurioconidiophore (BPI 893137). G-J. *M. norrisii* in culture (C3152, CBS 139737, BPI 893136, ex-type). G. detached single aleurioconidium. H. detached aleurioconidium with conidiophore cell attached. I, J. Branched aleurioconidiophores. K-O. *Phialophoropsis ferruginea* in galleries of *Trypodendron lineatum*. K-N. Specimen BPI 893130. K. Gallery growth. L-N. Deep-seated phialides. O. Deep-seated phialide (specimen BPI 407710). All photos by Nomarski interference microscopy of material stained with Cotton blue. Bar = 10 µm.

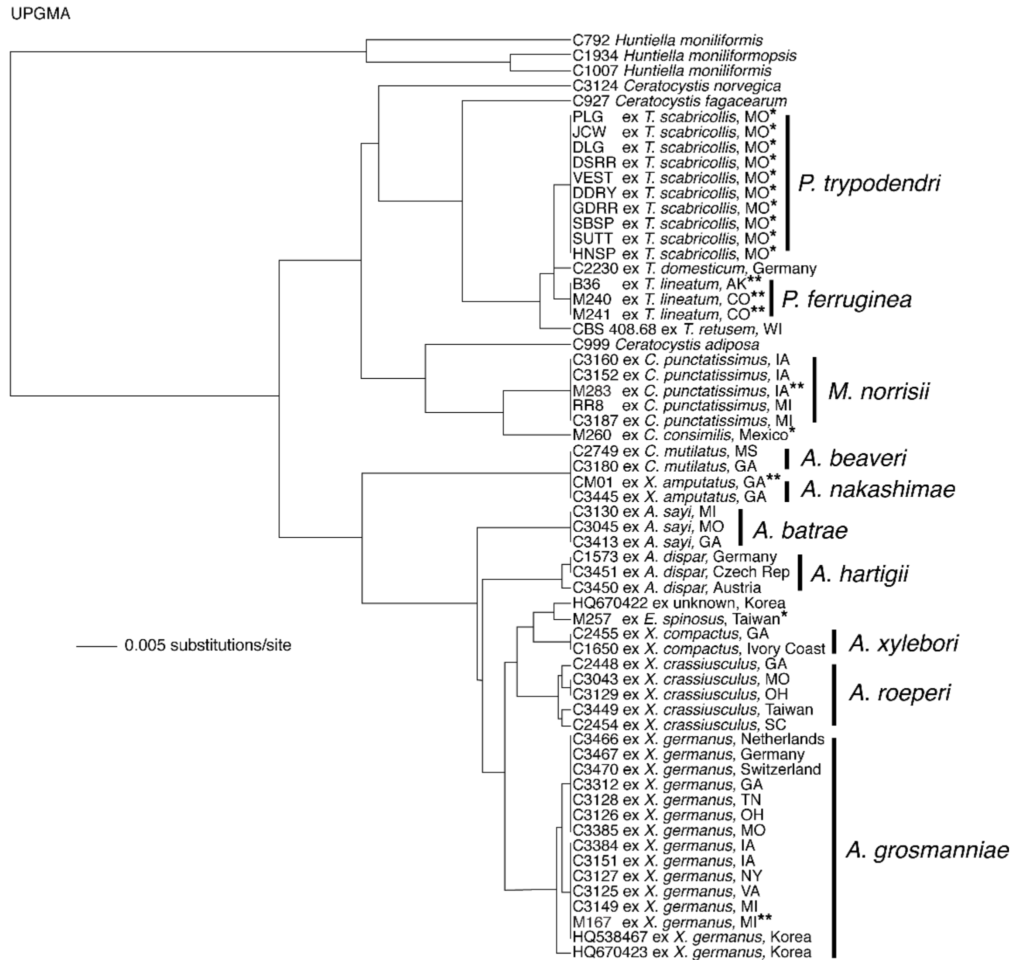

Figure 3. Unrooted UPGMA distance tree of ITS rDNA sequences of *Ambrosiella* spp. from *Cnestus, Xylosandrus,* and *Anisandrus* spp.; *Meredithiella* spp. from *Corthylus* spp.; *Phialophoropsis* spp. from *Trypodendron* spp.; and several representatives of the *Ceratocystidaceae*. Single asterisks indicate sequences obtained from DNA extracted from whole beetles or mycangial spore masses; double asterisks indicate sequences from DNA extracted from gallery growth. Sequences without asterisks are from DNA extracted from cultures. Country or USA state (two letter abbreviation) of origin of the beetle is indicated.

Xylosandrus germanus adults collected in eight different USA states (Georgia, Iowa, Michigan, Missouri, New York, Ohio, Tennessee, and Virginia) and Europe (Germany, the Netherlands, and Switzerland) yielded a unique *Ambrosiella* sp. A female beetle trapped in flight in Missouri was dissected to remove and observe the mycangium and its spore contents, and a culture of the *Ambrosiella* sp. was recovered from the removed spore mass. The mycangium sat just below the mesonotum and was attached to

the scutellum (Fig. 4A). When the mycangial contents were freed, the spore mass maintained the shape of the mycangium (Fig. 4B, C), and the spore mass did not disperse in water, lactic acid, or oil (Isopar M). When mounted and stained (Fig. 4D-F), contents of the mycangium were observed to be a homogenous mass of arthrospore-like cells, similar to those reported by Harrington et al. (2014) from mycangia of *X. crassiusculus*. The ITS rDNA sequences of all isolates from *X. germanus* were identical except at one base position near the end of ITS2, and these ITS sequences closely matched two GenBank accessions (HQ538467 and HQ670423) from Korean *X. germanus* specimens (Fig. 3).

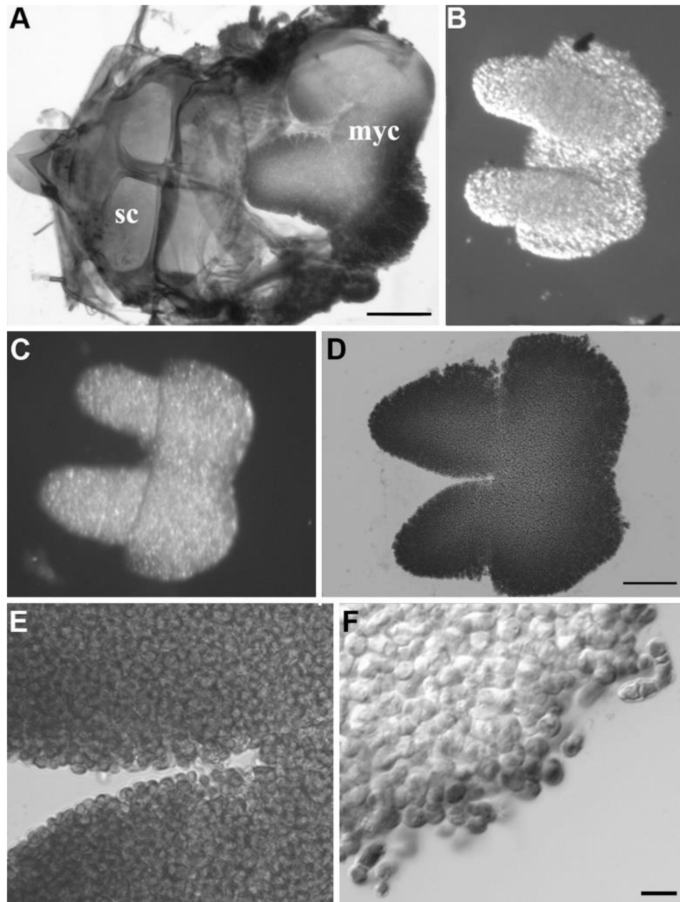

Figure 4. Mycangia and excised spore mass of *Ambrosiella grossmaniae* from a female *Xylosandrus germanus*. A. excised mycangium (myc) and attached scutellum (sc). B-F. mycangial spore mass. B. ventral aspect. C. dorsal aspect. D, E. pressed with coverslip. D. full spore mass. E. detail between lobes. F. edge of spore mass. B, C by stereo microscope, unstained material. All other photos by Nomarski interference microscopy of material stained with cotton blue. For A and D, bar = 100 µm; for F, bar = 10 µm.

Xylosandrus crassiusculus adult beetles from Georgia, Missouri, Ohio, and South Carolina yielded isolates with ITS sequences matching that of *A. roeperi* (Harrington et al. 2014). An additional adult *X. crassiusculus* was trapped in Taiwan in July 2014 and yielded an isolate of *A. roeperi* with an ITS sequence similar to other recovered

A. roeperi sequences, but the sequence most closely matched that of a South Carolina isolate from *X. crassiusculus* (Fig. 3).

Three *Xylosandrus compactus* beetles trapped in Georgia in 2007 yielded isolates of *A. xylebori*, and each had an ITS sequence identical to a culture (CBS 110.61 = C1650) from the holotype, which was from *X. compactus* in the Ivory Coast (Fig. 3).

Fresh *A. hartigii* isolates were obtained from *An. dispar* collected in Austria and the Czech Republic. These isolates (including CBS 139746 = C3450, from Austria) sporulated heavily, unlike the isolate from the holotype specimen (CBS 404.82 = C1573, from Germany). The ITS sequences of all *A. hartigii* isolates were identical, except for the Austrian isolate, which had one base substitution as well as an additional T near the end of the ITS2 region (Fig. 3).

An *Anisandrus sayi* adult trapped in Boone County, Missouri in May 2013 yielded a novel *Ambrosiella* sp. with branching aleurioconidiophores and disarticulating aleurioconidia (Fig. 1 J-N). Additional *An. sayi* adults trapped with a rotary net trap in Montcalm County, Michigan in May 2014 yielded the same fungal species. More specimens were trapped in Chattahoochee National Forest, Georgia in June and July 2014, and four beetles were ground and dilution plated, yielding the same *Ambrosiella* sp. The ITS sequences of isolates from all three locations were identical and most similar to that of *A. hartigii* (Fig. 3).

An *Eccoptopterus spinosus* adult was trapped in Taiwan in July 2014 and stored in ethanol. Although fungal isolation was not possible, the mesonotal mycangium was dissected and yielded a dual-lobed spore mass similar in morphology to that recovered from the mycangia of *X. germanus* (Fig. 4). DNA extracted from the spore mass from the

mycangium of *E. spinosus* yielded a unique ITS sequence (GenBank KR611325) somewhat close to that of *A. xylebori* but most closely matching a sequence (HQ670422) of an unidentified *Ceratocystis sp.* ("CspXapi1") from an ambrosia beetle in Korea, perhaps *An. apicalis* (formerly *X. apicalis*.)

Corthylini

Isolation from the galleries of *Corthylus punctatissimus* in young black maple (*Acer nigrum*) saplings in Iowa in August 2013 yielded a fungus with an ITS sequence close to but distinct from all known *Ambrosiella*. The sporulation in galleries and cultures formed terminal aleurioconidia on many short side branches (Fig 2 E-J). Surface-sterilized males taken from these Iowa galleries, as well as galleries and beetles collected in Michigan from *Acer saccharum* in October 2013, yielded isolates with the identical ITS sequence (Fig. 3).

Male *C. consimilis* beetles from La Esperanza, Mexico, collected in 2007, were stored in ethanol. DNA was extracted from the prothorax of one of the specimens and yielded an ITS sequence (KR611327) similar to the symbiont from *C. punctatissimus* but differing at 10 base positions (Fig. 3). A dissection of the prothorax revealed a long, coiled tube connected to the procoxal cavity and containing a homogenous spore mass of cells similar to those seen in mesonotal mycangia of *Xyleborini* females (Harrington et al. 2014).

Xyloterini

T. scabricollis beetles trapped in 2013 from 10 locations in Missouri were stored in ethanol. The DNA extractions from female beetles from each location yielded an identical ITS sequence (KR611329) using the *Ceratocystis*-specific primers, and the sequence was similar to that of the symbionts of other *Trypodendron* spp. (Fig. 3).

Trypodendon lineatum beetles were trapped in Alaska using Lineatin lure and collection cups that were dry and contained the insecticide Vapona (Reich et al. 2014). Several female specimens were dried and shipped to Iowa for isolations, but no *Ambrosiella* sp. was isolated. Galleries in an infested log of *Picea* sp. collected in Colorado in 2014 were also examined. The galleries (BPI 893129, 893130) were packed with conidiophores with deep-seated phialides but no aleurioconidia (Fig. 2 K-N). Attempts to isolate the fungus from the galleries were not successful, but DNA was extracted from the fungal growth. The ITS sequences from the extracted DNA from female beetles trapped in Alaska and from the gallery growth in Colorado were identical (KR611328) and similar to those of the fungal symbionts from other *Trpodendron* spp. (Fig. 3).

A culture deposited as *A. ferruginea* (CBS 460.82) was isolated in 1971 from *T. domesticum* in Germany. No conidiophores were seen in this culture, but its ITS sequence differed only slightly from that of the *T. lineatum* and *T. scabricollis* symbionts (Fig. 3).

The ITS sequence (KC305145) of another *A. ferruginea* isolate from *T. retusum* in Wisconsin (CBS 408.68 = MUCL 14520) was similar to, but distinct from, the ITS sequence from the three other *Trypodendron* associates (Fig. 3).

Phylogenetic analysis

A Bayesian consensus tree of the combined SSU and TEF1α dataset placed the fungal symbionts within the *Ceratocystidaceae* (Fig. 5). There was strong support for grouping the ambrosia beetle symbionts with *C. adiposa* (Butler) Moreau (1952), *C. fagacearum* (Bretz) J. Hunt (1956), *C. norvegica* J. Reid & Hausner (2010), and *Huntiella* spp. There appeared to be three lineages of ambrosia symbionts, and these three

lineages correlated with the three respective host beetle tribes: *Xyleborini*, *Corthylini*, and *Xyloterini* (Fig. 5).

There was strong support (1.0 prior probability, 100% bootstrap) for the *Xyleborini* associates as a monophyletic clade, and all of these species formed aleurioconidia on branching conidiophores (Fig. 5). The *X. amputatus* associate had sequences similar to those of *A. beaveri,* the *An. sayi* associate had sequences similar to those of *A. hartigii*, and the *X. germanus* associate had sequences most similar to those of *A. roeperi* and *A. xylebori* (Fig. 5). The single species from the *Corthylini* appeared to group separately from the *Ambrosiella* spp. from the *Xyleborini* and appeared to be more closely related to *C. adiposa* and *C. norvegica*, as well as to the genus *Huntiella* (formerly the *C. moniliformis* complex). The sequences of the *Trypodendron* symbionts were most similar to those of *C. fagacearum* (Fig. 5).

Comparison of marginal likelihoods from topological testing in MrBayes showed that it was more likely that the three lineages of ambrosia beetle symbionts were polyphyletic (-10,793.39) rather than a single, monophyletic group (-10,803.71). *Phialophoropsis* (*Xyloterini* associates) was more likely to be a separate clade distinct from the *Xyleborini* associates (i.e., *Ambrosiella*) (-10,793.91), rather than forming a single monophyletic group with it (-10,795.35). The *Corthylus punctatissimus* associate was clearly outside of *Ambrosiella sensu stricto* (Fig. 5), but the *C. punctatissimus* associate was found to be more likely grouped with the *Xyleborini* associates as a sister group (-10,789.59) than not (-10,794.99) in the marginal likelihood testing.

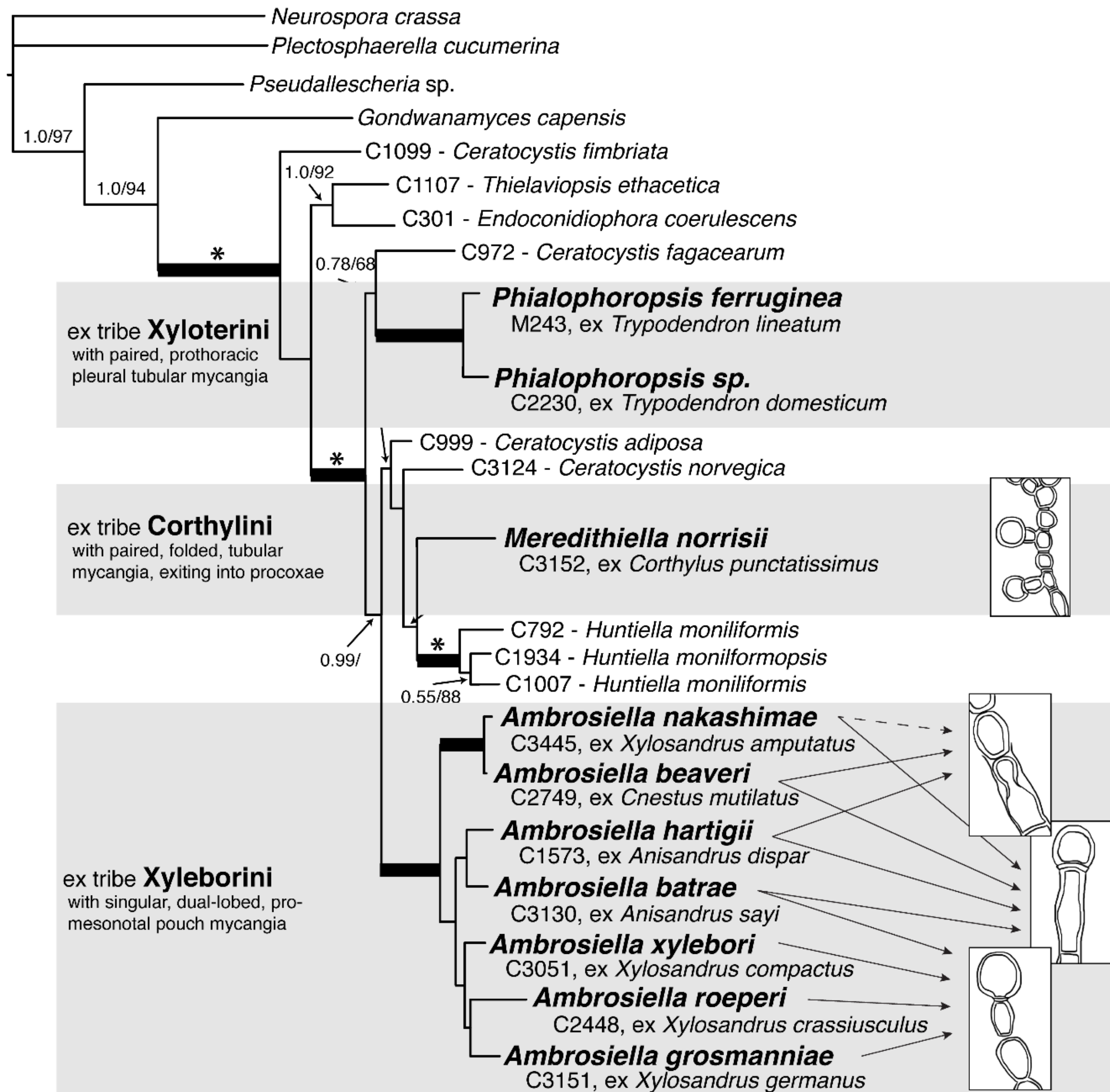

Figure 5. Phylogenetic tree from Bayesian analysis of the combined TEF1α and SSU (18S) rDNA dataset of ambrosia beetle symbionts and representatives of the *Microascales*. Posterior probabilities from Bayesian analysis and bootstrap support values (> 50%) from maximum parsimony analysis are indicated on branch labels. Thickened branches with asterisks indicate posterior probability of 1.0 and 100% bootstrap support. Tribes and the type of mycangium exhibited by the host beetles in the genera associated with *Ceratocystidaceae* are indicated in the three shaded boxes. Diagramatic illustrations of conidiophores of the ambrosia beetle symbionts are included; solid arrows indicate consistent observation in a species, and the dashed arrows indicate rare or ambiguous observations. The tree is rooted to *Neurospora crassa* and *Plectosphaerella cucumerina.*

Taxonomy

Phylogenetic analysis and morphological characters supported recognition of three new species of *Ambrosiella,* resurrection of the genus *Phialophoropsis* for the symbionts of *Trypodendron* spp.*,* and assignment of the *Corthylus punctatissimus* symbiont to a new genus.

AMBROSIELLA Arx & Hennebert emend. T.C. Harr., Mycotaxon 111: 354. 2010

Type species: *Ambrosiella xylebori* Brader ex Arx & Hennebert

Ambrosiella beaveri Six, De Beer and W.D. Stone, Antonie van Leeuwenhoek 96: 23. 2009

MycoBank MB 504757

Comments – This species was probably introduced to the Southeastern USA with its beetle host, *Cnestus mutilatus*, from Asia (Six et al 2009). The culture from the holotype of *A. beaveri* (CBS 121750) and two isolates (C3180 and C3181) from *C. mutilatus* in Georgia produce chains of phialoconidia from deep-seated phialides (Fig 1A) and terminal aleurioconidia on branched conidiophores (Fig 1B), as illustrated by Six et al. (2009).

Ambrosiella nakashimae McNew, C. Mayers, and T. C. Harr., **sp. nov.** (Fig. 1 C-F)

MycoBank MB 812571

Etymology. Named after Toshio Nakashima, who characterized fungi sporulating in the galleries of numerous ambrosia beetles in Japan.

Typus: **USA**: *Georgia*: Lowndes Co., 30° 49' 06.6" N, 83° 28' 48.3" W**,** *Xylosandrus amputatus* gallery in *Cinnamomum camphora*, Aug 2014, *S. Cameron,* holotype (BPI 893134); ex-type C3445 (CBS 139739).

Colonies on malt extract yeast agar 7-32 mm after 4 days and 25-50 mm after 6 days at 25° C, surface mycelium white, flat, becoming smoke gray to olive green with cream patches, reverse cream white, becoming dark green to black, odor weak, slightly sweet at 6-10 days. *Sporodochia* white to gray on surface, superficial. *Aleurioconidiophores* (Fig 1E) hyaline to brown with age, simple to branched, 12-115 (155) µm long, composed of one or more cells, producing a single terminal aleurioconidium or a chain of aleurioconidia, conidiogenous cell with a collarette. *Aleurioconidia* (Fig 1F) globose to subglobose, hyaline to light brown with age, thick-walled, aseptate, 6-10 (14) × 6-10 µm. *Phialoconidiophores* uncommon, with moderately seated phialides, producing basipetal chains of conidia by ring-wall building. *Phialoconidia* ellipsoidal to globose, hyaline to light olive brown with age, becoming thick-walled, aseptate 6-10 (12) × 5-9 µm. *Protoperithecia* (Fig 2B, 2C) superficial, spherical, tan to brown, 55-70 µm diam. *Gallery growth* dense, flat, white to grey, becoming brown with age. *Aleurioconidiophores* (Figs 1C) similar to those in culture, 9-70 µm long, ending in a phialide that may have a distinct collarette. *Aleurioconidia* in galleries (Fig 1D) larger than in culture, terminal or in chains, globose to suboblate, thick walled, aseptate, hyaline, may become light brown with age, (8) 9-12.5 (15) × (7.5) 8.5-11.5 (16) µm.

Other cultures examined: **USA**: *Georgia*: Lowndes Co., 30° 49' 06.6" N, 83° 28' 48.3" W, *Xylosandrus amputatus* gallery in *Cinnamomum camphora*, Aug 2014, *S. Cameron,* CBS 139740 (C3443).

Notes. The symbiont of *X. amputatus* is similar to *A. beaveri* both in DNA sequences and morphology, especially the aleurioconidiophores, but differs in growth rate (7-32 mm vs. 28-36 mm diam after 4 days at 25 C), rarity of phialoconidiophores, and the production of protoperithecia. This is the first report of protoperithecia in ambrosia beetle symbionts, which are thought to be asexual (Harrington et al. 2010). No perithecia or ascospores were seen, but the studied isolates were from galleries of a single infestation and may be of a single mating type. The beetle is native to Asia (Cognato et al. 2011b), and it is possible that only a single mating type of *A. nakashimae* was introduced. It also is possible that *A. nakashimae* is conspecific with *A. beaveri*, but phenotypic differences between the strains warrants distinction at this time.

Ambrosiella hartigii L.R. Batra, Mycologia 59: 998. 1968

MycoBank MB 326143

Comments – The ambrosial symbiont of *Anisandrus dispar* in Germany was originally named *Monilia candida* by Hartig (1844), but this name was found to be a homonym of an earlier species and was redescribed by Batra (1967) as *Ambrosiella hartigii*. The fungus has been reported from *An. dispar* in Michigan, Oregon, and Washington (Batra 1967, Roeper et al. 1980, Roeper and French 1981). We obtained DNA sequences (Fig. 4) from another German isolate from a mycangium of *An. dispar* (CBS 404.82 = C1573), though this isolate is no longer sporulating. Recently obtained European isolates from *A. dispar* from Austria (CBS139746 = C3450) and the Czech

Republic sporulated heavily on MYEA, and these isolates produced two spore types in culture: chained phialoconidia from moderately seated phialoconidiophores (Fig 1G) and terminal aleurioconidia (Fig 1I) on aleurioconidiophores (Fig 1H), as illustrated by Batra (1967). *A. hartigii* has been reported as the fungal symbiont of *An. sayi* and *An. obesus* (Hazen and Roeper 1980, Roeper and French 1981, Roeper et al. 1980) and *X. germanus* (Roeper 1996, Weber and McPherson 1984), but the symbionts of *An. sayi* and *X. germanus* are described here as new species of *Ambrosiella*.

Ambrosiella batrae **C.** Mayers, McNew & T.C. Harr., **sp. nov.** (Fig. 1 J-N)

MycoBank MB 812572

Etymology: Named after Lekh Raj Batra, who worked extensively on fungi symbiotic with ambrosia beetles.

Typus: **USA**: *Michigan*: Montcalm County, Alma College Ecological Station, 43° 23’ 32.31” N, 34° 53’ 40.91” W, isolated from *Anisandrus sayi* caught in flight, July 2013, *R. Roeper*, holotype (dried culture, BPI 893131); ex-type living culture C3130 (CBS 139735).

Colonies on malt yeast extract agar 13-51 mm diam. after 4 days at 25° C, odor sweet at 4-10 days, surface growth white to buff, fluffy to chalky, growth below surface dense, with irregular colony margins, coloring the media deep rust, darkening with age to chestnut, rust colored liquid drops occasionally seen on surface mycelium. *Conidiophores* scattered on aerial mycelium or concentrated in sporodochia, white to buff, spherical. *Aleurioconidiophores* hyaline to light brown, simple to branched, 25-105 µm tall, either composed of monilioid cells and with a terminal aleurioconidium with subtending collarette (Fig 1L), or rarely composed of non-monilioid cells with chained

aleurioconidia from shallow phialides (Fig 1J). *Aleurioconidia* globose to subglobose, thick-walled, aseptate, smooth, hyaline to rarely light brown, 11-16.5 (21) × 11-16 μm, either borne terminally on monilioid aleurioconidiophores, often tearing away along with one, two, or three conidiophore cells attached (Fig 1M, 1N), or borne on non-monilioid aleurioconidiophores in short chains of aleurioconidia that break off either singly (Fig 1K) or in short chains.

Other cultures examined: **USA**: *Georgia*: Chattahoochee National Forest, from *An. sayi* caught in flight, 1 July 2014, *S. Fraedrich* (C3415). *Missouri*: Boone County, isolated from *An. sayi* caught in flight, 20 May 2013, *S. Reed* (CBS 139736, C3045).

Notes. This species was isolated from a North American native (Wood and Bright 1992a, 1992b), *An. sayi*, females of which have mesonotal mycangia (Hazen and Roeper 1980). Cultures of *A. batrae* can be distinguished from other *Ambrosiella* species by their dark rust/chestnut staining of the medium, the scattered, spherical sporodochia, and the presence of both conidia borne in chains (Fig. 1 J) and terminal conidia that tear away with conidiophore cells attached (Fig. 1 M,N).

Ambrosiella xylebori Brader ex Arx & Hennebert, Mycopathologia et Mycologia Applicata 25: 314. 1965

MycoBank MB 326147

Comments – The genus *Ambrosiella* was invalidly described by Brader (1964) as *A. xylebori* from a *Xylosandrus compactus* gallery in *Coffea canephora* from the Ivory Coast, but no type was designated. Von Arx and Hennebert (1965) illustrated and designated a type for the genus and species based on Brader's isolate (CBS 110.61). The association of an *Ambrosiella* with *X. compactus* has been confirmed in India (Batra

1967, Bhat and Sreedharan 1988) and Japan (Kaneko and Takagi 1966). Brader's culture (CBS 110.61 = C3051) in our collection no longer sporulates, but an isolate from *X. compactus* collected in 2007 from Georgia (C2455) showed vigorous growth (62 – 63 mm after 4 days on MYEA) and sporulation. We observed the two types of aleurioconidiophores illustrated by Brader (1964) and von Arx and Hennebert (1965): one with disarticulating monilioid conidiophore cells (Fig 1V), breaking off with attached aleurioconidia (Fig 1U), and a second, straight, hyphoid aleurioconidiophore with a single, attached aleurioconidium (Fig 1W). The latter conidiophore type appears to be unique to *A. xylebori*. The fungus reported from *Corthylus colombianus* as *A. xylebori* (Batra 1967) is likely closely related to the new species we describe here from *C. punctatissimus,* which also was previously reported to be *A. xylebori* (Roeper 1996).

Ambrosiella roeperi T.C. Harr. & McNew, Mycologia 106: 841. 2014

MycoBank MB 805798

Comments – This recently described symbiont of the Asian species *Xylosandrus crassiusculus* (Harrington et al. 2014) produces aleurioconidiophores (Fig 1S) with terminal aleurioconidia (Fig 1T) that break off with one or more conidiophore cells attached; no phialoconidiophores have been observed. *A. roeperi* has been reported from Georgia, Ohio, Missouri, and South Carolina. A new isolate from *X. crassiusculus* in Taiwan is confirmed to be *A. roeperi* based on morphology and ITS sequence (Fig. 4). A culture from *X. crassiusculus* in Taiwan identified as *A. xylebori* by Gebhardt et al. (2005) was likely *A. roeperi.*

Ambrosiella grosmanniae C. Mayers, McNew & T.C. Harr., **sp. nov**. (Fig. 1 O,P)

MycoBank MB 812573.

Etymology. Named after Helene Francke-Grosmann for her pioneering work on ambrosia beetles and their mycangia.

Typus: **USA**: *Iowa*: Story County, Ames, Reactor Woods, 42° 02' 39.0" N, 93° 39' 40.5" W, isolated from *Xylosandrus germanus* caught in flight, 5 August 2013, *C. Mayers*, holotype (BPI 893132); ex-type C3151 (CBS 137359).

Colonies on malt yeast extract agar 45-60 mm diam. after 4 days at 25° C, surface covered with dense buff to olivaceous aerial mycelium, leading margin white, underside olivaceous to isabelline, becoming darker with age, odor sweet, noticeable at 4 days, fading after 8 days. *Aleurioconidiophores* (Fig. 1R) rare, occurring singly or grouped on white to buff sporodochia, hyaline, simple to branched, 20-60 µm long, composed of moniliod cells. *Aleurioconidia* produced terminally from a distinct subtending collarette, thick-walled, smooth, hyaline, aseptate, globose to subglobose, 7.5-12 × 7.5-12 µm, tearing away with attached conidiophore cells. *Gallery growth* a dense layer of aleuriocondiophores (Fig. 1O), producing aleurioconidia Fig 1P, 1Q) as in culture. *Growth in mycangium* composed of arthrospore-like cells 4.4-8.0 µm in diameter, irregular in shape, single or in septate chains of two to four cells (Fig. 4F).

Other specimens examined: **USA**: *Michigan*: Grand Traverse County, Traverse City, Ashton Park, 44° 46’ 10.77’ N, 85° 38’ 59 W, gallery of *X. germanus* in *Acer saccharum*, 24 July 2013, *R. Roeper* (BPI 893133).

Other cultures examined: **Germany**: *Waldeck*: near Jena, beech forest, from *X. germanus* caught in flight, 2014, *P. Biedermann* (C3467). **Netherlands**: *Gelderland:* near

Wageningen, from *X. germanus* caught in flight, 2014, *L. van de Peppel* (C3466). **USA**: *Georgia*: Clarke Co., Whitehall Forest, from *X. germanus* specimen caught in flight, 21 March 2014, *S. Fraedrich* (C3312). *Michigan*: Grand Traverse Co., Traverse City, Ashton Park, 44° 46' 10.77" N, 85° 38' 59" W, isolated from *X. germanus* taken from gallery (BPI 893133) in *Acer saccharum*, 24 July 2013, *R. Roeper* (CBS 137358, C3149). *Missouri*: St. Louis County, from *X. germanus* specimen caught in flight, 30 April 2014, *S. Reed* (C3385). *New York*: Tompkins Co., Ithaca, from *X. germanus* mycangium, April 2009, *L. Castrillo* (C3127). *Ohio*: Wayne Co., isolated from *X. germanus* mycangium, May 2010, *B. Anderson* (C3126). *Tennessee*: Warren Co., McMinnville, from *X. germanus* mycangium, April 2011, *N. Youssef* (C3128). *Virginia*: Princess Anne Co., Virginia Beach, isolated from *X. germanus* mycangium, June 2009, *P. Schultz* (CBS 137357, C3125). **Switzerland**: *Canton of Bern:* near Bern, beech forest, from *X. germanus* caught in flight, 2014, *P. Biedermann* (C3470).

Notes. This new species has been consistently isolated from *X. germanus* galleries, mycangia, and ground beetles from eight USA states and three European countries. Sequences of ITS rDNA (HQ538467 and HQ670423) from a "*Ceratocystis* sp." isolated from *X. germanus* in Korea matched closely to those of *A. grosmanniae* (Fig 3). The dense aerial mycelium of *A. grosmanniae* grows quickly in culture, but sporulation is rare, as previously reported for the *X. germanus* symbiont in Japan (Kaneko et al. 1965). It shares these qualities with *A. xylebori,* but the olivaceous to brown pigmentation of *A. grosmanniae* cultures distinguishes it from the white growth of *A. xylebori*. Growth in the mycangium is similar to that observed for *A. roeperi* in

mycangia of *X. crassiusculus*, though the mycangial spores of *A. grosmanniae* are somewhat smaller (Harrington et al 2014).

MEREDITHIELLA McNew, C. Mayers & T.C. Harr., gen. nov.

Mycobank MB 812574

Etymology. Named for Meredith Blackwell, whose work has included fungi associated with a wide array of insects.

Solitary, thick-walled, terminal aleurioconidia produced on short side branches from monillioid hyphae. Associated with ambrosia beetles.

Type species: *Meredithiella norrisii* McNew, Mayers, and T.C. Harr., sp. nov.

Though only one species of *Meredithiella* is described at this time, an ITS sequence from *Corthylus consimilis* and Batra's (1967) illustrations of a similar fungus from *C. columbianus* imply that there are other species in *Meredithiella* associated with *Corthylus* spp. The aleurioconidia of *Meredithiella* look similar to those of some *Ambrosiella* spp., but the aleurioconidiophores of the *C. punctatissimus* (Fig. 2 I, J) and *C. columbianus* symbionts are uniquely branched.

Meredithiella norrisii McNew, C. Mayers & T.C. Harr., **sp. nov.** **(**Fig. 2 F-J)

MycoBank MB 812575.

Etymology. Named after Dale Norris who, along with his students, studied ambrosia beetles and their fungi.

Typus: **USA**: *Iowa*: Story Co., McFarland Park, *Corthylus punctatissimus* gallery in *Acer nigrum* sapling, 8 August 2013, *T. Harrington,* holotype (BPI 893135); ex-type C3152 (living culture CBS 139737, dried culture BPI 893136).

Colonies on malt yeast extract agar 20-38 mm diam. after 4 days at 25 C, surface mycelium flat to aerial, white, becoming light gray brown, underside olivaceous, becoming dark brown, odor sweet at 4-10 days then fading. *Sporodochia* common, occurring singly or coalescing into dense, flat masses on the surface of mycelia, white to buff, spherical, sometimes exuding a light red liquid. *Aleurioconidiophores* (Fig. 2I, 2J) on sporodochia or in loose aerial tufts, hyaline to light brown, as one-celled or rarely multiple-celled side branches, 13.5 - 16 µm long, arising from long chains of monillioid hyphae, bearing a single, terminal aleurioconidium. *Aleurioconidia* terminal, subglobose to globose, thick-walled, aseptate, smooth, hyaline, 9-11 × 8-11 µm, breaking off with a conidiophiore cell attached (Fig. 2H) or rarely singly (Fig. 2G). *Gallery growth* with abundant aleurioconidiophores (Fig. 2F) bearing terminal aleurioconidia, 9-12.5 × 7.5-13 µm.

Other specimens examined: **USA**: *Michigan*: Grand Traverse Co., Traverse City, Ashton Park, 44° 46’ 11.60” N, 85° 38’ 34.89” W, *C. punctatissimus* gallery in *Acer saccharum,* 8 July 2013, *R. Roeper* (BPI 893137).

Other cultures examined: **USA**: *Iowa*: Story Co., McFarland Park, isolated from *C. punctatissimus* beetle in gallery of *Acer nigrum* sapling, 8 August 2013, *T. Harrington* (C3160). *Michigan*: Grand Traverse Co., Traverse City, Ashton Park, isolated from *C. punctatissimus* beetle in gallery of *Acer saccharum* sapling, October 2013, *R. Roeper* (C3187).

Notes. Meredithiella norrisii was recovered from galleries and from male *C. punctatissimus* beetles from Michigan and Iowa. Though the symbiont of both *C. punctatissimus* (Roeper 1995, 1996) and *C. colombianus* (Batra 1967) were previously

identified as *A. xylebori,* the *C. punctatissimus* symbiont is morphologically distinct and falls outside of *Ambrosiella* in phylogenetic analyses (Fig. 5). The ITS sequence from *C. consimilis* is likely that of a distinct species of *Meredithiella* (Fig. 3), and Batra's (1967) illustrations of *A. xylebori* from *C. colombianus* galleries look similar to the gallery sporulation of *M. norrisii*.

PHIALOPHOROPSIS L.R. Batra **emend.** T.C. Harr

Conidiophores hyaline, one-celled to septate, ending in deep-seated phialides, producing hyaline, aseptate conidia singly or in chains; and/or hyphae forming monilioid chains of chlamydospores, breaking apart singly or in groups. Aleurioconidia not present. Associated with ambrosia beetles.

Type species: Phialophoropsis trypodendri L.R. Batra

Batra (1967) originally created *Phialophoropsis* to accommodate *P. trypodendri* from *Trypodendron scabricollis*. Though he placed the *T. lineatum* associate in *Ambrosiella*, both *P. trypodendri* and *A. ferruginea* form deep-seated phialides, and aleurioconidia have not been noted in either species. Based on the distinctive phialides, Roeper (1972) suggested that *A. ferruginea* and *A. hartigii* should be transferred to *Phialophoropsis* or the genus *Ambrosiella* should be emended to include species with deep-seated phialides. The latter was done in a revision of *Ambrosiella* (Harrington et al. 2010). However, the morphological and phylogenetic evidence support retention of *Phialophoropsis* to accommodate ambrosia beetle symbionts with deep-seated phialides and the absence of aleurioconidia. The *T. lineatum* associate also forms thick-walled, hyphal swellings that disarticulate in the beetle galleries, as found in the *T. lineatum*

galleries from Colorado and as illustrated by Mathiesen-Käärik (1953). Thus far, only *Trypodendron* symbionts are accommodated in *Phialophoropsis*.

Phialophoropsis trypodendri L.R. Batra, Mycologia 59:1008. 1968

MycoBank MB 336297.

Synonym: Ambrosiella trypodendri (L.R. Batra) T.C. Harr., Mycotaxon 111:355. 2010 MycoBank MB 515299.

Comments – Batra (1967) described this species from cultures isolated from galleries of *T. scabricollis* in *Pinus echinata* collected in Arkansas. We examined the type material (BPI 422499, LRB-1952) of *P. trypodendri* and found a single microscope slide, which had short chains of what appear to be phialoconidia, as illustrated by Batra (1967) and redrawn by Seifert et al. (2011). No other material of *P. trypodendri* appears to be available, but the ITS rDNA sequence from DNA extracted from female *T. scabricollis* trapped at 10 locations in Missouri was similar to the ITS rDNA sequences of the symbionts of *T. lineatum, T. domesticum*, and *T. retusum* (Fig. 3).

Phialophoropsis ferruginea (Math.-Käärik) T.C. Harr, **comb. nov.**

MycoBank MB 812586.

Basionym: Monilia ferruginea Math.-Käärik, Meddelanden fran Statens Skogforskningsinstitut 43: 57 (1953)

MycoBank MB 474947

Synonym: Ambrosiella ferruginea (Math.-Käärik) L.R. Batra, Mycologia 59: 1000 (1968)

MycoBank MB 326141

Comments – An ambrosial fungus in the galleries of *T. lineatum* was observed by Hartig (1872) in Germany and later by Leach et al. (1940) in Minnesota. Mathiesen-Käärik (1953) described the fungus as *Monilia ferruginea*, and Batra (1967) moved the species to *Ambrosiella.* Other studies confirmed the relationship between *P. ferruginea* and *T. lineatum* (Francke-Grosmann 1967, Funk 1965), but this species was also thought to be the symbiont of *T. domesticum* and *T. retusum* (Batra 1967), *T. betulae* (Roeper and French 1981), and *T. rufitarsis* (French and Roeper 1972). We examined Batra's (1967) Oregon material (BPI 407710) from *T. lineatum* galleries and saw palisades of conidiophores with deep-seated phialides bearing phialoconidia singly or in chains. Isolations were attempted from galleries made by *T. lineatum* in a log of *Picea* sp. from Colorado (BPI 893129, 893130), but the attempts were unsuccessful. Microscopic examination of this gallery growth found phialoconidiophores with deep-seated phialides and chains of conidia (Fig. 2L, 2M) packed densely along the walls of the gallery (Fig. 2N). The ITS sequence from *T. lineatum* beetles trapped in Alaska matched the sequence from the *T. lineatum* galleries in Colorado. The ITS, SSU, and TEF1α sequences from the DNA extracted from *T. lineatum* galleries in Colorado were similar to those of the symbionts from *T. domesticum* and *T. retusum* (Fig. 3, 5), which appear to be different *Phialophoropsis* spp. awaiting further study.

Uncertain or excluded species of *Phialophoropsis*

Phialophoropsis cambrensis B.L. Brady & B. Sutton, Trans. Br. Mycol. Soc. 72: 337. 1979

MycoBank MB 319858

Comments – This species was described from a leaf lesion on *Embrothium lanceolatum* in Wales (Brady and Sutton 1979). The illustrations of the conidiogenous

cells are similar to those of *Phialophoropsis*. However, the cultures and conidia were reported to be salmon pink, there was no report of the odor of ripe bananas typical of *Phialophoropis* cultures, and *P. cambrensis* was not associated with an ambrosia beetle.

Phialophoropsis nipponica Matsush., Matsushima Mycological Memoirs 9: 19. 1996

MycoBank MB 415852

Comments – This species was isolated from the surface of a decaying branch and associated with apothecia (Matsushima 1996), which would not be consistent with the current placement of *Phialophoropsis* within the *Microascales*.

Discussion

This study is the first to associate lineages of ambrosia fungi with specific mycangial types and suggest a tighter co-evolutionary pattern between the fungal and beetle mutualists than has previously been recognized. However, the fungal associates of only a small percentage of the more than 3400 ambrosia beetle species have been studied (Batra 1967, Harrington et al. 2010), and the symbioses between ambrosia beetles with large, elaborate mycangia and the *Ceratocystidaceae* may prove to the exception rather than the rule. Ambrosia beetles typically feed on a mixture of fungi (Batra 1966, 1967, Kinnuura 1995), and adult beetles may be externally contaminated with a diversity of microorganisms, but growth in mycangia appears to be more specific. In the case of the more ubiquitous and relatively small mycangia found in most ambrosia beetle genera, mixtures of *Raffaelea* spp. and other fungi may be found in an individual beetle, and a single *Raffaelea* sp. may be associated with more than one beetle tribe (Harrington and Fraedrich 2010, Harrington et al. 2010, 2011, Kasson et al. 2013). There appears to be much more specific associations in those ambrosia beetle genera that have large, elaborate mycangia (Harrington et al. 2014). We examined 14 ambrosia beetle species in

six genera and three tribes with relatively large, elaborate mycangia and found that each species harbored a unique ambrosia species in the *Ceratocystidaceae*. The fungi recovered or detected included six previously recognized species, four new species, and four putative species. The 14 fungal species sorted into three lineages, suggesting that the symbiosis independentaly arose within each of the three respective beetle tribes: *Ambrosiella* within *Xyleborini*, *Meredithiella* within *Corthylini*, and *Phialophoropsis* within *Xyloterini*.

The large mycangia of beetles in these three tribes are found in different parts and sexes of the adult beetles and apparently arose independently. In each case, it appears that the genera with large, complex mycangia evolved from other genera with smaller and simpler mycangia that are known to harbor *Raffaelea* spp. (Harrington et al. 2014). In the *Xyleborini*, females of species in the genera *Xylosandrus, Anisandrus, Cnestus,* and *Eccoptopterus* have large mesonotal mycangia (Beaver 1989, Francke-Grosmann 1956, 1967, Happ et al. 1976, Hulcr and Cognato 2010, Hulcr et al. 2007) that harbor *Ambrosiella* spp., while other genera of *Xyleborini* with simple oral mycangia (e.g., *Xyleborus* and *Euwallacea*) or with small elytral mycangia (e.g., *Xyleborinus*) (Beaver 1989, Francke-Grosmann 1967) may harbor unrelated species of *Raffaelea* or *Fusarium* (Freeman et al. 2013, Harrington et al. 2010, 2011, Kasson et al. 2013, O'Donnell et al. 2015). In *Corthylini*, male *Corthylus* spp. have long, folded tubes opening into the procoxal cavity (Finnegan 1963, Giese 1967), which apparently harbor *Meredithiella* spp., while other genera of *Corthylini* (e.g., male *Gnathotrichus* spp. and female *Monarthrum* spp.) have simple enlargements of the procoxal cavity (Batra 1963, Farris 1963, Lowe et al. 1967, Schneider and Rudinsky 1969a) and have *Raffaelea* symbionts

(Batra 1967, Funk 1970, Harrington et al. 2010, Roeper and French 1981). Finally, in *Xyloterini*, *Trypodendron* spp. carry *Phialophoropsis* in their complex, tubular, pleural-prothoracic mycangia (Abrahamson et al. 1967, Batra 1967, Francke-Grosmann 1956, 1967, French and Roeper 1972, Schneider and Rudinsky 1969b), while *Xyloterinus politus* has a prothoracic cavity guided by hairs with an unidentified fungus and simple oral mycangia in both sexes that harbor *Raffaelea* spp. (Abrahamson and Norris 1966, 1969, Harrington unpublished).

Our initial hypothesis was that the fungal species associated with large, complex mycangia would form a monophyletic group, i.e., *Ambrosiella* (Harrington et al. 2010). Although members of the *Ceratocystidaceae* were consistently associated with the large mycangia, the fungal associates appeared to be in three phylogenetic lineages that correlate with the tribe of their host, suggesting three separate origins of the symbiosis without horizontal exchange of the fungal symbionts between tribes. The clearest phylogenetic distinction is seen between the *Xyleborini* associates (*Ambrosiella*) and the *Xyloterini* associates (*Phialophoropsis*) (Alamouti et al. 2009, de Beer et al. 2014, Harrington 2013, Harrington et al. 2010, Six et al. 2009). *Phialophoropsis* appears to be closest to *Ceratocystis fagacearum,* while our analyses suggest that *Ambrosiella* and the new genus *Meredithiella* are more closely related to *C. adiposa, C. norvegica,* and the genus *Huntiella* (formerly the *C. moniliformis* complex). *Huntiella* spp., *C. fagacearum* and *C. adiposa* form phialoconidia in deep-seated phialides, but only *C. adiposa* forms aleurioconidia (Harrington 2009, Nag Raj and Kendrick 1975). None of these *Ceratocystis* spp. are clearly associated with ambrosia beetles (Harrington 2009), but *C. norvegica* was recovered from galleries of a conifer bark beetle (Reid et al. 2010).

Ceratocystis fagacearum, *C. adiposa* and *C. norvegica* are distinct from *Ceratocystis sensu stricto* (the *C. fimbriata* complex) and need further phylogenetic and taxonomic study (de Beer et al. 2014), as do the ambrosia beetle symbionts in the family.

The ambrosia fungi in the *Ceratocystidaceae* appear to be obligate symbionts and may only be dispersed in mycangia, in which they produce arthrospore-like cells with schizogenous division, rather than yeast-like budding (Harrington et al. 2014). Conidia produced in the galleries would likely be the propagules that enter the mycangia of callow adults (Harrington et al. 2014), but the conidiophores and conidia appear to be important adaptations for beetle grazing. Many of these fungi produce dense palisades of conidiophores or columns of conidia in ambrosia beetle galleries, and disarticulating conidiophores appear to be a common feature. The conidia and cells of the conidiophores often contain large lipid bodies (Harrington et al. 2014), and fungal-produced lipids and sterols may be important for beetle development (Kok 1979, Norris 1979, Norris et al. 1969, Six 2012).

Production of phialoconidia from deep-seated phialides by ring-wall building is found throughout the *Ceratocystidaceae* (Harrington 2009, Paulin-Mahady et al. 2002), and moderately- to deep-seated phialides were found in species of *Phialophoropsis* and *Ambrosiella*. The simplest ambrosia growth was found in the galleries of *T. lineatum*, where the phialoconidiophores of *P. ferruginea* are only one or two cells long and arranged side-by-side in a hymenium, as illustrated by Batra (1967), and basipetal chains of oily phialoconidia are produced in dense columns. The chlamydospore-like hyphal swellings of *P. ferruginea* described by Mathiesen-Käärrick (1953) were not observed in

our cultures of *Phialophoropsis*, nor were aleurioconidia seen in galleries or cultures of *Phialophoropsis* spp.

Gebhardt et al. (2005) reported phialidic conidiogenous cells in *P. ferruginea, A. hartigii*, and *A. xylebori*, but we have found only aleurioconidiophores with inconspicuous collarettes in *A. xylebori*. Moderately-seated phialides were observed in *A. beaveri*, *A. hartigii*, and perhaps *A. nakashimae*, but these may be the least specialized conidiophores produced by *Ambrosiella* spp. All three of these species form a second type of conidiophore, with an inconspicuous, subtending collarette and aleurioconidia that break off singly or in chains, but the conidia do not disarticulate with conidiophore cells attached. This second type of conidiophore also was observed in *A. batrae*, which also displays a third conidiophore type, which appears to be the most advanced and best adapted for insect grazing. The conidiophore is made up of branching, monillioid cells that may break off with attached, terminal aleurioconidia (Harrington et al. 2014). Distinctive, monillioid conidiophores have been illustrated in *A. xylebori* (Batra 1967, Brader 1964, Kaneko 1967, von Arx and Hennebert 1965), *A. roeperi* (Kaneko 1967, Harrington et al. 2014), and *A. grosmanniae* (Nakashimae et al. 1992), and we observed them in *A. batrae.* In addition to disarticulating aleurioconidiophores, *A. xylebori* produces single aleurioconidia from simple, hyphae-like aleurioconidiophores (Batra 1967, von Arx and Hennebert 1964), which likely do not disarticulate (Harrington et al. 2014).

Meredithiella norrisii produces terminal aleurioconidia on monillioid hyphae that tear away with one or more conidiophore cells attached, similar to the third type of *Ambrosiella* conidiophore. However, the *M. norrisii* aleurioconidia are borne on short

side branches that arise from a central monillioid hypha, as opposed to the branched aleurioconidiophores of *Ambrosiella* spp.

Most species in the family *Ceratocystidaceae* are weak to aggressive plant pathogens (Harrington 2009, 2013), but the ambrosia beetle symbionts in this family appear to be strictly nutritional symbionts. Ambrosia beetles have a broad array of fungal associations and have been implicated as vectors of tree pathogens, but rather than acting directly as vectors, they more commonly facilitate spread of pathogens, such as *C. fimbriata,* via expelled frass containing aleurioconidia (Harrington 2009, 2013). An exception is the invasive *Xyleborus glabratus,* whose mycangial symbiont is the laurel wilt pathogen, *Raffaelea lauricola* (Fraedrich et al. 2008, Harrington et al. 2011). Other *Raffaelea* spp. and *Fusarium* spp. associated with ambrosia beetles may aid their beetles in killing trees, but these fungi are not wilt fungi and do not systemically colonize their hosts (Harrington and Fraedrich 2010, Harrington et al. 2011, Kessler Jr 1974, Kusumoto et al. 2015, Mendel et al. 2012, Ploetz et al. 2013). In isolations from ambrosia beetles with large mycangia, we rarely recovered other fungi, such as *Fusarium* spp. However, *Ambrosiella* spp. dominated the ambrosia growth in fresh galleries of the *Xyleborini* and were consistently associated with the mycangia in microscopic observations, in isolations, and in PCR amplifications. The limited observations of galleries and mycangia of *Corthylus* spp. and *Trypodendron* spp. found similarly tight associations with *Meredithiella* and *Phialophoropsis* spp., respectively.

Ambrosiella spp. appear to be associated with only the genera of *Xyleborini* with large, mesonotal mycangia. In a possible exception, Kostovcik et al. (2014) detected DNA of an *Ambrosiella* sp. from oral mycangia of both *Xyleborus ferrugineus* and *X.*

affinis using PCR. The amplified ITS sequence from *X. ferrugineus* matched most closely to the ITS sequence of *A. grosmanniae* from *X. germanus* (HQ538467), referred to as "*Ceratocystis* sp., CspXger3" (Kostovcik et al. 2014). It is possible that contaminating DNA of an *Ambrosiella* sp., such as *A. roeperi* from *X. crassiusculus*, was amplified by Kostovcik et al. (2014). They failed to detect a *Raffaelea* sp. in *X. ferrugineus* or *X. affinis* mycangia, but we (Harrington, unpublished) have isolated *Raffaelea* spp. but not *Ambrosiella* spp. from both of these beetle species, which have small, oral mycangia.

Evidence suggests that mycangial symbionts in the *Ceratocystidaceae* are species-specific and consistently found in both the native and introduced populations of their respective beetle symbionts. Examples include the respective *Ambrosiella* spp. associated with intercontinental populations of *Xylosandrus germanus*, *X. crassiusculus*, and *X. compactus*. Also, related species within beetle genera carry related fungal symbionts, even if the beetle species are native to different continents. The American *An. sayi* harbors *A. batrae*, while the closely related *A. hartigii* is associated with the European *An. dispar*. Vertical, linear transfer of asexual fungal symbionts from parent to offspring within galleries and severe inbreeding in the haplo-diploid *Xyleborini* (Cognato et al. 2010) may lead to tight co-evolution between the beetle and fungal lineages, perhaps foretelling an evolutionary dead end. However, the discovery of what appears to be protoperithecia in *A. nakashimae* suggests that these mycangial symbionts are not strictly asexual, and their sexual spores may be transmitted on the exoskeleton of insects, facilitating horizontal transfer among beetle species.

There is likely much unexplored diversity among the fungi associated with ambrosia beetles with large, complex mycangia. Study of additional *Corthylus* spp. and

Trypodendron spp. is required to determine the degree of specificity to their fungal symbionts as compared to the *Xyleborini*. Of particular interest are *Scolytoplatypus* spp., which have large mycangial pockets in the pronotum (Schedl 1962) and have been associated with fungi with monillioid chains of spores (Kinuura 1995; Nakashima et al. 1987, 1992; Nakashima 1989). *Microcorthylus* spp. apparently have mycangia similar to *Corthylus* spp. (Schedl 1962) and may harbor species similar to *M. norissii*. On the other hand, further studies of ambrosia beetles with simpler mycangia may find that symbionts in the *Ceratocystidaceae* have other, less-specific associations with ambrosia beetles.

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Tables

Table 1. Cultures, specimens, and GenBank accessions for representative species.

	Specimen ID (Collection ID)	Associated ambrosia beetle	GenBank Accession No		
			ITS	SSU	TEF-1a
Ambrosiella					
A. batrae	C3130 (CBS 139735)	*Anisandrus sayi*	KR611322	KR673881	KT290320
A. beaveri	C2749 (CBS 121750)	*Cnestus mutilatus*	KF669875	KR673882	KT318380
A. nakashimae	C3445 (CBS 139739)	*Xylosandrus amputatus*	KR611323	KR673883	KT318381
A. grosmanniae	C3151 (CBS 137359)	*Xylosandrus germanus*	KR611324	KR673884	KT318382
A. hartigii	C1573 (CBS 404.82)	*Anisandrus dispar*	KF669873	KR673885	KT318383
A. roeperi	C2448 (CBS 135864)	*Xylosandrus crassiusculus*	KF669871	KR673886	KT318384
A. xylebori	C3051 (CBS 110.61)	*Xylosandrus compactus*	KF669874	KR673887	KT318385
Ambrosiella sp.	M257	*Eccoptopterus spinosus*	KR611325		
Meredithiella					
M. norrisii	C3152 (CBS 139737)	*Corthylus punctatissimus*	KR611326	KR673888	KT318386
Meredithiella sp.	M260	*Corthylus consimilis*	KR611327		
Phialophoropsis					
P. ferruginea	M243 (BPI 893129)	*Trypodendron lineatum*	KR611328	KR673889	KT318387
Phialophoropsis sp.	C2230 (CBS 460.82)	*Trypodendron domesticum*	KC305146	KR673890	KT318388
Phialophoropsis sp.	CBS 408.68	*Trypodendron retusem*	KC305145		
P. trypodendri	SUTT	*Trypodendron scabricollis*	KR611329		
Ceratocystis* and other *Ceratocystidaceae					
C. adiposa	C999 (CBS 183.86)		=JN604448	KR673891	HM569644
C. fagacearum	C927 (CBS 129242)		=KC305152	KR673892	KT318389
C. fimbriata	C1099 (ICMP 8579)		AY157957	KR673893	HM569615
C. norvegica	C3124 (UAMH 9778)		DQ318194	KR673894	KT318390
Endoconidiophora coerulescens	C301 (CBS 100.198)		KC305116	KR673895	HM569653
Huntiella moniliformis	C1007 (CBS 204.90)		=DQ074739	KR673896	KT318391
Huntiella sp.	C792		KR611330	KR673897	KT318392
H. moniliformopsis	C1934 (DAR 74609)		=NR119507	KR673898	HM569638
Thielaviopsis ethacetica	C1107		=KJ881375	KR673899	HM569632

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CHAPTER 3. FIRST REPORT OF A SEXUAL STATE IN AN AMBROSIA FUNGUS: *AMBROSIELLA CLEISTOMINUTA* SP. NOV. ASSOCIATED WITH THE AMBROSIA BEETLE *ANISANDRUS MAICHE*

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Abstract

Genera of ambrosia beetles in the tribe Xyleborini with large, mesonotal mycangia host unique fungal symbionts in the genus *Ambrosiella.* The symbiont of a recent invasive to the USA from Asia, *Anisandrus maiche,* had not been previously characterized. We found the mycangium anatomy of *An. maiche* collected in Ohio to be similar to that of *Anisandrus dispar* and consistently isolated a novel fungus, *Ambrosiella cleistominuta* sp. nov., from *An. maiche* mycangia and galleries. The fungus was distinguished from other named *Ambrosiella* by morphological characters and DNA sequences (ITS rDNA and *TEF-1a*). The mycangial symbionts of ambrosia beetles had been assumed to be strictly asexual, but *A. cleistominuta* produces cleistothecious ascomata with ascospores in beetle galleries and in culture. In contrast to ascomata of other *Ceratocystidaceae*, the relatively small ascomata of *A. cleistominuta* are neckless

and without ostioles. The ascospores are relatively large, and single ascospore colonies produced ascomata and ascospores in culture, showing that *A. cleistominuta* is homothallic.

Introduction

Anisandrus maiche (*Coleoptera: Curculionidae: Scolytinae*) is an Asian ambrosia beetle that has recently invaded the USA and Europe (Rabaglia et al. 2009; Terekhova & Skrylnik 2012). Adult female *A. maiche* have been recovered from flood-stressed *Cornus florida* L. trees attacked in Ohio, USA (Ranger et al. 2015), and could present a similar risk to ornamental and horticultural trees as other invasive Xyleborini (Ranger et al. 2016). Based on studies of other *Anisandrus* spp. (Mayers et al. 2015), a species of *Ambrosiella* Arx & Hennebert emend. T.C. Harr. (*Microascales*: *Ceratocystidaceae*) would be expected to serve as a mycangial symbiont of *An. maiche.*

Ambrosia beetles cultivate fungal gardens along the walls of galleries tunneled in sapwood, and larvae and adults feed on crops of conidia and conidiophores as their food source (Batra 1967; Harrington et al. 2010). Ambrosia beetles are polyphyletic (Kirkendall et al. 2015), and tribes of ambrosia beetles have independently evolved special organs (mycangia) to transport their symbionts to new trees (Batra 1967; Francke-Grosmann 1967; Beaver 1989; Six 2012). The adult beetles secrete nutrients into the mycangium to support active growth of the symbiont, and the overflow of fungal propagules leads to inoculation of newly excavated galleries (Beaver 1989). Mycangia of various genera and tribes of ambrosia beetles vary considerably in size, shape, and location on the body (Francke-Grosmann 1967; Beaver 1989), and their primary fungal symbionts also vary (Harrington et al. 2010, 2014; Mayers et al. 2015).

The Xyleborini genera *Anisandrus, Cnestus, Eccoptopterus, Hadrodemius,* and *Xylosandrus* form a monophyletic group with relatively large mesonotal (mesothoracic) mycangia (Hulcr & Cognato 2010). These mycangia are formed by a deep invagination of the intersegmental membrane between the scutellum and pronotum (Francke-Grosmann 1956; Happ et al. 1976; Stone et al. 2007). Species of *Ambrosiella* have proven to be the primary fungal symbionts of Xyleborini with large mesonotal mycangia, such as *Anisandrus dispar* F., *Anisandrus sayi* Hopkins, *Cnestus mutilatus* (Blandford), *Eccoptopterus spinosus* (Oliver), *Xylosandrus compactus* (Eichhoff), *Xylosandrus crassiusculus* (Motschulsky), and *Xylosandrus germanus* (Blandford) (Six et al. 2009; Harrington et al. 2010, 2014; Mayers et al. 2015).

The goals of this study were to characterize the mycangium and identify the mycangial symbiont of an invasive population of *An. maiche* established in Ohio, USA. We hypothesized that *An. maiche* would have a mesonotal mycangium similar to that of other *Anisandrus* (Francke-Grosmann 1956, 1967; Happ et al. 1976; Hulcr et al. 2007). Further, we expected the mycangium to harbor budding spores of *Ambrosiella*, which would serve as the primary food source of the larvae (Mayers et al. 2015).

Materials and Methods

Beetle collection

Live *Anisandrus maiche* females were collected using ethanol-baited bottle traps deployed at four locations in Wayne County, Ohio: Barnard Road Site: Lat. 40º45'41.43"N, Long. 81º51'16.88"W; Davey Farm Site: Lat. 40º51'53.41"N, Long. 82º3'8.80"W; Badger Farm Site: Lat. 40º46'38.62"N, Long. 81º51'9.34"W; Metz Road Site: Lat. 40º52'19.87"N, Long. 81º56'26.06"W. Bottle traps were assembled according to Ranger et al. (2010), but moist paper towels were placed in the lower collection bottle

rather than low-toxicity antifreeze in order to maintain beetle and fungal viability (Ranger et al. 2015). Female adults were stored refrigerated in parafilm-sealed Petri dishes with moist filter paper, then killed by crushing the exoskeleton and shipped overnight in glass vials with or without sterile moist filter paper. Male *A. maiche* are flightless and were not collected.

Additional adult females were excavated from naturally-infested *Gleditsia triacanthos* L. trees growing in a commercial ornamental nursery in Ohio (Lat. 41°49'35.41"N; Long. 81°2'27.40"W). Stem sections were refrigerated and then split using a sterilized hand pruner. Adult female *A. maiche* collected from their host galleries were stored, killed, and shipped as described above.

Mycangia

Intact mycangia were dissected from beetles and separated from the scutellum with sterile needles, forceps, and razors on glass slides in a manner similar to that described by Batra (1985). Intact mycangia and spore masses teased from intact mycangia were mounted in cotton blue on a microscope slide and observed with Nomarski interference contrast (BH-2 compound microscope, Olympus, Melville, NY) and digitally photographed (Leica DFC295 camera and Leica Application Suite V3.6, Leica Camera Inc., Allendale, NJ). For some images, composites of several images taken at the same magnification and focus level were stitched together with the Photomerge function in Photoshop CS6 (Adobe, San Jose, CA) in "reposition" mode with blending enabled.

Fungal isolations

Intact *A. maiche* females were first surface-sterilized by submerging in 75% ethanol for 10s, then submerging in two successive baths of sterile deionized water and

allowed to dry on paper towels. Beetles were then pulled apart with sterile forceps and the portions containing the prothorax/mesothorax, scutellum, and mycangium were separately plated directly on SMA (1% malt extract, Difco Laboratories, Detroit, MI; 1.5% agar, Sigma-Aldrich, St. Louis, MO; and 100 ppm streptomycin sulfate added after autoclaving). Fungal colonies were subcultured to MYEA (2% Difco malt extract, 0.2 % Difco yeast extract, 1.5% agar).

Hyphal tip and single spore cultures

Round, pigmented structures resembling cleistothecia were observed in galleries with ambrosia growth and in one of the cultures on MYEA. Individual spherical structures were removed from galleries, cleaned by dragging across the surface of sterile MEA (1.5% malt extract, 1.5% agar), and DNA was extracted from these cleaned structures using PrepMan® Ultra (Applied Biosystems, Foster City, CA).

Single hyphal tip and single ascospore colonies were obtained from the culture (C3843) that produced spherical bodies on MYEA. Isolated hyphal tips beyond the advancing margin of growth were identified on MYEA using a dissecting scope (at 25× – 40× magnification and substage lighting), excised, and transferred to MYEA. Single ascospore cultures were obtained by crushing a single, spherical structure in a drop of sterile water on a flame-sterilized glass slide under a sterile coverslip, confirming the presence of the putative ascospores but absence of conidia at 500×, carefully raising the coverslip, and transferring the liquid containing spores to MEA with a micropipette. Individual spores were separated at 25× – 40× using a sterile needle. The isolated spores were allowed to germinate, and spores with a single germ tube were transferred to fresh MYEA plates.

Artificially-infested stem segments

Live *An. maiche* trapped in-flight at the Badger Farm and Davey Farm locations were allowed to infest *Cornus florida* stem sections under laboratory conditions that were 1.0–2.5 cm diam. and 9–10 cm long. Stem sections taken from live trees were soaked in distilled/deionized water for ~18 hrs, blotted and air-dried for 5 min, and placed in closed plastic containers (13 cm in diam., 9 cm tall) with moist paper towels. About 12 punctures were placed in the lid for ventilation. Stems were held at room temperature for 14 d at 23 °C and stored refrigerated until dissection. The infested stems were then split open and ambrosia growth within galleries was removed with sterile needles and plated on SMA for isolation or mounted in cotton blue for microscopic observation.

Culture description

Isolates from *A. maiche* and *C. florida* were grown at room temperature on MYEA. Agar plugs cut with a #1 cork borer (approximately 3mm diameter) were transferred from the leading margin of growth to three MYEA plates, grown at 25 °C for 7 d in the dark, and the diameter of the colonies measured and averaged for each isolate. Culture pigmentation/colors are in accordance with Rayner (1970).

DNA sequencing and analysis

Extractions of mycelia and spores were as previously described (Mayers et al. 2015), but extractions from some *Ambrosiella* cultures with excessive pigment were performed with the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-tek, Norcross, GA). The internal transcribed spacer (ITS) region of the ribosomal DNA, small subunit rDNA (SSU, 18S rDNA), and translation elongation factor 1-alpha (*TEF-1a*) were amplified and sequenced as per Mayers et al. (2015). Forward and reverse reads were compared using Sequence Navigator v 1.0.1 (Applied Biosystems, Foster City, CA). The SSU and

TEF-1a sequences were used as queries in NCBI's (National Center for Biotechnology Information) BLASTn tool.

The ITS sequences of the new species were aligned with those of eight other putative and named *Ambrosiella* (Mayers et al. 2015) in PAUP 4.0bb10 (Swofford 2002). The outgroup taxon was *Ceratocystis adiposa* (E.J. Butler) C. Moreau (DQ318195), a close relative to *Ambrosiella* within the *Ceratocystidaceae* (de Beer et al. 2014; Mayers et al. 2015). The dataset had 557 aligned characters, including gaps, and 106 of the characters were parsimony-informative. Gaps were treated as a fifth state. The analyses used stepwise addition and the tree-bisection-reconnection branch-swapping algorithm. Bootstrap support values were obtained by a full heuristic, maximum parsimony, 10,000-replicate bootstrap analysis in PAUP.

Results

Mycangium observations

Each of the seven examined female *Anisandrus maiche* (three trapped in flight, and four infesting *G. triacanthos*) had a mesonotal mycangium that opened between the scutellum and pronotum. The prominent dual lobes described for the mycangia of related genera, such as *Cnestus, Eccoptopterus,* and *Xylosandrus* (Stone et al. 2007; Harrington et al. 2014; Mayers et al. 2015), were not observed in the *An. maiche* mycangia. Instead, spores were observed in a small, unlobed pouch below the scutellum (Fig. 1), as illustrated in *Anisandrus dispar* by Francke-Grosmann (1956, 1958, 1967) and Happ et al. (1976). The scutellum curves ventrally on its anterior side, as in *An. dispar* (Francke-Grosmann 1958), and the dorsal surface is covered with pits, each ornamented on its rim

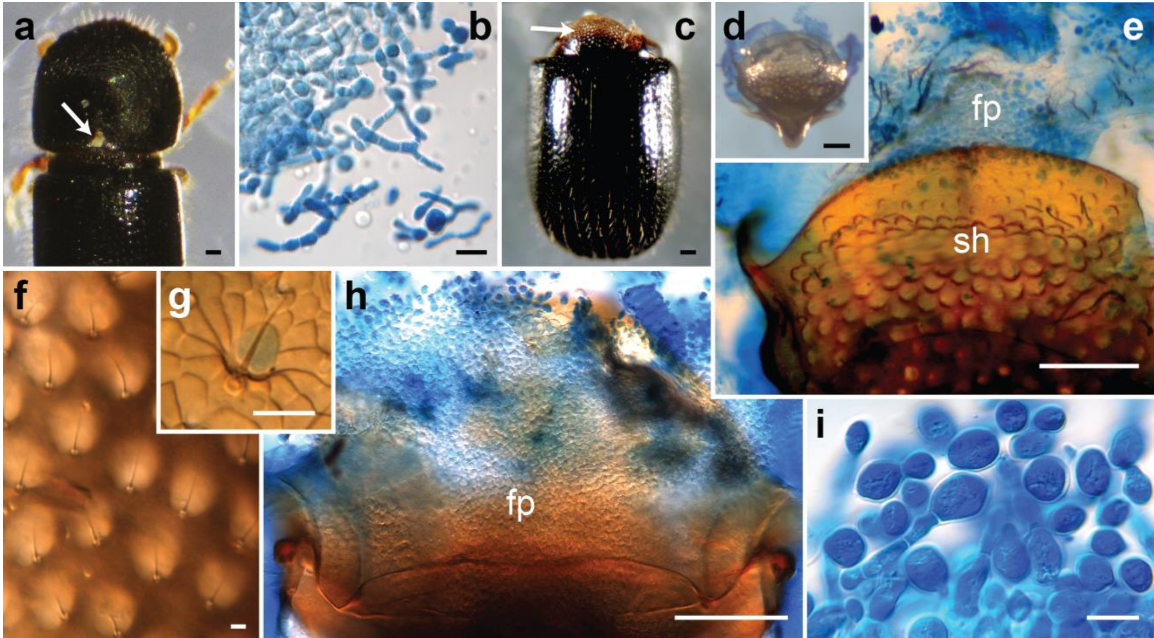

Figure 1. Mycangium of *Anisandrus maiche* and mycangial propagules of ***Ambrosiella cleistominuta*** sp. nov. (a) Exterior spore mass on the posterior margin of the pronotum (arrow). (b) Exterior spore mass, with germinating propagules. (c) Female with pronotum removed, revealing the light brown scutellum (arrow) protruding from below the mesonotum. (d) Dorsal aspect of excised scutellum. (e) Pits covering scutellum, scutellum hinge (sh), and fungal spores (fp) exiting from the mycangium below. (f) Detail of scutellum pits with rim setae. (g) Fungal propagule in shallow pit, with setae above. (h) Ventral aspect of scutellum, showing mycangium pouch full of fungal spores (fp). (i) Fungal propagules growing in mycangium. Photos a, c, and d by dissecting microscope. All other photos by Nomarski interference microscopy of material stained with cotton blue. Bar = 10 µm in b, f, g, i. For all other photos, bar = 100 µm.

with a single seta (Figs. 1d–f). The pit setae posterior to the hinge of the scutellum point anteriorly and medially, while the pit setae anterior to the scutellum hinge are not as uniform in their direction. The pits often contained one or more fungal propagules (Fig. 1g). A tuft of hairs at the base of the pronotum, often associated with mesonotal mycangia (Hulcr et al. 2007), was present in all females of *An. maiche*, as illustrated by Rabaglia et al. (2009).

The material teased from inside the mycangium was a dense, homogenous mass of fungal propagules (Figs. 1h, i), similar in appearance to that of other *Ambrosiella* (Francke-Grosmann 1956; Kaneko & Takagi 1966; Harrington et al. 2014, Mayers et al. 2015). The propagules appeared to proliferate by schizogenous, arthrospore-like growth.

Some beetles had external masses of spores associated with the tuft of hairs on the posterior edge of the pronotum (Fig. 1a). The external mass appeared to be composed of germinating propagules with branching hyphae (Fig. 1b).

Fungal isolation and identification

Isolations from dissected mycangia of surface-sterilized *An. maiche* trapped in flight or taken from infested *G. triacanthos* stems consistently yielded cultures of a fast-growing fungus that produced red-brown aerial hyphae with rust-colored liquid drops. The cultures had a sweet, fruity-ester smell and only rarely sporulated on MYEA.

Twelve of the 13 crushed beetles caught in flight and shipped with moist filter paper yielded the new fungal species, but only one of the nine beetles shipped without moist filter paper yielded the fungus. Only the new species grew from the mycangia of beetles shipped with moist filter paper, though in some cases other fungi grew from other plated parts of the beetle, such as unidentified yeasts from pieces of the gut. Three of the four plated beetles excavated from *G. triacanthos*, all shipped with moist filter paper, yielded the new fungal species.

Each of the 12 sequenced isolates from *An. maiche* adults and three from galleries in *C. cornus* yielded the same ITS sequence (GenBank KX909940), which differed from *Ambrosiella hartigii* L.R. Batra, the symbiont of *An. dispar*, by an additional T near the end and a repeated AATT at the very end of ITS2. The new species formed a strongly-supported clade with *A hartigii* separate from other *Ambrosiella* in phylogenetic analysis (Fig. 2).

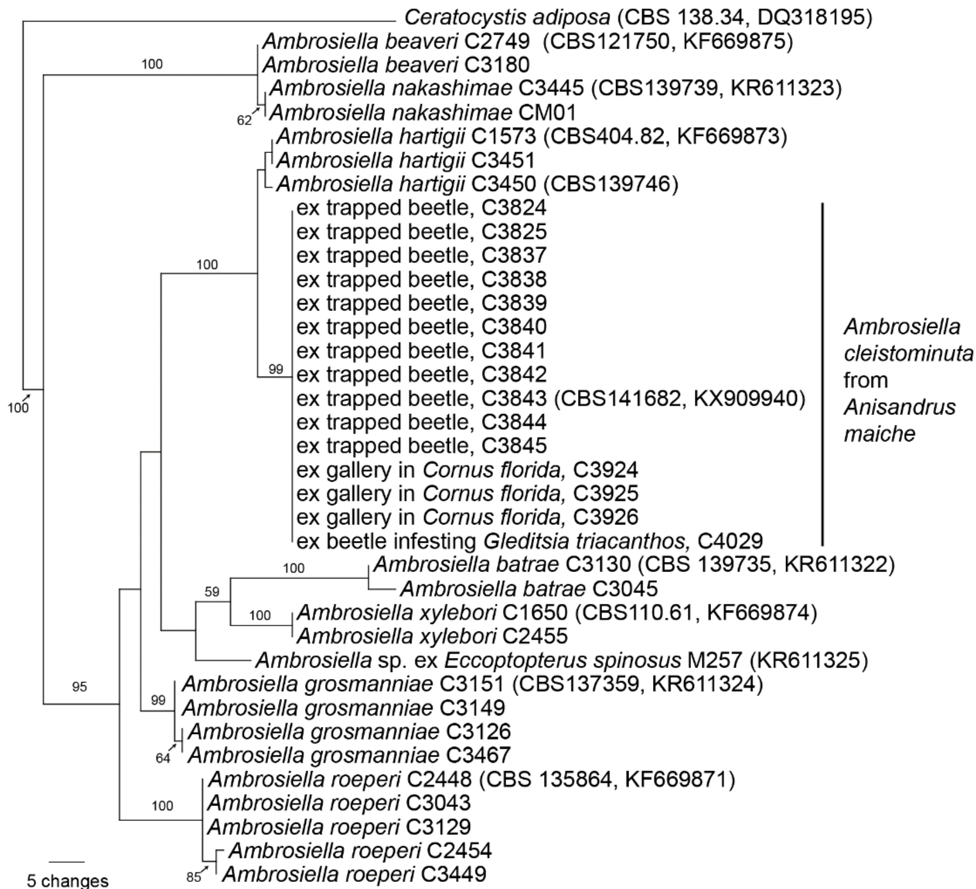

Figure 2. One of 12 most parsimonious trees of *Ambrosiella* spp. produced by unweighted maximum parsimony analysis of an ITS rDNA dataset of 557 aligned characters, 106 of which were parsimony-informative. Branch support values are from 1000 bootstrap replications. The outgroup was *Ceratocystis adiposa*. Species names or sources are followed by isolate numbers from the Iowa State University collection. Accession numbers for the Centraalbureau boor Schimmelcultures and GenBank are given in parentheses, where available.

Isolates C3843 and C3924 from *An. maiche* had identical *TEF-1a* and SSU sequences. Sequences for both gene regions confirmed placement of the new species within *Ambrosiella.* The trimmed *TEF-1a* sequence (KX925309) was 1168 bases long and included a 107-bp intron; it was most similar (1154/1167 bp matching) to the *TEF-1a* sequence of *A. hartigii* (KT318383.1). The trimmed SSU sequence of the new species (KX925304) was 1657 bases long and was most similar to the SSU sequence of *Ambrosiella grosmanniae* C. Mayers, McNew, & T.C. Harr. (KR673884, 1655/1655 bp matching) and *A. hartigii* (KR673885, 1653/1655 bp matching).

Culture morphology

Conidiophores (Figs. 3a–h) of the new species were rare in culture but were morphologically similar to those of *A. hartigii* and *Ambrosiella batrae* C. Mayers, McNew, & T.C. Harr. (Mayers et al. 2015). Two types of conidiophores were observed, but intermediate forms were seen. Phialoconidiophores (Figs. 3a, 3b) were usually composed of multi-branched, monilioid hyphae and moderately-seated phialides that produced cylindrical to barrel-shaped phialoconidia in chains. Aleurioconidiophores (Figs. 3c–h) produced globose, thick-walled aleurioconidia from what appeared to be very shallow phialides, often with inconspicuous collarettes. Aleurioconidiophores usually produced a single terminal aleurioconidium (Figs. 3c–e), but occasionally, chains of lightly pigmented aleurioconidia surrounded by a membranous sheath (Fig. 3f) and/or red-brown pigment (Figs. 3g, h) were seen.

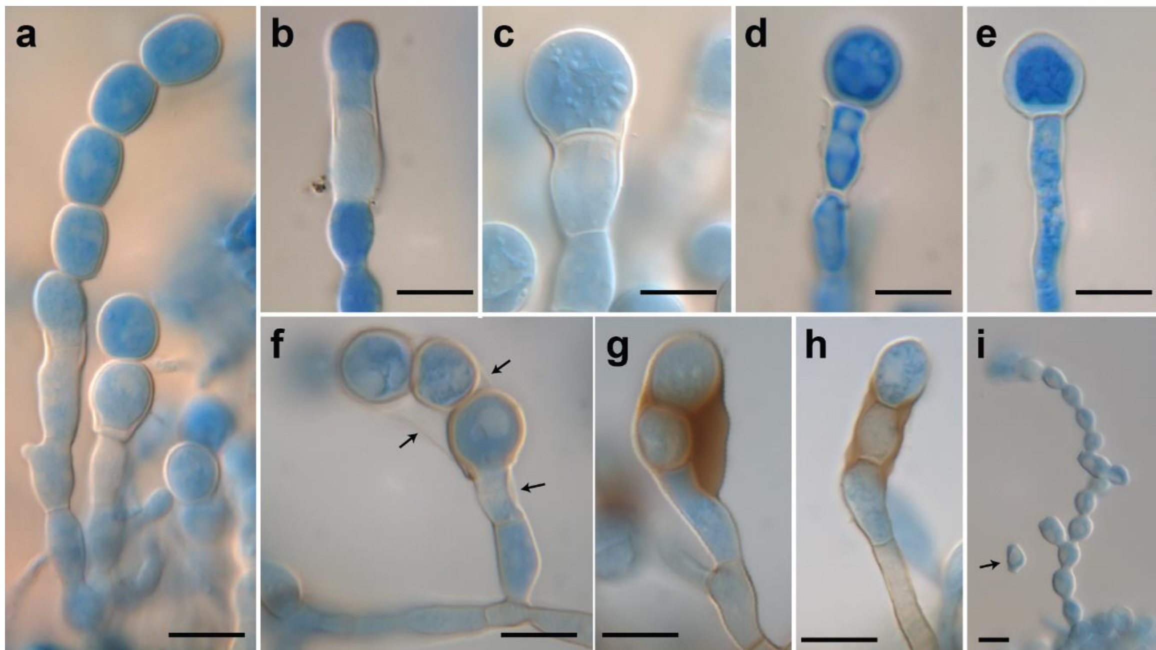

Figure 3. Conidiophores and conidia of ***Ambrosiella cleistominuta*** sp. nov. (a, b). Phialoconidiophores. (a) Bearing chained phialoconidia. (b) Deeply-seated phialide on monillioid hyphae. (c–h) Aleurioconidiophores. (e) On simple hyphae. (f) With membranous sheath (arrows). (g, h) With pigment. (i) Arthrospore (arrow) disarticulating from monillioid chain. All photos by Nomarski interference microscopy of material stained with cotton blue of ex-holotype isolate C3843 (CBS 141682). Bar = 10 µm.

Ascomata

None of the studied isolates of the new species initially produced ascomata. However, after several serial transfers of isolate C3843, a sector produced thick, white, fluffy aerial mycelia with many small, brown spherical structures in the aerial mycelium (Fig. 4). The fluffy white phenotype and the production of the spherical structures persisted through several serial transfers when grown on MYEA, but not on MEA. The

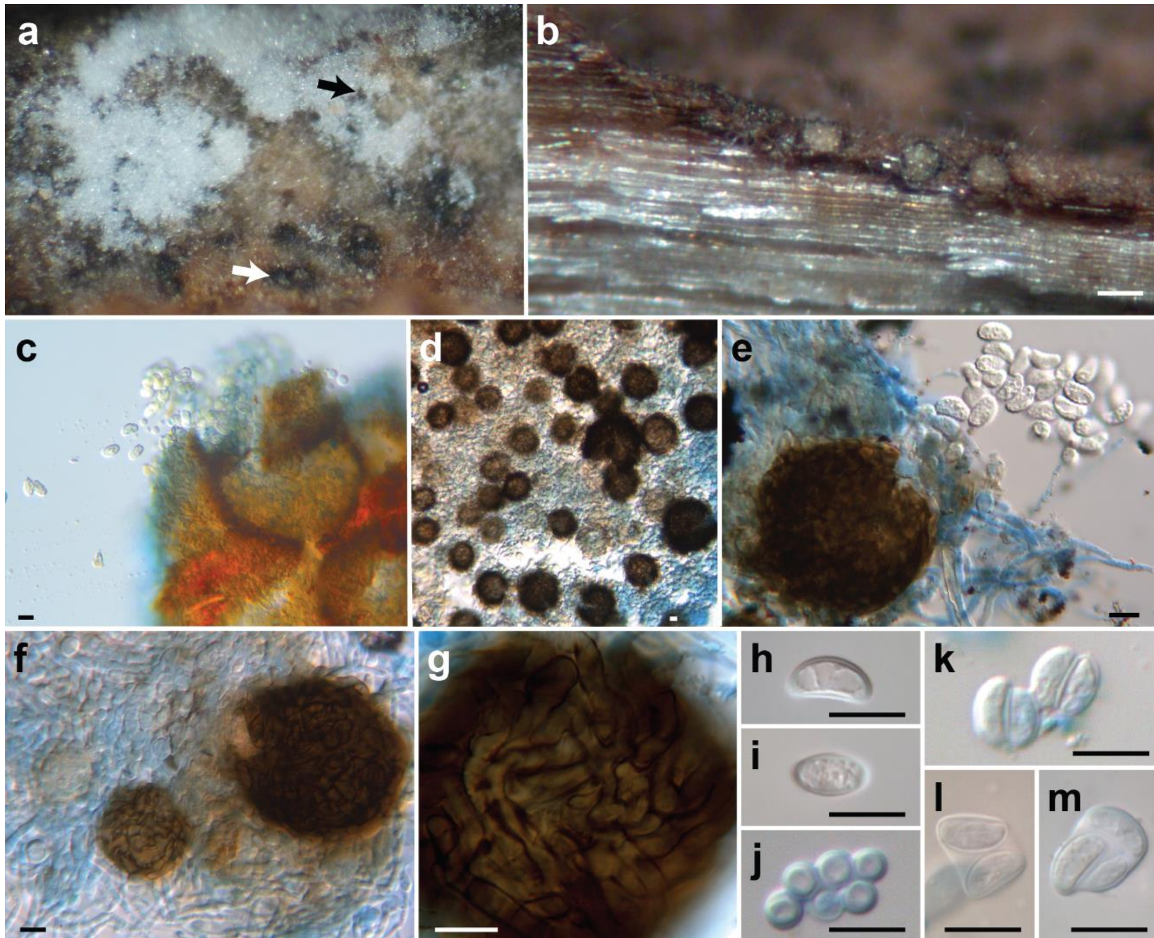

Figure 4. Gallery growth and sexual state of ***Ambrosiella cleistominuta*** sp. nov. (a–d) In gallery of *Anisandrus maiche.* (a) Opened cleistothecia (white arrow) in grazed area of the ambrosia growth and unopened cleistothecia (black arrow) in ungrazed areas. (b) Longitudial-sections of three cleistothecia embedded in ambrosia growth showing lighter ascospore masses inside. (c) Crushed cleistothecium and ascospores. (d–m) From cultures of the ex-holotype, isolate C3843 (CBS 141682). (d) Dense cluster of cleistothecia in white, aerial mycelium. (e) Ascospores from cracked cleistothecia. (f) Three developmental stages of cleistothecia. (g) Outer texture of cleistothecium. (h–m) Ascospores. (h) Side view. (i) Top view. (j) End view. (k) Paired ascospores. (l, m) Ascospores in membranous material. All photos by Nomarski interference microscopy of material stained with cotton blue. Bar = 100 µm in a and b.; = 10 µm in c–m.

spherical structures were small (40 – 80 µm diam) and lacked ostioles or necks, and were first assumed to be protoperithecia, as reported in *Ambrosiella nakashimae* McNew, C. Mayers & T. C. Harr. (Mayers et al. 2015). However, microscopic examination of crushed spheres revealed reniform spores of uniform size and shape (Figs. 4h–m), sometimes found in pairs (Fig. 4k), as has been found with ascospores of other *Ceratocystidaceae* (Van Wyk et al. 1993). Five hyphal tip colonies from C3843 and five colonies derived from single spores teased from the spherical structures each produced white, fluffy mycelia and the spherical fruiting bodies with reniform spores. The ITS sequence obtained from two cleaned ascomata, a hyphal tip colony, and a single-ascospore colony were identical to the original C3843 culture and to the other isolates of the new species.

Gallery growth

Some of the *C. florida* stem segments infested by *An. maiche* had only short, abandoned galleries, which contained neither brood nor ambrosia growth. However, three stem segments had one or more galleries running along the pith, and each gallery had larvae and/or pupae with luxurious, white ambrosia growth (Fig. 4a). Phialoconidiophores and aleurioconidiophores, identical to those in culture, dominated the galleries, though there was also limited sporulation of unidentified contaminating molds. Isolates obtained from the ambrosia growth in each of the three stems had the ITS sequence and culture morphology of the isolates from individual beetles (Fig. 2).

Buried in the ambrosia growth of the three stem segments were spherical fruiting structures without necks or ostioles, identical to those seen in culture but slightly larger, bearing reniform spores (Figs. 4a–c). Where the ambrosial growth had been grazed by

larvae, the spheres were open, irregular hemi-spheres, with edges of the dark, pigmented outer walls flush with the surrounding grazed mycelium. Pale yellow-brown spore masses were visible inside the cup-like remains of the spheres (Fig. 4a). It appeared that the spheres were broken or chewed open by the grazing of larvae because the white, ungrazed growth had only intact spheres, which were buried in the ambrosia growth (Fig. 4a).

Taxonomy

Morphological characters and DNA sequence analyses supported the recognition of the symbiont of *An. maiche* as a new species of *Ambrosiella.*

Ambrosiella cleistominuta C. Mayers & T.C. Harr. **sp. nov.** Figs. 1, 3, 4

MYCOBANK NUMBER: 819507.

TYPUS: United States of America. Ohio: Wayne county, near Barnard Rd, a dried culture isolated from an *Anisandrus maiche* female caught in flight, 40°45'41.43"N, 81°51'16.88"W, 8-Jul-2015, coll. *C. Ranger* (BPI 910177, holotype; CBS 141682 = C3843, ex-type culture). GenBank ITS rDNA sequence accession No. KX909940.

ETYMOLOGY: (L.) *cleistominuta,* in reference to its small cleistothecia.

DESCRIPTION: *Colonies:* on malt yeast extract agar 45–75 mm diam. after 4 d at 25 °C, odor sweet at 3–5 d, fading by 7 d, surface growth aerial, white to buff, dense and matted or sparse and in tufts, with small, wet, rust-colored clumps sometimes suspended on aerial hyphae, older cultures producing amber- to rust-colored liquid drops, margin hyaline, submerged, coloring the agar medium deep rust to chestnut. *Ascomata:* dark brown, spherical, texture intricata, suspended in aerial hyphae, 40–80 µm in diameter at maturity (Fig. 4d–g), lacking necks or any apparent opening. *Asci:* not observed. *Ascospores:* 9.0–12.0 µm × 4.5–7.0 µm in side view, 7.0–12.0 µm × 4.5–6.5

µm in top view, thick-walled, reniform, occasionally in pairs or groups (Figs. 4h–m). *Sporodochia:* rare in cultures, white, spherical, superficial, bearing conidiophores. *Conidiophores* (Figs. 3a–h) often branching, scattered in tufts on media surface, in clusters near plate edges, or on sporodochia, single- or many-celled, of two types: *Phialoconidiophores:* (Figs. 3a, b) hyaline, bearing single or chained phialoconidia from moderately- to deeply-seated phialides. *Aleurioconidiophores:* (Figs. 3c–h) hyaline to dark red-brown, bearing single or chained aleurioconidia, apparently from shallow phialides with inconspicuous collarettes. *Phialoconidia:* cylindrical, aseptate, smooth, hyaline, 8.0–14.0 µm × 6.0–14.0 µm, usually longer than wide (Fig. 3a, b), detaching singly or in chains. *Aleurioconidia:* globose to ellipsoidal, generally thick-walled, 7.0–10.5 µm × 8.0–12.0 µm, not detaching easily, hyaline to red-brown, borne singly and/or a red-brown pigment (Figs. 3f–h). *Arthrospores:* (Fig. 3i) rare in culture, exogenous, derived from disarticulating chains of monilioid cells, globose to ellipsoidal, 8.5–10.0 µm × 6.5–8.0 µm. *Mycangial growth:* (Figs. 1c, d) composed of irregular to globose, thick-walled cells 4.5–10.5 µm × 5.5–14.0 µm, with polar growth and dividing schizogenously, germinating with short, branching hyphae upon exiting the mycangium (Figs. 1a, b). *Gallery growth:* as in cultures, but cleistothecia somewhat larger, 70.0–110.0 µm diam. (Fig. 4c).

ECOLOGY AND DISTRIBUTION: In galleries and mycangia of *Anisandrus maiche*.

OTHER SPECIMENS EXAMINED: **USA:** *Ohio*: Wayne Co., ambrosia growth in *Cornus florida* artificially infested by *Anisandrus maiche* that were caught in flight in Wayne Co., August 2015, *C. Ranger*, OHAnma1-3 gal1, BPI 910176.

OTHER CULTURES EXAMINED: **USA:** *Ohio:* Lake Co., isolated from *Anisandrus maiche* infesting saplings of *Gleditsia triacanthos*, 15 August 2015, *C. Ranger,* C4029. Wayne Co., culture isolated from gallery with ambrosia growth (OHAnma1-3 gal1) in *Cornus florida* artificially infested by *Anisandrus maiche* caught in flight in Wayne Co., August 2015, *C. Ranger*, C3926.

COMMENTS: Based on DNA analyses, the mycangial symbiont of the Asian species *Anisandrus maiche* is most closely related to *Ambrosiella hartigii*, which is the mycangial symbiont of the related European species, *Anisandrus dispar* (Mayers et al. 2015). While *Ambrosiella cleistominuta* produces two types of conidiophores that could be classified as phialoconidiophores or aleurioconidiophores, there was a gradient of conidiophore morphologies, similar to what has been found in *A. hartigii* (Mayers et al. 2015). Cultures of *A. cleistominuta* are a darker red-brown, lacking the white, chalky surface growth sometimes seen in cultures *A. hartigii*. Of all known *Ambrosiella*, only *A. cleistominuta* is known to produce ascomata and ascospores.

Discussion

As hypothesized, *Anisandrus maiche* has a mesonotal mycangium like other *Anisandrus*, and the mycangium harbors budding spores of *Ambrosiella*. While *Ambrosiella* spp. can be difficult to isolate because the mycangium and gallery propagules are intolerant of desiccation (Zimmermann & Butin 1973; Beaver 1989), *Ambrosiella cleistominuta* was consistently isolated from beetles shipped with moist filter paper. *Ambrosiella cleistominuta* was the only ambrosia fungus isolated from the mycangium of *An. maiche,* supporting the conclusion that *Ambrosiella* is tightly associated with Xyleborini species with mesonotal pouch mycangia (Harrington et al. 2014; Mayers et al. 2015). Surprisingly, *A. cleistominuta* produced ascomata in cultures

and in galleries. Aside from associated yeasts (Batra 1963), a sexual state has never been reported from a mycangial symbiont of an ambrosia beetle.

Mycangium

The large mesonotal mycangia of *Anisandrus, Cnestus, Eccoptopterus, Hadrodemius,* and *Xylosandrus* are formed by an invagination of the intersegmental membrane between the scutellum and posterior base of the pronotum (Francke-Grosmann 1956, 1967; Happ et al. 1976; Stone et al. 2007). Francke-Grosmann (1956,1958) noted morphological differences in the mycangia of *X. germanus* and *Anisandrus dispar*. *Xylosandrus germanus* has two large bilateral lobes forming the left and right sides of the mycangium, as illustrated by Mayers et al. (2015), and its posterior membrane is attached to the anterior edge of the scutellum. The mycangium of *An. dispar* lacks the large bilateral lobes, is somewhat smaller, and its posterior membrane is attached further back on the ventral scutellum. Like *An. dispar*, the mycangium of *An. maiche* is relatively small and lacks the dual lobes reported for *Cnestus, Eccoptopterus,* and *Xylosandrus* (Francke-Grosmann 1967; Stone et al. 2007; Harrington et al. 2014; Mayers et al. 2015)

The conspicuous pits on the dorsal side of the scutellum are ornamented with setae on their rims, which sometimes hold fungal spores. Stone et al. (2007) illustrated similar pits holding propagules of *Ambrosiella beaveri* Six, de Beer & W.D. Stone on the scutellum of *Cnestus mutilatus.* We (unpub. data) have also noted scutellum pits with setae in *Anisandrus sayi, C. mutilatus,* and *E. spinosus*; setae with no pits in *Xylosandrus amputatus* (Blandford); and the absence of pits or setae on the completely smooth scutella of *X. compactus, X. crassiusculus,* and *X. germanus.* The biological significance of these pits and setae is unclear. In *An. maiche,* the setae that are posterior to the hinge of the scutellum generally point towards the opening of the mycangium, perhaps assisting

movement of spores into the mycangium of a callow female, or setae may spread or filter the spore mass exiting the mycangium of a tunneling female.

Culture morphology

Like the symbionts of *An. dispar* and *An. sayi, A. cleistominuta* produces phialoconidiophores bearing chains of barrel-shaped conidia and aleurioconidiophores that generally produce larger, single, globose, thick-walled conidia. The former apparently produces conidia via ring wall-building and the latter via replacement wall-building, following the terminology of Nag Raj & Kendrick (1993). The delicate chains of phialoconidia may be better adapted to enter the mycangium of their beetle hosts, while the aleurioconidia may be better adapted as food for grazing by the larvae and adults. Associates of *Xylosandrus,* such as *Ambrosiella roeperi* TC Harr. & McNew and *A. grosmanniae*, only produce aleurioconidiophores (Harrington et al. 2014; Mayers et al. 2015). Females of *Xylosandrus* have been reported to evert their large, lobed mycangia to acquire aleurioconidia from the ambrosia growth along the gallery walls (Kaneko 1967), and these species may not need phialoconidiophores for sowing of their mycangia.

Aleurioconidia are common in the family, but the unusual membranous sheaths around chains of aleurioconidia of *A. cleistominuta* have been described for only one other species in the *Ceratocystidaceae, Ceratocystis adiposa* (Hawes & Beckett 1977a, 1977b), a close relative of *Ambrosiella* (Mayers et al. 2015). In *C. adiposa*, the sheath surrounding the first aleurioconidium is formed within the phialide neck from the inner wall of the conidiogenous cell, and the sheath elongates along with the developing spore chain (Hawes & Beckett 1977b).

Sexual state of *Ambrosiella*

This is the first report of a sexual stage in a mycangial symbiont of an ambrosia beetle, but spherical structures assumed to be proto-perithecia were previously seen in cultures and galleries of *Ambrosiella nakashimae* associated with *X. amputatus* (Mayers et al. 2015). Beauverie (1910) also illustrated spherical structures with textured, darkened walls that may have been ascocarps of *Ambrosiella hartigii* embedded in ambrosia growth in galleries of *An. dispar*. Recently, Musvuugwa et al. (2015) reported sexual states for some species of *Raffaelea,* and *Raffaelea* are generally associates of ambrosia beetles (Harrington et al. 2010). However, the three reported *Raffaelea* spp. with sexual states may not be ambrosia beetle symbionts. *R. vaginata* T. Musvuugwa, Z.W. de Beer, L.L. Dreyer, & F. Roets was isolated from a *Lanurgus* (*Coleoptera: Curculionidae*) (Musvuugwa et al. 2015), but beetle species in this genus are herbiphagous or phloeophagous scolytids (Kirkendall et al. 2015) and do not have mycangia (Hulcr et al. 2015). *Raffaelea deltoideospora* (Olchow. & J. Reid) Z.W. de Beer & T.A. Duong was isolated from wood and from pupal chambers of cerambycid beetles, not ambrosia beetles (Musvuugwa et al. 2015). *Raffealea seticollis* (R.W. Davidson) Z.W. de Beer & T.A. Duong was reported from an abandoned beetle gallery in a hemlock stump (Davidson 1966). Cryptic sex was hypothesized for *Raffaelea lauricicola* T.C. Harr., Fraedrich, & Aghayeva, the mycangial symbiont of *Xyleborus glabratus* Eichhoff., but ascomata were not identified (Wuest et al. 2017).

Ambrosia fungi have been assumed to be strictly asexual, clonal lineages (Farrell et al. 2001; Normark et al. 2003; Harrington 2005; Harrington et al. 2010) because of the yeast-like reproduction in the mycangium during dispersal. Additionally, vertical transmission from mothers to daughters in the haplo-diploid lifestyle of the Xyleborini

(Cognato et al. 2011) limits opportunities for effective heterothallic mating. While asexual species are widespread in ascomycetes, it may be a transient or unstable state in nature, even in ancient lineages (Taylor et al. 1999). Truly strict asexual lineages are hypothesized to accumulate deleterious mutations over time, leading to evolutionary dead-ends (Haigh 1978; Taylor et al. 1999). *A. cleistominuta* formed fertile ascomata in nature and in culture, and all single-ascospore and hyphal tip colonies also produced ascomata and ascospores, indicating homothalism. The discovery of a sexual state in *A. cleistominuta* and ascocarp initials in *A. nakashimae* (Mayers et al. 2015) suggests that other ambrosia fungi may maintain cryptic sexual states despite the nature of their obligate mutualisms. Male Xyleborine beetles, which are flightless, travel to other galleries in heavily-infested trees, which may allow for contact between fungal strains and genetic recombination. Homothalism may facilitate sexual reproduction in spite of limited contact between thalli, but it may also limit outcrossing.

Cain (1956) argues that adaptations from ostiolate to cleistothecious ascomata are common in the ascomycetes and not taxonomically informative, but the unique cleistothecia of *A. cleistominuta* are noteworthy. Ascomata of other *Ceratocystidaceae* have spherical bases within which ascospores are produced, and the spores travel through long necks, exiting from ostioles and forming a wet mass of ascospores at the tip, which is an adaptation for contamination of the exoskeleton for insect-based dispersal (Malloch & Blackwell 1993; Harrington 2005, 2009). Ascomata of other families of *Microascales* also are typically ostiolate, though some species of *Kernia, Pithoascus,* and *Pseudallescheria* in the *Microascaceae* are known to be cleistothecious (Malloch 1970; von Arx 1978; von Arx et al. 1988; Barr 1990).

The ascomata of *A. cleistominuta* have no apparent opening, and the only exposed ascospore masses were observed in grazed galleries. The ascomata appeared to be broken open following grazing by the larvae or adults, despite the fact that pigmented ascomata are thought to be resistant to grazing by insects (Malloch & Blackwell 1993). The ascospores may be eaten by the beetles as a supplemental food source, or dispersed on the beetle exoskeleton or passed through the gut. The ascospores produced by *A. cleistominuta* are relatively large and are most similar in shape to those produced by its close relative, *C. adiposa* (Van Wyk & Wingfield 1990), which also produces reniform ascospores but has perithecia with very long necks (Malloch & Blackwell 1993).

Other lineages of mycangial symbionts of ambrosia fungus may harbor cryptic sexual states. Fruiting bodies buried in gallery growth may have been missed, overlooked as contaminants, or ignored due to a lack of evidence that they were produced by the fungal symbionts. Sexual fruiting structures in ambrosia beetle galleries may be rare, as in the fungal cultivars of some attine ants (Taylor et al. 1999). Future studies should take special note of spherical bodies found in ambrosial growth.

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CHAPTER 4. NEW *MEREDITHIELLA* SPECIES FROM MYCANGIA OF *CORTHYLUS* AMBROSIA BEETLES SUGGEST GENUS-LEVEL COADAPTATION BUT NOT SPECIES-LEVEL COEVOLUTION

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Abstract

Meredithiella norrisii (Microascales, Ceratocystidaceae) is an ambrosia fungus carried in mycangia of the North American ambrosia beetle, *Corthylus punctatissimus*. Reports on the identity of the fungal symbionts of other species of *Corthylus* have been inconsistent. This study tested the hypothesis that *Meredithiella* spp. are the primary symbionts of *Corthylus* spp. Cultures and/or ITS rDNA barcode sequences of *Meredithiella* spp. were obtained consistently from beetles and galleries of nine *Corthylus* spp. The ITS sequences of three putative species of *Meredithiella* were associated with *C. consimilis* and *C. flagellifer* in Mexico and *C. calamarius* in Costa Rica. The symbiont of *C. columbianus* in the USA was identified as *M. norrisii*. Two new *Meredithiella* spp. are described: *M. fracta* from *C. papulans* in Florida and Honduras, and *M. guianensis* associated with *C. crassus* and two unidentified *Corthylus* spp. in French Guyana. The *Meredithiella* spp. propagate in the mycangia of adult females by thallic-arthric growth, and the ambrosia growth in larval cradles comprises

bead-like hyphal swellings or conidiophores, with or without terminal aleurioconidia. Bayesian phylogenetic analysis of a combined 18S and 28S nuc rDNA, and *TEF*1-α dataset demonstrated that *Meredithiella* is a distinct monophyletic clade within the Ceratocystidaceae, but its phylogenetic placement with regard to the other ambrosial genera in the family remains ambiguous. The mycangia of *C. punctatissimus* and *C. papulans* are also compared using light microscopy and micro CT imaging, revealing that they differ in both size and shape, but these differences may not correlate with different lineages of *Meredithiella*.

Introduction

Like other ambrosia beetles (Kirkendall et al. 2015; Hulcr and Stelinski 2017), species of *Corthylus* (Coleoptera, Curculionidae, Scolytinae, Corthylini, subtribe Corthylina) farm and feed upon fungal gardens along the walls of their galleries and larval cradles in wood. One or both sexes of adult ambrosia beetles use organs called mycangia to store and transport actively-growing propagules of their fungal symbionts to establish fungal gardens in new trees (Francke-Grosmann 1967; Six 2003). Usually the adult female has the mycangia, but in *Corthylus* males have the mycangia, which consist of a pair of coiled, reticulated tubes in the prothorax that open into the procoxal cavities (Schedl 1962; Finnegan 1963; Giese 1967; Nord 1972). *Meredithiella norrisii* was described as the mycangial symbiont of *C. punctatissimus* (Mayers et al. 2015), but the fungal species associated with mycangia of other *Corthylus* have not been clearly identified.

Corthylus species are only found in the Americas, ranging from Canada to Argentina (Wood 1982; Wood and Bright 1992), but many of the species are uncommon and understudied (Wood 2007). Some species construct galleries in living hosts and are

important pests. For example, *C. columbianus* causes a discoloration known as flagworm in hardwoods (Nord 1972), and *C. zulmae* may facilitate disease in *Alnus acuminata* in Colombia (Gil et al. 2004; Jaramillo et al. 2011). Of the four *Corthylus* species in the USA, *C. punctatissimus* and *C. columbianus* are widespread in the eastern USA, while *C. petilus* is native to Arizona and Mexico; *C. papulans* (=*C. spinifer*) is an introduced species in Florida (Wood 1977, 1982; Wood and Bright 1992).

Ambrosia beetles are associated with a variety of fungi, including mutualistic primary ambrosia fungi that serve as their main food source and have co-adapted to be transmitted in mycangia (Batra 1985). Auxiliary fungi may be transmitted on the cuticle or in the gut (Batra 1985; Biedermann et al. 2013), or potentially in mycangia (Bateman et al. 2016), but such auxiliary fungi may not be important symbionts (Harrington 2005). Many primary ambrosia fungi are species of the genus *Raffaelea* (Ophiostomatales) (Harrington et al. 2010; Dreaden et al. 2014; Simmons et al. 2016), but some ambrosia beetles with relatively large and specialized mycangia host ambrosia fungi in one of three genera in the Microascales: *Ambrosiella*, *Phialophoropsis*, and *Meredithiella* (Harrington et al. 2010; Mayers et al. 2015).

A variety of fungi have been suggested to be the primary ambrosia fungi of *Corthylus*. A yeast species in *Pichia* was isolated from galleries, observed in mycangia, and interpreted as a primary nutritional symbiont of *C. columbianus* (Wilson 1959; Kabir and Giese 1966; Giese 1967; Gil et al. 2004). Batra (1967) identified the primary symbiont of *C. columbianus* as *Ambrosiella xylebori*, although illustrations suggest that the symbiont was similar to *M. norrisii*, the recently described symbiont of *C.*

punctatissimus (Mayers et al. 2015). Mayers et al. (2015) also detected DNA of another putative species of *Meredithiella* in mycangia of *C. consimilis.*

Several species of *Corthylus* were encountered in ongoing studies by the authors and collaborators, presenting opportunities to clarify the primary ambrosia fungi of *Corthylus*. The goal of this study was to characterize *Corthylus* mycangia, identify their fungal contents, isolate symbionts from the beetles, and characterize ambrosia growth in their galleries and larval cradles.

Materials and Methods

Sample Collection

The following beetle and gallery specimens were studied: *Corthylus papulans*: USA: Florida, Gainesville, Austin Cary Forest (29.732161, -82.219386), ethanol-baited light trap, two males kept alive on moistened tissue paper until dissection, Spring 2016, *C. Bateman*; Honduras, Francisco Morazán, campus of Zamerano Pan-American Agricultural School, ethanol-baited light trap, one male immediately preserved in 95% ethanol, Summer 2013, *C. Storer*; *C. crassus*: French Guiana, near Kaw Mountain (4.55892, -52.19662), gallery with male in unidentified tree host immediately preserved in 97% ethanol, June 2015, *C. Bateman*; *Corthylus* sp. A: French Guiana, near Kaw Mountain (4.55892, -52.19662), gallery with male in unidentified tree host immediately preserved in 97% ethanol, June 2015, *C. Bateman*; *Corthylus* sp. B: French Guiana, near Kaw Mountain (4.55892, -52.19662), gallery with male in unidentified tree host immediately preserved in 97% ethanol, June 2015, *C. Bateman*; *C. flagellifer*: Mexico, Michoacán, Ario de Rosales, six females in single gallery in *Persea americana,* 23 September 2014, *S. Ochoa-Ascencio*; and *C. calamarius*: Costa Rica, Cerro de la Muerte, single male in *Chusquea subtessellata,* 26 June 2010, *S. Smith.*

Two herbarium specimens deposited in KANU (University of Kansas Herbarium) as *C. columbianus* gallery material were studied: KANU 376401 and KANU 376400, cited by Batra (1967) as 1893-LRB and 2032-LRB, respectively. Hand-written notes (presumably Batra's) on the single packet in KANU 376401, which contained galleries in wood, identified the contents as 1893-LRB but gave collection information (October 1963, beech, Deer, Arkansas) that was not consistent with Batra's (1967) published notes for 1893-LRB (May 1965, *Ulmus* sp., Deer, Arkansas), so the material inside could not be verified. The other specimen (KANU 376400) contained three packets, collected by J.C. Nord in June 1966 from *Acer rubrum* wood with *C. columbianus* galleries, as well as a dried-down culture isolated from the gallery tunnels; the written notes matched Batra's (1967) published notes for 2032-LRB.

Males of *C. consimilis* and males and galleries of *C. punctatissimus*, as well as *M. norrisii* cultures, were available from a previous study (Mayers et al. 2015).

Mycangium dissection and observation

Mycangia were dissected and removed from freshly killed males or ethanol preserved specimens. For light microscopy, the prothorax was separated and bisected longitudinally, and excess body tissue was trimmed carefully with a scalpel until only the front leg, procoxa, and mycangium remained. The leg/coxa/mycangium was then mounted in cotton blue for imaging with Normarski interference contrast microscopy (BH-2 compound microscope, Olympus, Melville, New York). Images were captured using a Leica DFC295 camera and Leica Application Suite 3.6 (Leica Camera Inc., Allendale, New Jersey). Some images taken at different focus levels and combined into a single focus-stacked composite image using CombineZP (Alan Hadley).

Three-dimensional images of mycangia were rendered using non-destructive X-ray tomography (micro-CT scans) using a Pheonix v|tome|x m (GE, Boston, Massachusetts) at the University of Florida Nanoscale Research Facility. Settings were as described in Bateman et al. (2017), and data were visualized post-scan using VG StudioMAX 3.0 (Volume Graphics, Heidelberg, Germany). Most of the tissues surrounding the mycangium, such as tracheoles, were collapsed in the dried specimens and were easy to differentiate from the rigid, tubular mycangia. The tubular process was highlighted manually and appeared to denote the mycangium wall, including gland cells, and the fungal cells within.

Fungal Isolation from *Corthylus papulans*

Fresh males were surface-sterilized by immersing in 75% ethanol for 10 s followed by two washes in sterile deionized water, then dried on paper towels. The prothorax was separated using a sterile scalpel, crushed with sterile forceps, and plated directly onto malt extract agar with streptomycin (SMA: 1% malt extract, Difco Laboratories, Detroit, Michigan; 1.5% agar, Sigma-Aldrich, St. Louis, Missouri; 100 ppm streptomycin sulfate added after autoclaving). Fungi growing from the procoxal region or from fragmented sections of the tubular mycangium were subcultured onto plates of malt yeast extract agar (MYEA: 2% Difco malt extract, 0.2 % Difco yeast extract, 1.5% agar).

Microscopic examination of fungi and species description

Ambrosia growth in galleries or in pure culture was scraped with a sterile needle and mounted in cotton blue on a sterile microscope slide. Slides were viewed by light microscopy and imaged as described above.

For the novel species from *C. papulans,* agar plugs with mycelia were cut with a #1 cork borer (3 mm diam.) taken from the leading margin of growth on MYEA, placed

onto the center of fresh MYEA, and grown at 25 C in the dark for 5 d. Color designations are those of Rayner (1970).

For the novel species associated with *C. papulans,* a representative culture was deposited in the culture collection of the Westerdijk Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS), and a dried culture was deposited in the U.S. National Fungus Collections (BPI) as a holotype specimen. For the novel species associated with multiple *Corthylus* spp. in French Guiana, a representative specimen was deposited in BPI as a holotype specimen.

DNA extraction and sequencing

DNA from mycangia was extracted using either PrepMan® Ultra (Applied Biosystems, Foster City, California) or the DNeasy® Blood and Tissue Extraction Kit (QIAGEN, Valencia, California). In some cases, several mycangia from a collection were combined for a single extraction. DNA from fungal cultures (MYEA at room temperature, 4–14 d) was extracted using PrepMan® Ultra, the ProMega Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin) or the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-tek, Norcross, Georgia). DNA was obtained from gallery specimens using PrepMan® Ultra. Some PrepMan® Ultra extractions were concentrated using Amicon® Ultra-0.5 Centrifugal Filter Devices (EMD Millipore, Billerica, California).

Amplification and sequencing of the nuc rDNA internal transcribed spacer barcode (ITS1-5.8S-ITS2 = ITS), nuc 18S rDNA (18S), and translation elongation factor 1-α (*TEF*1-α) were as described in Mayers et al. (2015). For nuc 28S rDNA (28S), amplification used primers LR0R and LR5, and sequencing used LR0R and LR3 (Vilgalys 2005), with the ITS PCR conditions described in Mayers et al. (2015). For ITS

sequencing from extracted DNA that was potentially mixed with DNA of contaminating fungi or in low-yield (such as galleries, dried specimens, whole beetles, or dissected beetle mycangia), the Ceratocystidaceae-specific primer pairs Cerato1F/ITSCer3.7R and ITSCer3.1/ITS4 were used for amplification and sequencing (Mayers et al. 2015). Complementary and overlapping DNA reads were checked and assembled using Sequence Navigator 1.0.1 or AutoAssembler 1.3.0 (Applied Biosystems, Foster City, California).

Phylogenetic analyses for ITS

New ITS sequences were manually aligned using Notepad++ 6.5.5 (Notepad++ Team). The dataset (TreeBase URL: http://purl.org/phylo/treebase/phylows/study/TB2:S20925) had 492 aligned characters, including gaps, of which 38 were parsimony-informative, 53 were variable but parsimony-uninformative, and 401 were constant. To illustrate sequence differences among isolates, including single-base indels, a maximum parsimony (MP) analysis was performed with PAUP 4.0b10 (Swofford 2003) with gaps treated as a fifth state, stepwise addition, and the tree-bisection-reconnection (TBR) branch-swapping algorithm. The outgroup taxa were *Ceratocystis norvegica, Ceratocystis adiposa*, and *Ambrosiella xylebori* (Table 1), which are close relatives to *Meredithiella* within the Ceratocystidaceae (Mayers et al. 2015). The tree was midpoint rooted. Bootstrap branch support values were obtained with 1000 replications in PAUP.

Phylogenetic analyses of multigene dataset

A combined dataset (TreeBase URL: http://purl.org/phylo/treebase/phylows/study/TB2:S20924) of sequences of 18S (aligned length 1600 bp), 28S (592 bp) and *TEF*1-α (1226 bp), total aligned length 3478

characters, was aligned manually in Notepad++ and included sequences from Mayers et al. (2015, 2017) and newly generated sequences (Table 1). The *TEF*1-α alignment included an intron of 124 bp that could not be unambiguously aligned across all taxa, and a single-codon (3 bp) insert found only in the two outgroup taxa. These 127 characters were eliminated before analyses, leaving 3351 characters in the final combined dataset. Outgroup taxa were *Plectosphaerella cucumerina* and *Knoxdaviesia capensis* (Table 1).

For Bayesian analysis of the combined dataset, optimum models and partitions were found using PartitionFinder 2 (Lanfear et al. 2016) with linked branch lengths, all models, "aicc" (converted Aikaike Information Criterion) model selection, and a greedy algorithm (Lanfear et al. 2012), powered by PhyML (Guindon et al. 2010). The best models and partitions were found to be: TRN+G for 18S; F81+I+G for *TEF*1-α codon positions 1 and 2; GTR+G for *TEF*1-α codon position 3; and SYM+G for 28S. A Bayesian consensus tree was produced with MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003) using these models, and a single MCMC run with four chains (one cold, three heated) for 1 000 000 generations, after which a burn-in of 15% was applied. A consensus tree was generated using the SUMT function.

To obtain further branch support for the Bayesian tree, maximum likelihood (ML) and maximum parsimony (MP) bootstrap analyses were run on the same multigene alignment. The ML analysis was performed with RAxML 8.2.7 (Stamatakis 2014), using the model GTR+I+G selected with PartitionFinder 2 in "raxml" mode. Bootstrap values were obtained with 1000 replicates and ML non-parametric analysis, with each replicate starting from a randomized MP starting tree. The MP analysis was performed with PAUP 4.0b10 by running 1000 bootstrap replicates of a full heuristic analysis with gaps treated

as a fifth state; the multi-gene alignment had 436 parsimony-informative, 284 variable but parsimony-uninformative, and 2631 invariant characters.

Results

Mycangium observations

Male specimens of *C. punctatissimus, C. consimilis, C. papulans,* and *C. crassus* had pairs of tube-shaped mycangia on the lateral sides of the interior of the prothorax that fed into the procoxal cavities (Figs. 1A, B, 2A, B). The mycangia of all four species had reticulated walls (Fig. 1B, F) and were packed with homogenous masses of irregularly-shaped, budding spores. The spores persisted as a tightly-packed columnar mass when expelled from the mycangium by pressing with a glass cover slip (Figs. 1D, F, G).

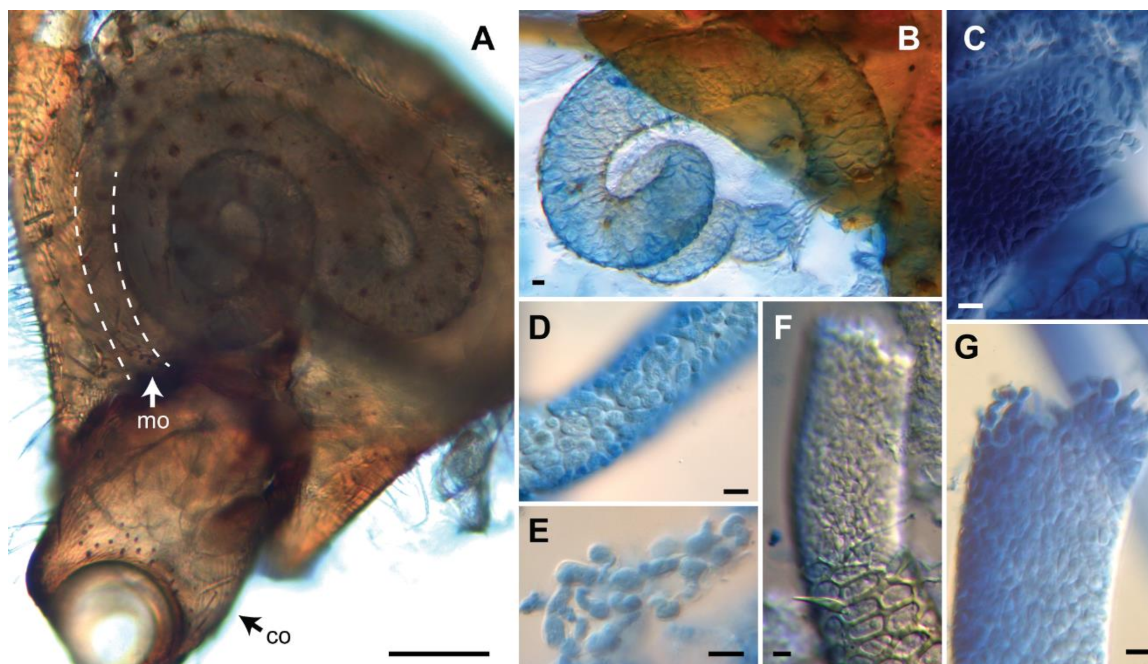

Figure 1. Mycangia and spores from *Corthylus papulans* (A–C), *Corthylus crassus* (D, E), and *Corthylus consimilis* (F, G). A. Mycangium seen through exoskeleton of the dissected left half of prothorax; dotted lines trace the rest of the tube exiting into the mycangium opening (mo) into coxal cavity above the coxa (co); beetle anterior is to the upper left. B. Spiraling mycangium terminus that travels medially at center of spiral in A, showing reticulated walls. C. Spore mass squeezed from the mycangium. D, G. Columnar mass of spores squeezed from the mycangium. E. Spores from mycangium. F. Spore mass exposed after the rigid, reticulated mycangium has broken away. All photos by Nomarski interference microscopy of stained material (cotton blue). Bar = 100 µm for A, 10 µm for others.

The spores appeared to vary in both size and shape, perhaps a consequence of poorly developed cell walls.

As previously illustrated (Finnegan 1963), the mycangia of *C. punctatissimus* wound back and forth horizontally within the prothorax (Fig. 2A), as did the mycangia of *C. consimilis*. The mycangia of *C. crassus* (not pictured) were similar to those of *C. punctatissimus* but were significantly smaller and wound vertically rather than horizontally. In *C. papulans,* the mycangia folded posteriorly once and then tightly spiraled (Fig. 1A, B, 2B). At the center of this spiral, the tubes turned medially and ended in a loose spiral, like a pig's tail (Fig. 1B).

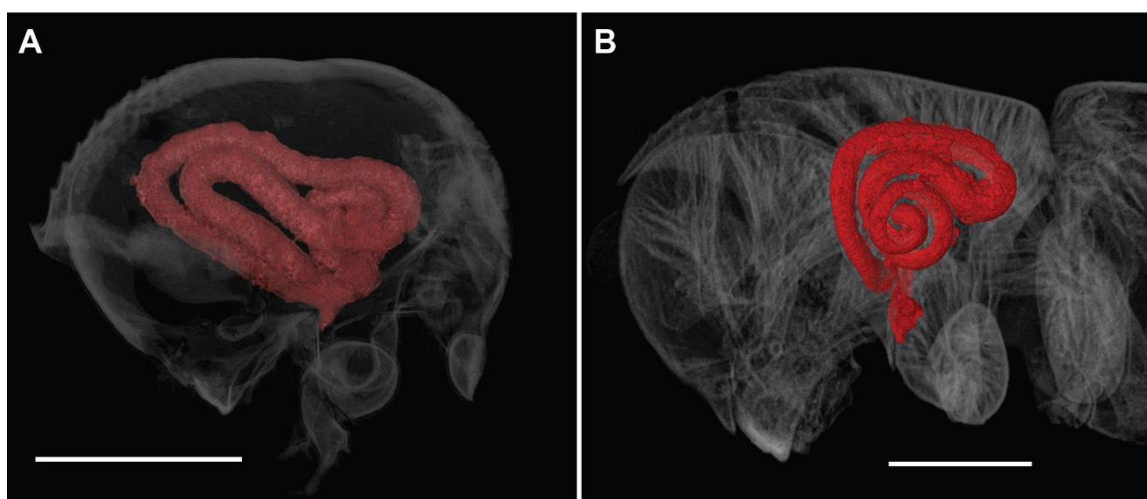

Figure 2. Micro-CT scans of adult male (A) *Corthylus papulans* and (B) *Corthylus punctatissimus* showing tubular mycangium structure highlighted in red. Bar = 0.5 mm.

All observed mycangia had large tracheoles nearby, perhaps suppling oxygen to secretory gland cells. The tracheoles could be mistaken for the mycangial tubes, but the tracheoles were ribbed rather than reticulated, and the tracheoles did not have spores within.

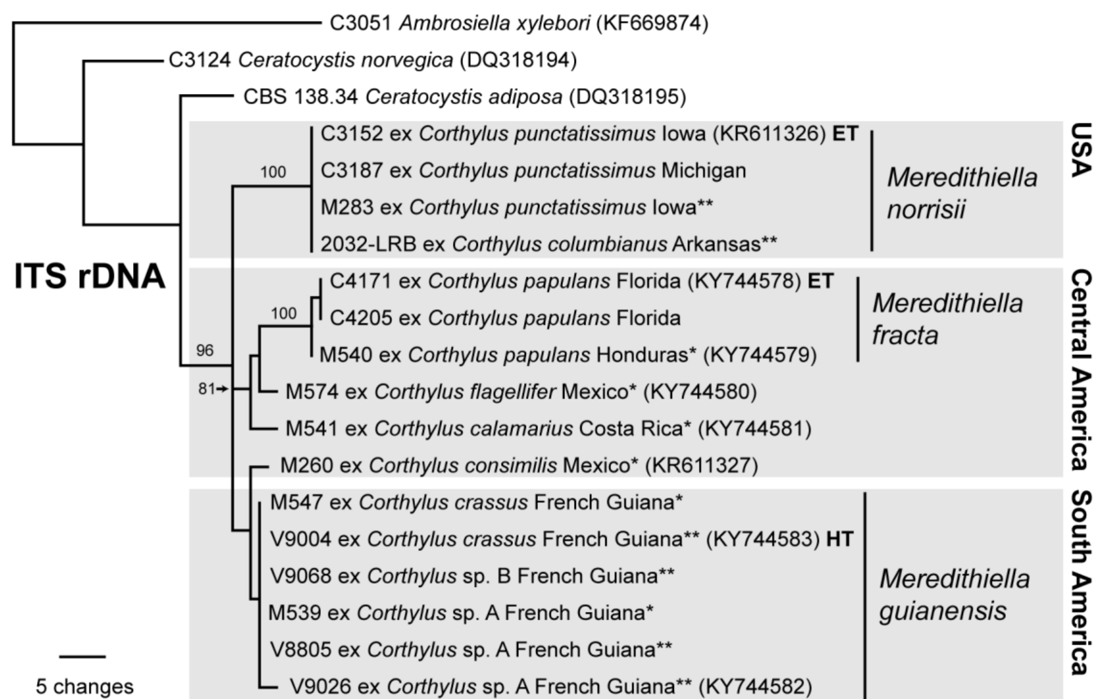

Figure 3. One of 12 most parsimonious trees of representative *Ceratocystis, Ambrosiella,* and *Meredithiella* spp. based on sequences of the internal transcribed spacer (ITS) regions 1 and 2 and 5.8S rDNA. Bootstrap support values from 1000 replications indicated on branches. GenBank accession numbers in parentheses (when available) and isolate numbers are given for each sequence. Single asterisk indicates sequences obtained from DNA extractions of whole beetles or dissected beetle parts (i.e. mycangia); double asterisks are from galleries; sequences without asterisks are from pure cultures. Type status of sequences designated by bold acronyms: **HT** = holotype; **ET** = ex-type.

Fungal associations

Adult specimens and/or galleries of nine *Corthylus* spp. from five countries were studied for the presence of Ceratocystidaceae, and each of the nine species were associated with one of six putative species of *Meredithiella* based on ITS DNA barcoding (Fig. 3).

Corthylus punctatissimus and *C. columbianus* specimens

Meredithiella norrisii was consistently associated with *C. punctatissimus* (Mayers et al. 2015), and cultures of this species produced characteristic aleurioconidia on short side-branches of conidiophores on MYEA (Figs. 4A–D). Batra's (1967) herbarium specimens of *C. columbianus* galleries contained sparse ambrosia growth that consisted

of monilioid chains with short, two-celled side branches (Fig. 4E), identical to the ambrosia growth of *M. norrisii* in galleries of *C. punctatissimus*. Amplification of ITS rDNA was unsuccessful from the DNA extracted from *C. columbianus* galleries. However, PCR was successful with DNA extracted from the dried-down culture included in KANU 376400, which was isolated from the same galleries. The DNA extracted from a single sporodochium taken from this dried culture yielded an ITS1 sequence (using Ceratocystidaceae-specific primers) that was identical to that of *M. norrisii* (Fig. 3).

***Corthylus papulans* specimens**

The dissected mycangial contents of the two male specimens of *C. papulans* trapped in-flight in Florida yielded a pigmented fungus with a fruity odor on SMA (Fig. 4F). The ITS sequences from the two isolates were identical and similar to that of *M. norrisii* (Fig. 3). One of the mycangia from the ethanol-preserved *C. papulans* male that was trapped in-flight in Honduras was filled with a homogenous mass of irregularly-shaped fungal propagules (Fig. 1C). The DNA extracted from the other mycangium of this beetle yielded an ITS sequence nearly identical to the sequence from the cultures from Florida, differing by only a single base substitution (a “C” for a “T”) in ITS2 (Fig. 3).

The isolates from the two *C. papulans* beetles trapped in Florida produced branched monilioid hyphae in culture (Figs. 4G, H), similar to the conidiophores of *M. norrisii* (Figs. 4B–E). However, thick-walled, terminal aleurioconidia were not seen in the new species. The monilioid cells appeared to disarticulate into short chains or individual cells (Fig. 4J, K). Some of the cells had golden inclusions of an unknown substance (Fig. 4I), perhaps carotenoids (Avalos and Limón 2015). One isolate (C4171)

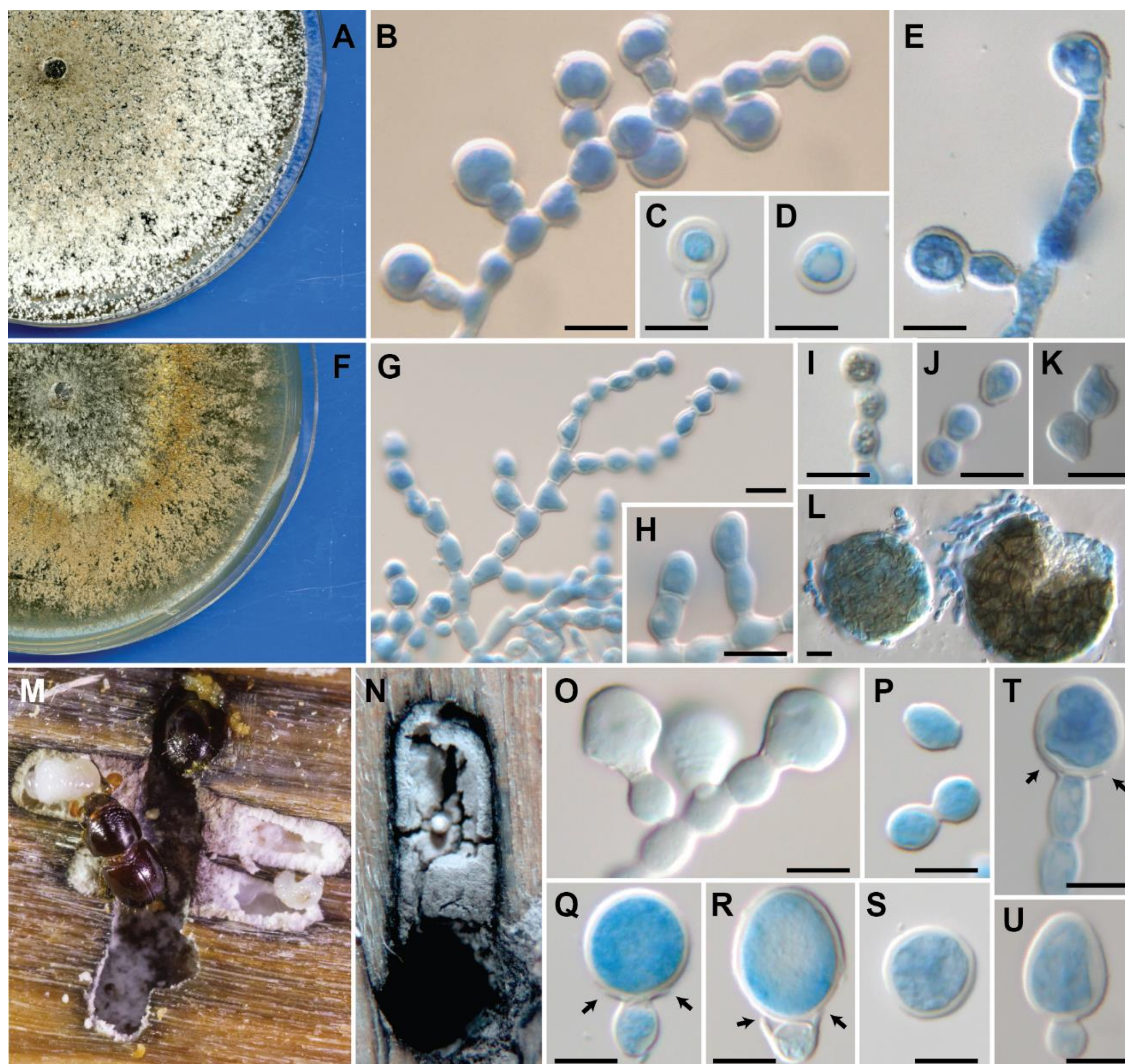

Figure 4. Cultures, ambrosia growth in galleries, conidiophores, and conidia of *Meredithiella* spp. (A–E) *Meredithiella norrisii.* A. Growth at 13 d on MYEA. B. Conidiophore bearing side-branches and terminal aleurioconidia. C. Detached aleurioconidium with conidiophore cell attached. D. Detached aleurioconidium. E. Conidiophore from *C. columbianus* gallery bearing side-branch and terminal aleurioconidia. (F–L) *M. fracta*. F. Growth at 13 d on MYEA. G. Branching monillioid chains of arthroconidia. H. Short side branches. I. Chain of arthrospores with golden inclusions. J, K. Disarticulated arthrospores. L. Immature spherical ascomata. (M–U) *M. guianensis*. M. Gallery of *Corthylus crassus* showing adults in the parent gallery and larvae with ambrosia growth in larval cradles. N. Ambrosia growth in larval cradle. O. Conidiophore bearing side-branch and terminal aleurioconidia. P. Disarticulated arthrospores. Q, R. Detached aleurioconidia with conidiophore cell attached; collar marked with arrows. S. Detached aleurioconidium. T. Terminal aleurioconidium on conidiophore; collar marked with arrows. U. Detached aleurioconidium with conidiophore cell attached. A–D from ex-type culture from *Corthylus punctatissimus* (C3152, CBS 139737). E from *Corthylus columbianus* gallery specimen 2032-LRB (KANU 376400). F–L from ex-type culture (C4171, CBS 142645) from *Corthylus papulans*. M–S from holotype (BPI 910532) from *Corthylus crassus* gallery. T, U from gallery V8805 of an unidentified *Corthylus* sp. All photos except A, F, M, N by Nomarski interference microscopy of stained material (cotton blue). A, F imaged with Epson 10000XL scanner with blue background. M, N by digital photography. Bar = 10 µm.

produced a V-shaped sector in culture that produced small brown spheres (Fig. 4L) that were similar to the immature ascomata reported in *Ambrosiella nakashimae* and *A. cleistominuta* (Mayers et al. 2015, 2017). Production of the immature ascomata persisted after transfer of the C4171 sector to new media, but no asci or ascospores were observed.

French Guiana specimens

Six nearly-identical ITS sequences were obtained from DNA extracted from mycangia or gallery growth of *C. crassus* and two unidentified *Corthylus* spp. in French Guiana. These included sequences from *C. crassus* mycangia (M547) and gallery (V9004), *Corthylus* sp. A mycangia (M539, ITS1 only) and galleries (V8805 and V9026), and a *Corthylus* sp. B gallery (V9068). The sequence from gallery V9026 differed by two single-base substitutions from the other five ITS sequences. Ambrosia growth from two areas of each of the four galleries were separately extracted, amplified, and sequenced, and in each case the same ITS sequence was obtained from the two samples. The ITS sequences from the French Guiana specimens were most similar to the sequence from an adult *C. consimilis* collected in Mexico (Fig. 3).

Gallery specimens V9026 (*Corthylus* sp. A) and V9068 (*Corthylus* sp. B) contained tunnel sections with only sparse hyphal growth, but gallery specimens V8805 (*Corthylus* sp. A) and V9004 (*C. crassus*) included larval cradles with thick, white, luxurious ambrosia growth of tightly packed, monilioid conidiophores (Figs. 4M–O). Entrances of the larval cradles were plugged with mycelium (Figs. 4M, N). A black rind of pigmented hyphae lay beneath the thick white ambrosia growth in the cradles and the superficial, white growth of the parent galleries (Figs. 4M, N). Aleurioconidia formed on small, side-branches of the conidiophores (Fig. 4 O–U) and were subtended by inconspicuous collarettes as seen in *Ambrosiella* (Harrington et al. 2014; Mayers et al.

2015). Aleurioconidia usually detached with a single conidiogenous cell or rarely detached singly (Fig. 4Q–S). The monilioid conidiophores also disarticulated into individual cells or chains of cells (Fig. 4P).

***Corthylus flagellifer* and *C. calamarius* specimens**

Females identified as *C. flagellifer* were excavated from a dying avocado tree in Mexico. Females lack mycangia, but the dissected foregut, especially the crop, anterior to the proventriculus (Rubio et al. 2008), of one female appeared to have fungal cells. An ITS sequence recovered from the extracted DNA of the foregut material was unique and similar to that of other *Meredithiella* spp. (Fig. 3). The DNA extract from the foregut also yielded a 28S sequence (KY748664) similar to *Cyberlindnera fabianii* (Wick) Minter (904/913 bp matching KY107356) using BLASTn (NCBI). The DNA extracted from the *C. calamarius* mycangia yielded a unique ITS sequence that was most similar to the sequence from the DNA extracted from *C. flagellifer* (Fig. 3).

Phylogenetic analyses

There was limited variation among the ITS sequences of the symbionts associated with the *Corthylus* spp. Analyses yielded 12 most parsimonious trees, with some support for separating the *Meredithiella* spp. from other related genera in the Ceratocystidiaceae (Fig. 3), but most of the branches within the genus were unsupported. The ITS sequences associated with *C. columbianus* and *C. punctatissimus* were identical, which appears to represent *M. norrissi*. A second group comprised the fungi associated with *C. papulans, C. flagellifer,* and *C. calamarius.* A third unsupported group contained the fungi associated with *C. consimilis* and the three *Corthylus* spp. from French Guiana.

A Bayesian consensus tree of the combined 18S, 28S, and *TEF*1-α dataset placed the *Corthylus* symbionts within the Ceratocystidaceae in a monophyletic group with

strong support (Fig. 5). The other genera of ambrosial beetle symbionts (*Ambrosiella, Phialophoropsis*) each had strong support, but there was only weak support for the relationships of the three genera of ambrosia beetle symbionts with *Huntiella, Ceratocystis adiposa, Ceratocystis norvegica,* and *Ceratocystis fagacearum*.

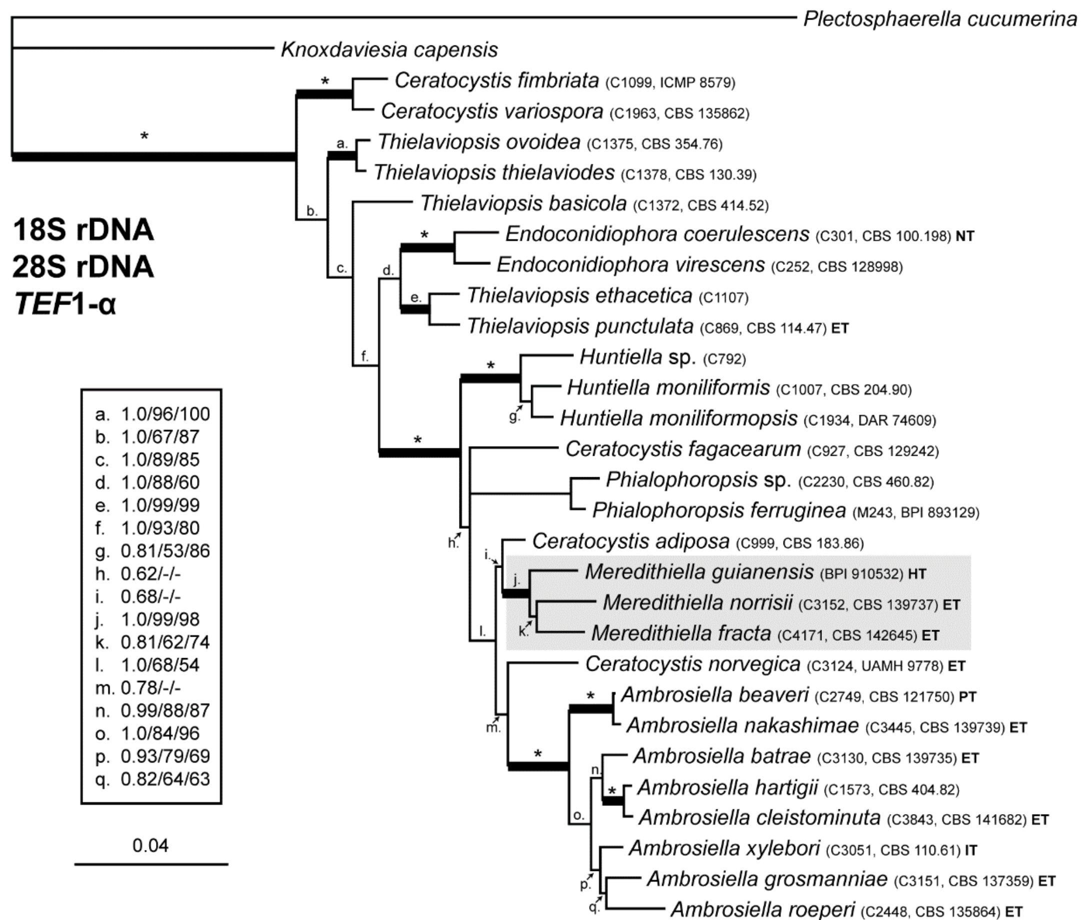

Figure 5. Multigene (18S rDNA, 28S rDNA, and *TEF*1-α) Bayesian tree of Ceratocystidaceae representatives including three species of *Meredithiella* (in gray box). Support values for branches labelled with lowercase letters are given in the key and in the following order: MrBayes posterior probability/RAxML bootstrap support/maximum parsimony bootstrap support. Branches labelled with asterisks have support values of 1.0, 100, and 100, respectively; thick branches have support values of 1.0, >90, and >90, respectively. Isolate numbers and sequence accessions are given after species names. Bar = 0.04 estimated substitutions per site. Type status of sequences designated by bold acronyms: **HT** = holotype; **ET** = ex-type; **IT** = isotype; **PT** = paratype; **NT** = neotype.

Taxonomy

Phylogenetic analyses and differences in morphology between described taxa and those observed here supported the description of two new species of *Meredithiella* from *Corthylus* spp.

Meredithiella fracta C. Mayers, C. Bateman & T.C. Harr. sp. nov. Figs. 1C, 4E–J

MycoBank MB823267

Typification: USA. FLORIDA: Alachua County, Gainesville (29.732161, -82.219386), dried culture isolated from mycangium of *Corthylus papulans*, 23 Feb 2016, *C. Bateman C4171* (**holotype** BPI 910531). Ex-holotype culture CBS 142645.

Etymology: "fracta" (Latin), nominative, feminine of *fractus*, "broken/fragmented."

Colonies on MYEA 35–55 mm diam after 5 d at 25C; margin hyaline, submerged, later superficial, becoming white to pale mouse grey, aerial mycelium clumped, becoming olivaceous, with hyaline, to cinnamon, to rust colored exudate that may eventually soak the aerial mycelium and form craters in white clumps of mycelium and stain the medium rust; reverse buff with isabelline tendrils, becoming olivacious black; odor sweet, fading by 3 wk. *Sporodochia* rare, clustered near center of plate, white to pale mouse gray, irregular. *Conidiophores* on sporodochia or in loose aerial tufts, 15–75 µm tall, hyaline to light brown, branching, monilioid, produced by acropetal budding, disarticulating into thallic-arthric conidia. *Conidia* oblate-spheroidal to globose to broadly ellipsoidal, thin- or thick-walled, 5.5–10 × 6–7.5 µm, sometimes with golden inclusions, breaking off singly, in short chains, or rarely in branched aggregates, truncate on one or both ends. *Immature ascocarps* spherical, dark brown, 15–45 µm diam., walls composed of textura angularis, lacking ostiole or neck. *Ascospores* not observed. *Growth*

in mycangium composed of thick-walled, arthrospore-like cells singly or in short chains, sometimes branched, produced by thallic-arthric growth, irregular, 4.5–11.5 × 3–9 µm.

Other cultures examined. USA. FLORIDA: Alachua County, Gainesville (29.732161, -82.219386), isolated from mycangium of *C. papulans*, 23 May 2016, *C. Bateman C4205*.

Notes. The new species was associated with the mycangia of male *C. papulans* from both Florida and Honduras (Fig. 3). Unlike other known *Meredithiella* spp., no terminal aleurioconidia were seen in culture. Instead, *M. fracta* produces simple, branched, monilioid conidiophores that disarticulate. It grows much faster than *M. norrisii* on MYEA at 25 C, and cultures of *M. fracta* at 5 d have less white-chalky surface growth, less pigment in the center of the colony, and a stronger, fruity odor.

Meredithiella guianensis C. Mayers, C. Bateman & T.C. Harr. sp. nov.

Figs. 1G, H, 4K–R

MycoBank MB823268

Typification: FRENCH GUIANA**.** CAYENNE: near Kaw Mountain (4.55892, -52.19662), gallery of *Corthylus crassus* in unknown host, 5 Jun 2015, *C. Bateman V9004* (**holotype** BPI 910532).

Etymology: *guianensis,* after the country of origin, French Guiana.

Gallery growth thick, white, with black layer below; *Aleurioconidiophores* hyaline, monilioid, breaking apart easily to form arthrospores or forming long central strands with many side branches, one- or multiple-celled, 20–50 µm long, each bearing a single, terminal aleurioconidium with an inconspicuous collarette. *Aleurioconidia* terminal, oblate-spheroidal to globose to broadly ellipsoidal, 11–22 × 10.5–20 µm,

generally taller than wide, usually spherical, often ovoid, rarely pyriform when constrained by collar, thick-walled, aseptate, smooth, hyaline, breaking off with conidiogenous cell attached, sometimes with multiple conidiophore cells attached, or rarely singly. *Arthrospores* formed from disarticulating conidiophores, 7.5–11 × 6–8 µm, globose to fusoid, truncate on one or both ends. *Growth in mycangium* composed of irregular cells, 5–16 × 3.5–11 µm, propagating by schizogenous division, single or in septate chains of a few cells, rarely branching.

Additional specimen examined: FRENCH GUIANA**.** CAYENNE: near Kaw Mountain (4.55892, -52.19662), gallery of an unidentified *Corthylus* sp. in unknown host, 5 Jun 2015, *C. Bateman V8805.*

Notes. *Meredithiella guianensis* is very similar in morphology to *M. norrisii*, but the aleurioconidia are much larger in *M. guianensis*. The holotype specimen of *M. guianensis* was from a gallery of *C. crassus,* but similar ITS sequences were also obtained from DNA extracts of ambrosia growth in galleries of two unknown *Corthylus* spp., also found in French Guiana. The gallery material and beetles were preserved in ethanol in the field, and no attempt was made to isolate *M. guianensis* in pure culture.

Discussion

This study confirms that *Meredithiella* spp. are primary symbionts of *Corthylus*. Nine different *Corthylus* spp. from five countries were associated with named or unnamed *Meredithiella* spp. Like *Ambrosiella*, which are associated with *Xylosandru*s spp. and close relatives in the Xyleborini that have large, mesonotal mycangia (Harrington et al. 2014; Mayers et al. 2015, 2017), species of *Meredithiella* appear to be exclusively associated with *Corthylus* spp., which have unique large, tubular mycangia.

Each of the studied *Corthylus* spp. carries *Meredithiella,* just as each of the studied Xyleborini with large, mesonotal mycangia carries *Ambrosiella* (Mayers et al. 2015, 2017). A similarly exclusive association appears to occur between the unique, large, prothoraxic mycangia of *Trypodendron* and *Phialophoropsis* (Mayers et al. 2015). These exclusive associations suggest coadaptation, in which each evolutionary event leading to a large, well-developed mycangium corresponds with a domestication of a distinct fungal lineage, each adapted to produce luxuriant ambrosia growth in galleries and arthrosporic type growth in mycangia (Harrington et al. 2014; Mayers et al. 2015). However, species level co-evolution within genera is less clear. There is a suggestion in *Corthylus* that there is horizontal transfer of fungal symbionts among sympatric beetle species.

Tubular mycangia opening into the procoxae were first described in *Corthylus schaufussi* and *C. punctatissimus* by Schedl (1962) and Finnegan (1963), respectively. Similar tubular mycangia were later reported in *C. columbianus* (Giese 1967) and *C. fuscus* (Orañegui and Atkinson 1984). Our microscopic examinations revealed that the mycangia of *Corthylus* spp. have reticulated walls and are rigid, and they fracture rather than flex when manipulated. Each examined mycangium held tightly packed, homogeneous masses of fungal spores. The tubes appeared to wind back and forth in *C. punctatissimus, C. consimilis,* and *C. crassus*, as do the mycangia of *C. columbianus* (Giese 1967; Nord 1972). In contrast, the mycangia of *C. papulans* form a spiral. The mycangia of *C. schaufussi* may be intermediate between these two morphologies, with a broadly-folded lateral portion that terminates in an intricate spiral (Schedl 1962). There are too few observations to test the hypothesis that *Corthylus* spp. with similar mycangia carry closely-related *Meredithiella* spp.

Blandford (1895) placed *Corthylus* spp. into two groups ('division I' and 'division II') based primarily on morphology. Division I included *C. papulans, C. flagellifer,* and other species whose females have long cirri on their antenna. Of the species we studied, C. *calamarius, C. crassus,* and both unknown *Corthylus* spp. from French Guiana would have been placed in division I based on antenna cirri. Blandford's division II included *C. punctatissimus, C. fuscus,* and other species whose females have small or nonexistent antenna cirri; *C. columbianus, C. consimilis,* and *C. schaufussi* would probably be considered members of division II. The two mycangia shapes do not appear to divide into Blandford's (1895) divisions as his division I includes the spiral-shaped mycangia of *C. papulans* and the winding mycangia of *C. flagellifer*, *C. calamarius*, and *C. crassu*s.

It is not known if a molecular phylogeny of *Corthylus* would match Blandford's divisions, and it is not clear if Blandford's placement of *Corthylus* spp. into the two divisions correlates to the relationships of their fungal mutualists. The ITS analysis of *Meredithiella* spp. is tenuous, but *M. fracta* associated with *C. papulans* appears to be related to the symbionts *of C. flagellifer* from Mexico and *C. calamaris* from Costa Rica, and each of these beetle species have the long antenna cirri of division I. The division II species *C. punctatissimmus* and *C. columbianus* carry *M. norrisii*, while the other studied division II species, *C. consimilis*, appears to have a symbiont more closely related to *M. guianensis*, which was associated with the division I species from French Guiana.

The ITS relationships among the described and putative *Meredithiella* spp. suggest that there may be three geographic groups within *Meredithiella:* eastern USA, Central American and South American. *Meredithiella norrisii* is the associate of the USA natives *C. punctatissimus* and *C. columbianus*. *Corthylus papulans* is an invasive in

Florida but native to Central America (Wood 1977), and the ITS sequences of the *M. fracta* from Florida and Honduras were similar to that of the unidentified mycangial symbiont of *C. calamarius*, only reported in Costa Rica (Wood 1974), and to the *Meredithiella* detected in the foregut of female *C. flagellifer* (Guatemala to Central Mexico) (Wood 1982). *Meredithiella guianensis* may represent a South American clade; *C. crassus* has only been reported from French Guiana (Wood 2007), and the other two *Corthylus* spp. sampled there are unidentified or undescribed. The implied relatedness of the symbiont of the Mexican *C. consimilis* to *M. guianensis* may contradict the hypothesis that there are geographic groupings within *Meredithiella*. However, the ITS tree was not robust, and more thorough sampling and multigene analyses are needed.

Phylogenetic relationships among the genera of Ceratocystidaceae are not well resolved, but the grouping of the genera of ambrosia beetle symbionts (*Ambrosiella, Phialophoropsis,* and *Meredithiella*) with *Huntiella, Ceratocystis adiposa, C. fagacearum,* and *C. norvegica* was also evident in earlier analyses (de Beer et al. 2014; Harrington et al. 2014; Mayers et al. 2015). *Huntiella*, *C. adiposa, C. fagacearum,* and *C. norvegica* are non-ambrosial and unrelated to each other, and it appears unlikely that they represent four reversions from ambrosia mutualists to free-living fungi. The ambrosial species appear to be obligate symbionts and highly derived, but phylogenetic analyses fail to support a monophyletic grouping of the ambrosial genera. Mayers et al. (2015) suggested that *Meredithiella* represents an independent adaptation to ambrosia beetle symbiosis, distinct from the convergent adaptations of *Ambrosiella* (to Xyleborini with large mesonotal mycangia) and *Phialophoropsis* (to Xyloterini with large, prothoraxic pleural mycangia). However, the aleurioconidia of *M. norrisii* and *M. guianensis* are

similar to the aleurioconidia found in some species of *Ambrosiella,* and *C. adiposa* also forms aleurioconidia (Mayers et al. 2017). *Meredithiella* and *Ambrosiella* may produce chains of monilioid cells that break apart, which is thought to be an adaptation that facilitates beetle grazing (Harrington et al. 2014; Mayers et al. 2015). The recently-described ambrosia fungus *Afroraffaelea ambrosiae* (in the Ophiostomatales) has similar disarticulating, monilioid conidiophores (Bateman et al. 2017).

There have been conflicting reports on the primary mutualists of *Corthylus* spp. Hubbard (1897) illustrated monilioid strings in galleries of *C. punctatissimus* that disarticulated into short chains of "dumb-bell shaped" cells, consistent with the disarticulating conidiophore cells of *M. norrisii* (Mayers et al. 2015). Batra (1967) and Nord (1972) identified the primary symbiont of *C. columbianus* to be *A. xylebori,* but the ITS1 sequence generated from the DNA extraction of Batra's culture (from Nord's material) in KANU proved to be *M. norrisii*. Wilson (1959) and Kabir and Giese (1966) reported a yeast (*Pichia* sp.) with hat-shaped ascospores in galleries of *Corthylus columbianus* and suggested that it was the primary food source for the larvae. Giese (1967) illustrated hat-shaped ascospores, presumably of the same *Pichia* sp., inside the mycangium of *C. columbianus*. More recent studies of associates of *C. zulmae* in Colombia (Gil et al. 2004; Jaramillo et al. 2011) also found a *Pichia* sp. among gallery associates. However, we did not see hat-shaped ascospores or other indications of yeasts in mycangia of *C. punctatissimus, C. consimilis, C. papulans*, or *C. crassus*, and we did not isolate yeasts from the dissected mycangia of *C. papulans*.

Mycangial association was one of Batra's (1985) qualifications for a primary ambrosia fungus, and we consistently found homogeneous spore masses of *Meredithiella*

spp. within mycangia of male *Corthylus* beetles. *Meredithiella* species also dominated the ambrosia growth of fresh, active galleries and larval cradles. *Meredithiella* spp. may have been overlooked in other studies because propagules of *Meredithiella* from galleries and mycangia are very difficult to germinate, grow slowly, and can be easily missed or overrun by other fungi. *Fusarium* spp. were consistently reported as contaminants of *C. columbianus* galleries (Wilson 1959; Kabir and Giese 1966; Giese 1967; Gil et al. 2004), and Wilson (1959) reported that a sparsely-distributed but fast-growing *Fusarium* sp. overran other fungi in isolation attempts. The mycoflora of ambrosia beetle galleries changes over the life stages of the beetle (Kajimura and Hijii 1992; Kinuura 1995), but the *Corthylus* galleries that we studied had healthy ambrosia growth of *Meredithiella*.

Yeasts such as *Pichia* have been frequently associated with ambrosia beetles, especially in the gut (Suh et al. 2005; Harrington and Fraedrich 2010). Common yeast genera associated with ambrosia beetles include *Ambrosiozyma* (Walt 1972; Endoh et al. 2008; Kurtzman and Robnett 2013), *Wickerhamomyces* (Ninomiya et al. 2013; James et al. 2014), *Cyberlindnera* (Ninomiya et al. 2013), and various unclassified *Pichia* and *Candida* spp. (Haanstad and Norris 1985; Gil et al. 2004; Harrington and Fraedrich 2010; Suh and Zhou 2010). Yeasts have only occasionally been associated with ambrosia beetle mycangia (Batra and Francke-Grosmann 1964; Giese 1967; Kurtzman 2000; Six et al. 2009). Yeasts may not form thick layers of ambrosia growth for beetle grazing (Harrington 2005), but they are certainly fed upon by ambrosia beetles, as evidenced by the 28S sequence obtained from the foregut of *Corthylus flagellifer* (KY748664), which is similar to that of *Cyberlindnera fabianii* (KY107356). This does not necessarily imply a mutualistic association with the beetle.

Corthylus is rich in unstudied species, and their mycangial symbionts need further investigation. Of the other genera in the American sub-tribe Corthylina, *Microcorthylus castaneus* has mycangia similar to *C. schaufussi*, but the mycangia are smaller and less-spiraled (Schedl 1962). A *Microcorthylus* sp. in Costa Rica was found to have a *Geosmithia* sp. forming ambrosial growth in its galleries (Kolařík and Kirkendall 2010), but mycangial symbionts of *Microcorthylus* have not been identified. *Gnathotrichus* and *Monarthrum* have small, coxal mycangia (Farris 1963; Lowe et al. 1967; Schneider and Rudinsky 1969) and *Raffaelea* mycangial symbionts (Harrington et al. 2010). Intriguing patterns of evolution of mycangial types and fungal symbionts are just now emerging, and fungal symbioses within the Corthylina warrant further study.

Acknowledgements

The advice and assistance of Doug McNew (Iowa State U) is greatly appreciated. Salvador Ochoa-Ascencio (Universidad Michoacana de San Nicolás de Hidalgo), Sarah Smith (Michigan State U), and Caroline Storer (U Florida) provided beetles preserved in ethanol, and Thomas Atkinson (U Texas) supplied specimens of *C. consimilis* and helped identify other ambrosia beetle specimens. Edward Stanley and Gary Scheiffele of the Nanoscale Research Facility (U Florida) helped to CT-scan and visualize *Corthylus* mycangia. The herbarium specimens provided by the University of Kansas Herbarium (KANU) with the help of collections manager Caleb Morse are greatly appreciated. Chase Mayers was supported in part by fellowship funds from the Office of Biotechnology, Iowa State University (ISU). Craig Bateman and Micro-CT scans were supported by the National Science Foundation, the USDA-APHIS, and the USDA Forest Service. Other financial support was provided by the U.S. Forest Service, Special Technology and Development Program

Tables

Table 1. Collection information and GenBank accessions for studied cultures and specimens of Ceratocystidaceae and outgroup taxa.

	Ambrosia beetle host	Locality	Culture or specimen number(s)[1]	ITS	LSU	SSU	TEF-1α
Ambrosiella Arx & Hennebert emend. T.C. Harr.							
A. batrae C. Mayers, McNew & T.C. Harr.	*Anisandrus sayi*	Michigan, USA	C3130 (CBS 139735)	KR611322	KY744584	KR673881	KT290320
A. beaveri Six, de Beer & W.D. Stone	*Cnestus mutilatus*	Mississippi, USA	C2749 (CBS 121750)	KF669875	KF646765	KR673882	KT318380
A. cleistominuta C. Mayers & T.C. Harr.	*An. maiche*	Ohio, USA	C3843 (CBS 141682)	KX909940	KY744585	KX925304	KX925309
A. nakashimae McNew, C. Mayers & T.C. Harr.	*Xylosandrus amputatus*	Georgia, USA	C3445 (CBS 139739)	KR611323	KY744586	KR673883	KT318381
A. grosmanniae C. Mayers, McNew & T.C. Harr.	*X. germanus*	Iowa, USA	C3151 (CBS 137359)	KR611324	KY744587	KR673884	KT318382
A. hartigii L.R. Batra	*An. dispar*	Germany	C1573 (CBS 404.82)	KF669873	KY744588	KR673885	KT318383
A. roeperi T.C. Harr. & McNew	*X. crassiusculus*	Georgia, USA	C2448 (CBS 135864)	KF669871	KF646767	KR673886	KT318384
A. xylebori Brader ex Arx & Hennebert	*X. compactus*	Ivory Coast	C3051 (CBS 110.61)	KF669874	KM495318	KR673887	KT318385
Meredithiella McNew, C. Mayers & T.C. Harr.							
M. norrisii McNew, C. Mayers & T.C. Harr.	*Corthylus punctatissimus*	Iowa, USA	C3152 (CBS 139737)	KR611326	KY744589	KR673888	KT318386
	C. columbianus	Arkansas, USA	KANU 376400	ITS1 =KR611326	—	—	—
M. fracta	*C. papulans*	Florida, USA	C4171 (CBS 142645)	KY744578	KY744590	KY744594	KY773179
	C. papulans	Francisco Morazan, Honduras	M540	KY744579	—	—	—
M. guianensis	*C. crassus*	Kaw Mountain, French Guiana	BPI 910532	KY744583	KY744223	KY744227	KY773180
	Corthylus sp. A	Kaw Mountain, French Guiana	M544	KY744582	—	—	—
	Corthylus sp. B	Kaw Mountain, French Guiana	M546	=KY744583	—	—	—
Meredithiella sp.	*C. consimilis*	La Esperanza, Mexico	M260	KR611327	—	—	—
Meredithiella sp.	*C. calamarius*	Costa Rica	M541	KY744581	—	—	—
Meredithiella sp.	*C. flagellifer*	Michoacán, Mexico	M574	KY744580	—	—	—

Table 1 continued

Phialophoropsis L.R. Batra emend. T.C. Harr.							
P. ferruginea (Math.-Käärik) T.C. Harr.	*Trypodendron lineatum*	Colorado, USA	M243 (BPI 893129)	KR611328	KY744224	KR673889	KT318387
Phialophoropsis sp.	*T. domesticum*	Germany	C2230 (CBS 460.82)	KC305146	KF646766	KR673890	KT318388
Other Ceratocystidaceae							
Ceratocystis adiposa (Butler) C. Moreau		Ontario, Canada	C999 (CBS 183.86)	=DQ318195	=KM495320	KR673891	HM569644
C. fagacearum (Bretz) J. Hunt		Iowa, USA	C927 (CBS 129242)	=KC305152	=AF222483	KR673892	KT318389
C. fimbriata Ellis & Halst	.	Papua New Guinea	C1099 (ICMP 8579)	AY157957	=KR347445	KR673893	HM569615
C. norvegica J. Reid & Hausner		Norway	C3124 (UAMH 9778)	DQ318194	KY744591	KR673894	KT318390
C. variospora (R.W. Davidson) C. Moreau emend. J.A. Johnson & T.C. Harr.		Iowa, USA	C1963 (CBS 135862)	—	KF646773	KX925305	KR347450
Endoconidiophora coerulescens Münch.		Minnesota, USA	C301 (CBS 100198)	KC305116	AF275510	KR673895	HM569653
E. virescens R.W. Davidson		New York, USA	C252 (CBS 128998)	—	=KM495385	KX925306	HM569645
Huntiella moniliformis (Hedgc.) Z.W. de Beer, T.A. Duong & M.J. Wingf	.	India	C1007 (CBS 204.90)	=DQ074739	AF222487	KR673896	KT318391
H. moniliformopsis (Yuan & Mohammed) Z.W. de Beer, T.A. Duong & M.J. Wingf.		Warra, Tasmania	C1934 (DAR 74609)	=NR119507	KF646769	KR673898	HM569638
Huntiella sp.		Minnesota, USA	C792	KR611330	KY744592	KR673897	KT318392
Thielaviopsis basicola (Berk. and Broome) Ferraris		Netherlands	C1372 (CBS 414.52)	AF275481	AF222458	KX925307	HM569628
Thielaviopsis ethacetica Went		South America	C1107	=KJ881375	KY744593	KR673899	HM569632
Thielaviopsis ovoidea (Nag Raj & W.B. Kendr.) A.E. Paulin, T.C. Harr. & McNew		Baarn, Netherlands	C1375 (CBS 354.76)	AF275483	AF275502	KY744595	HM569625
Thielaviopsis punctulata (Hennebert) A.E. Paulin, T.C. Harr. and McNew		California, USA	C869 (CBS 114.47)	AF275495	AF275513	KX925308	KX925310
Thielaviopsis thielavioides (Peyronel) A.E. Paulin, T.C. Harr. & McNew		Oklahoma, USA	C1378 (CBS 130.39)	AF275486	AF222480	AF222518	HM569627

[1]Collections include: Iowa State University collection (beginning with C or M), Westerdijk Fungal Biodiversity Institute (CBS), U.S. National Fungus Collections (BPI), New South Wales Plant Pathology Herbarium (DAR), UAMH Centre for Global Microfungal Diversity (UAMH), International Collection of Microorganisms from Plants (ICMP), University of Kansas McGregor Herbarium (KANU). [2]GenBank accession numbers preceded by '=' represent identical (100% identity) match.

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CHAPTER 5. DIFFUSE COEVOLUTION BETWEEN FUNGAL CULTIVARS IN THE FAMILY CERATOCYSTIDACEAE AND THE MYCANGIA OF THEIR AMBROSIA BEETLE FARMERS

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Abstract

Ambrosia beetles farm fungi in sapwood and use pocket-like organs called mycangia to carry propagules of their fungal cultivars. Fungi selectively grow in the mycangia, which are central to the symbiosis, but the temporal dynamics of evolution between fungal cultivars and mycangia are poorly understood. We studied ambrosia fungi in the family Ceratocystidaceae from four unrelated tribes of ambrosia beetles with four different types of mycangia, including the uncharacterized symbionts of tribe Scolytoplatypodini. Fungal species were delineated using ITS rDNA barcoding, and a concatenated dataset of six loci (28S rDNA, 18S rDNA, *tef*1-α, *tub*, *mcm7*, and *rpl1*) was used to produce a phylogeny of ambrosia fungi in the family Ceratocystidaceae. Three of

the four mycangium types each consistently carried one of three genera of ambrosia fungi: *Ambrosiella*, *Meredithiella*, and *Phialophoropsis*. The pronotal disc mycangia of tribe Scolytoplatypodini had three minor morphological variants (in African *Scolytoplatypus,* Asian *Scolytoplatypus*, and Malagasy *Remansus mutabilis*) each associated with symbionts in one of three genera of Ceratocystidaceae (two new species of *Wolfgangiella* gen. nov.*,* three new species of *Toshionella* gen. nov., and *Ambrosiella remansi* sp. nov., respectively). *Catunica adiposa* gen. nov. et comb. nov. and *Solaloca norvegica* gen. nov. et comb. nov. accommodate closely-related species that are not symbionts of ambrosia beetles. The phylogenetic placement and divergent morphology of the ambrosial genera when compared with related non-ambrosial taxa, and discordance between the estimated fungal divergence dates and the estimated origins of their associated mycangia, suggest that a single origin of ambrosia symbiosis in the Ceratocystidaceae was unlikely. The Scolytoplatypodini may have been the first tribe to establish a symbiosis with the Ceratocystidaceae, and the Xyleborini with mesonotal mycangia may have acquired their symbiont lineage from a *Remansus*-like ancestor. Although beetle-fungus associations are not necessarily coevolving at the species level, ambrosia fungus genera in the Ceratocystidaceae appear to be locked into co-adapted associations with specific types of mycangia and show a pattern of diffuse coevolution.

Introduction

Multiple groups of wood-boring weevils, collectively known as ambrosia beetles, cultivate fungal gardens in sapwood in a mutualistic partnership (Hulcr and Stelinski 2017). These ambrosia beetles avoid competition that occurs in the nutritious inner bark (phloem) by exploiting fungal agriculture in the nutrient-poor sapwood (Harrington 2005). Most ambrosia beetle lineages are derived from phloem-feeding bark beetles

(Kirkendall et al. 2015) and share three critical components: first, the development of mycangia, any of a wide spectrum of physical adaptations the beetles use to carry and transport viable fungal propagules (Francke-Grosmann 1956, 1963, 1967; Hulcr and Stelinski 2017); second, the acquisition of a domesticated fungal cultivar (ambrosia fungus) that can grow in the mycangia and provide nutrition to the beetles and larvae by forming a dense layer of sporulation ('ambrosia') in the beetles' tunnels ('galleries') (Harrington et al. 2010, 2014; Mayers et al. 2015); and third, a behavioral change in the beetles to tunnel into and lay eggs in sapwood and to actively cultivate their fungal gardens, often involving sub-sociality (Biedermann et al. 2013; Biedermann and Rohlfs 2017). The reciprocal co-adaptation of mycangia and ambrosia fungi presents an intriguing model system for the evolution of obligate mutualisms. Recent studies suggest patterns of interdependence, specificity, and co-adaptation vary greatly across ambrosia beetles and their fungi, and these patterns may correspond with the diversity of mycangia (Harrington et al. 2010; Mayers et al. 2015; Skelton et al. 2018).

Some bark beetles use fungi to supplement their diets, and a few have well-developed mycangia in which their fungal symbionts can grow (Harrington 2005). In contrast, ambrosia beetles rely on fungi to survive in sapwood (Harrington et al. 2010). At least eleven separate lineages of bark-dwelling weevils in the subfamilies Scolytinae and Platypodinae (Coleoptera: Curculionidae) made the evolutionary leap to fungus farmers (i.e. ambrosia beetles) (Hulcr and Stelinski 2017). Several lineages of fungi have been identified as their primary ambrosia symbionts, making both ambrosia beetles and ambrosia fungi polyphyletic. A review of the literature suggests that each ambrosia beetle lineage originated with a novel mycangium type (Hulcr and Stelinski 2017), and some

lineages gave rise to sublineages with new mycangium types. The term 'mycangium' is applied to a wide array of unrelated physical crevices and cavities whose only common feature is the storage and transport of fungal spores (Six 2003), but the most effective and selective mycangia are 'glandular sac mycangia,' which are associated with gland cells that secrete nutrients into the mycangium lumen (Francke-Grosmann 1967; Schneider 1975; Six 2003). These secretions support the growth and overflow of fungal inoculum for colonization of the gallery (Schneider 1975; Beaver 1989). Some mycangia are relatively small and simple in relation to the beetle's body size, such as the oral pouch mycangia that have convergently developed in multiple ambrosia beetle lineages (Hulcr and Stelinski 2017). Four unrelated ambrosia beetle lineages have mycangia that are relatively large and complex, representing significant energy commitments by the beetles (Mayers et al. 2015, 2018).

Three of the lineages of ambrosia beetles with large and complex mycangia are each associated with one of three genera of ambrosia fungi in the family Ceratocystidaceae (Microascales) (Mayers et al. 2015, 2018). Ambrosia beetles in a fourth lineage, tribe Scolytoplatypodini, have large and complex disc-shaped pronotal mycangia (Schedl 1962; Nakashima et al. 1987; Beaver and Gebhardt 2006), but their fungal partners have not been well studied. Multiple Asian species of *Scolytoplatypus* were reported to have a symbiont in the genus *Ambrosiella* (Ceratocystidaceae) (Nakashima et al. 1987, 1992; Nakashima 1989; Kinuura et al. 1991; Kajimura and Hijii 1994; Beaver and Gebhardt 2006; Ito and Kajimura 2017). Tribe Scolytoplatypodini is thought to be older than the previously-studied ambrosia beetle lineages with large mycangia (Jordal 2013; Gohli et al. 2017; Pistone et al. 2017), and fungal symbionts of

this tribe could provide a missing link in the unresolved evolutionary history of ambrosia fungi in the Ceratocystidaceae (Mayers et al. 2015).

We hypothesized that there was a single origin of ambrosia beetle symbiosis in the fungal family Ceratocystidaceae, and that this origin corresponded with the evolution of the large, pronotal disc mycangium of the Scolytoplatypodini. Our first aim was to isolate ambrosia fungi from all three lineages in this understudied tribe: Asian *Scolytoplatypus*, African *Scolytoplatypus,* and *Remansus* (Jordal 2013). We used phylogenetic analyses to test the hypothesis that the fungal symbionts would comprise a single, novel, monophyletic group in the Ceratocystidaecae. The second aim was to construct a rigorous phylogeny of all ambrosia fungi in the Ceratocystidaceae, estimate their divergence dates, and compare those dates to available estimated divergence dates for beetle lineages with large mycangia. We hypothesized that the crown divergence date of each mycangium type would approximate or predate the crown divergence date of its specific, domesticated fungal lineage. Further, we hypothesized that all ambrosia fungi in the family are derived from a single domestication by tribe Scolytoplatypodini, that is, that the crown divergence date of the pronotal disc mycangium of the Scolytoplatypodini ambrosia beetles would approximate or predate the origin of ambrosia beetle symbiosis in the Ceratocystidaceae. The crown ages of each of the other large mycangium types should thus be coincidental with, or closely followed by, a separate symbiont capture of Scolytoplatypodini associates.

Materials and Methods

Specimen collection and fungal isolation

In addition to ambrosia fungus cultures and specimens available from previous studies (Mayers et al. 2015, 2017, 2018), additional fungal material was collected from

ambrosia beetles in tribes Scolytoplatypodini, Xyleborini, and Xyloterini and their galleries. Fungal cultures were isolated from beetles by surface-sterilizing, dissecting, and directly plating portions of the prothorax containing mycangia on fungal culture media (Mayers et al. 2015, 2017, 2018). Not all isolation attempts were successful from galleries and beetles, often due to desiccation of the insect or gallery material, but we were often able to extract DNA and identify the fungal symbionts by ITS-rDNA barcoding. Collection information for all material from which fungal isolates or DNA sequences were obtained are included in Table 1.

Microscopic observations

Fungal material or dissected mycangia were scraped from gallery walls or the surface of pure cultures with sterile tools and mounted in lactophenol and cotton blue or lactic acid and viewed with an Olympus BH-2 compound microscope (Mayers et al. 2017, 2018). Photographs were captured with a Leica DFC295 camera and Leica Application Suite V3.6 (Leica Camera Inc., Allendale, NJ). Contrast and brightness levels of some images were adjusted with Leica Application Suite or Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA). Some images are composites of multiple photographs taken at different focus planes and combined with CombineZP (Hadley 2010). Some mycangium illustrations are composites of multiple images taken at the same magnification and stitched together with the Photomerge function in Adobe Photoshop CS6.

Species descriptions

Isolates were first grown on malt yeast extract agar (MYEA; 2% malt extract, Difco Laboratories, Detroit, MI, USA; 0.2% yeast extract, Difco; 1.5% agar, Sigma-Aldrich, St. Louis, MO, USA) at room temperature. Agar plugs cut with a #1 cork borer

(3mm diam.) were transferred from the leading margin of growth to three new MYEA plates, incubated at 25° C for 7 d in the dark, and the diameter of the colonies measured. Colors are per Rayner (1970). Representative cultures were deposited in the Westerdijk Fungal Biodiversity Institute (CBS), and representative dried specimens were deposited in the U.S. National Fungus Collections (BPI).

DNA extraction, amplification, and sequencing

When working with small amounts of gallery growth, dissected mycangia, or whole beetles, DNA extractions generally used the PrepMan® Ultra kit (Applied Biosystems, Foster City, CA, USA), and extracts were concentrated when needed with Amicon® ultra-0.5 Centrifugal Filter Devices (EMD Millipore, Billerica, CA, USA). Extractions from pure cultures generally used the Promega Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) or, when pigments inhibited PCR amplification, the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, GA).

For routine species identification, amplification and sequencing of the nuclear rDNA ITS1-5.8S-ITS2 (internal transcribed spacer region, ITS barcode) used fungal primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990). The *Ceratocystidaceae*-specific ITS primer sets Cerato-1F/ITSCer3.7R and ITSCer3.5/ITS4 (Mayers et al. 2015) were also used to obtain sequences from DNA extracts from galleries, mycangia, and whole beetles. All ITS amplification used the same PCR conditions (85°C for 2m; 95°C for 1m35s; 36 cycles of 58°C for 1m, 72°C for 1m20s, and 95°C for 1m10s; 52°C for 1m; 72°C for 15m; and 4°C hold).

Six nuclear genes were sequenced for multi-locus analyses. For nuclear large subunit ribosomal DNA (28S rDNA), primers LR0R and LR5 were used for amplification, with the same PCR conditions as the ITS barcode, and primers LR0R and

LR3 were used for sequencing (Rehner and Samuels 1994; Vilgalys and Hester 1990). For nuclear small subunit ribosomal DNA (18S rDNA) amplification and sequencing, a combination of general primers (NS1, NS3, NS6, NS7, and NS8, SR1R, and SR9R) were used (Elwood et al. 1985; White et al. 1990; Vilgalys and Hester 1990), as well as a new primer designed for Ascomycetes ("NS4Asco", 5'-CTTCCGTCAATTTCTTTAAG-3') and two primers designed to specifically amplify fungal 18S rDNA from mycangia ("NS4Cer", 5'-CACTTTGATTTCTCGAAAG-3', used in place of NS4; and "SR9RCer", 5'-GGCATCAGTATTCAGCTGTC-3', used in place of SR9R). All 18S rDNA PCR used the same PCR conditions (94°C for 2m; 36 cycles of 94°C for 30s, 52°C for 30s, and 72°C for 1m; 72°C for 5m; and 4°C hold), but annealing temperature was lowered to 49°C in some cases. For translation elongation factor 1-alpha (*tef*1-α), primers EFCF1 and EFCF6 were used for amplification using the PCR conditions of Oliveria et al. (2015), though we generally replaced EFCF1 with a new primer designed to avoid a problematic intron in many isolates ("EFCF1.5", 5'-GCYGAGCTCGGTAAGGGYTC-3'). Internal primers EFCF2 (Oliveira et al. 2015) and another new primer ("EFCer3", 5'-CARACHCGTGAGCAYGCTCT-3') were occasionally used for supplemental internal sequencing. Amplification and sequencing of beta-tubulin (*tub*) used the primers (Bt1a and Bt1b) and conditions of Glass and Donaldson (1995). Amplification and sequencing of 60S ribosomal protein L1 (*rpl*1) and DNA replication licensing factor minichromosome maintenance complex component 7 (*mcm*7) used the primers (60S-506F/60S-908R and Cer-MCM7F/Cer-MCM7R) and conditions of de Beer et al. (2014), but for some isolates the annealing temperature was reduced to 55°C. Sequencing was performed by the Iowa State University DNA Sequencing Facility, and complementary

and overlapping DNA reads were checked and assembled using Sequence Navigator v 1.0.1 or AutoAssembler v 1.3.0 (Applied Biosystems, Foster City, California).

Molecular phylogeny

The ITS rDNA sequences of the Ceratocystidaceae have numerous indels that are ambiguously aligned, so for barcoding purposes, ITS sequences were manually aligned in two separate datasets: one for *Ambrosiella* and close relatives (TreeBASE URL: http://purl.org/phylo/treebase/phylows/study/TB2:S22558) and one for *Phialophoropsis* and close relatives (TreeBASE URL: http://purl.org/phylo/treebase/phylows/study/TB2:S22559). In addition to new ITS sequences obtained in this study (Table 1), both alignments also included additional ITS sequences (Table S1) from previous studies (Harrington et al. 2014; Lin et al. 2017; Mayers et al. 2015, 2017, 2018), newly-generated sequences of *Ceratocystis adiposa,* and sequences of *Phialophoropsis* isolates (Chapter 6). To illustrate identity among sequences, a separate maximum parsimony tree was created from each of the two alignments with PAUP 4.0b10 (Swofford 2002) via heuristic searches with gaps treated as fifth character (which preserved the important diagnostic signals present in indels), starting trees obtained via stepwise addition, and the tree-bisection-reconnection branch-swapping algorithm. Using *C. adiposa* and *Meredithiella norrisii* as a monophyletic sister group to the ingroup, the *Ambrosiella* alignment had 552 characters, of which 135 were parsimony-informative, 43 were variable but parsimony-uninformative, and 374 were constant. The *Phialophoropsis* alignment used *Bretziella fagacearum* as an outgroup and midpoint rooting, with 507 characters, of which 39 were parsimony-informative, 31 were variable but parsimony-uninformative, and 437 were constant. A representative tree was chosen from the trees produced from each alignment for illustration, and branch support

values were generated for each tree via 1000-replicate bootstrap maximum parsimony analysis in PAUP.

All multi-locus analyses used a manually-aligned, concatenated alignment (TreeBASE URL: http://purl.org/phylo/treebase/phylows/study/TB2:S22560) of 18S rDNA, *tef*1-α, 28S rDNA, *tub*, *mcm*7, and *rpl*1. Accession numbers for newly-generated sequences and those from previous studies (de Beer et al. 2014; Lin et al. 2017; Mayers et al. 2015, 2017, 2018) are listed in Table S2. Representatives of the major lineages in the Ceratocystidaceae were included (de Beer et al. 2014, 2017; Nel et al. 2017), but a close relative of the family, *Cornuvesica* (Marincowitz et al. 2015), was not included in analyses due to inadequate sequence data. Sequences also included those of a Michigan isolate of an unnamed Microascales sp. (C3547 = CBS 142647) from a gallery of the ship timber beetle, *Elateroides lugubris*. Sequences for the six genes were also extracted from genome assemblies available in the NCBI database (National Center for Biotechnology Information, Bethesda, Maryland): *Huntiella moniliformis* JMSH00000000 (van der Nest et al. 2014), *Huntiella bhutanensis* MJMS00000000 (Wingfield et al. 2016b), and *Scedosporium boydii* NJFT00000000 (unpublished). The resulting six-gene alignment had 5276 characters. Introns were eliminated from *tef*1- α, *tub*, *mcm7*, and *rpl1*, and ambiguously-aligned regions were eliminated (618 characters total) from 18S rDNA and 28S rDNA. The final alignment consisted of 4658 characters, 3347 of which were constant, 261 were variable but parsimony-uninformative, and 1050 were parsimony-informative.

A Bayesian analysis of the six-gene alignment was performed with MrBayes 3.2.2 (Ronquist et al. 2012), with a single MCMC run with four chains (one cold, three heated)

for 1,000,000 generations, which was sufficient to achieve an average standard deviation of split frequencies less than 0.002. Models and partitions were selected by PartitionFinder 2 (Lanfear et al. 2016) in "mrbayes" mode, using "aicc" (converted Aikaike Information Criterion) model selection and a greedy algorithm (Lanfear et al. 2012) and powered by PhyML (Guindon et al. 2010). As suggested by PartitionFinder 2, GTR+I+G was applied to the first partition (18S rDNA), fifth partition (28S rDNA and *tub* codon position 1), sixth partition (*rpl1* codon position 2 and *tub* codon position 2), seventh partition (*mcm7* codon position 1), tenth partition (*rpl1* codon position 1), and eleventh partition (*rpl1* codon position 3); GTR+G was applied to the third partition (*tef*1-α codon position 3 and *tub* codon position 3) and ninth partition (*mcm7* codon position 3); GTR+I was applied to the eighth partition (*mcm7* codon position 2); and F81+I+G was applied to the second partition (*tef*1-α codon position 2) and fourth partition (*tef*1-α codon position 1). A consensus tree was generated using the function 'sumt' with a burnin value of 150,000 and visualized with FigTree 1.4.0. In addition to posterior probability values provided by MrBayes, additional branch support values were generated for the tree via 1000-replicate bootstrap parsimony analysis in PAUP and 1000-replicate bootstrap maximum likelihood analysis in RAxML (Mayers et al. 2018).

Divergence date estimates

A Bayesian tree with estimates of divergence dates (Analysis A) was generated using BEAST v. 2.4.7 (Bouckaert et al. 2014) and an .xml file created in BEAUti 2 from the same six-gene alignment used to create the consensus BI tree of the Ceratocystidaceae. Most genera were pruned to two representative species, and the sequences from three outgroup taxa were extracted from the genomes of *Aspergillus niger* ASM285v2 (Pel et al. 2007), *Sclerotinia sclerotiorum* AAGT01000000 (Amselem

et al. 2011), and *Xylaria* sp. JS573 JWIU00000000 (unpublished). In the absence of fossils of Ceratocystidaceae, secondary calibrations were applied in uniform distributions (Schenk 2016) to the Leotiomycetes-Sordariomycetes crown (267–430 Ma) and the Sordariomycetes crown (207–339 Ma), defined as the 95% highest posterior density range from the 5-fossil-calibrated analysis of Beimforde et al. (2014). Site models were unlinked, and the tree and relaxed log normal clock were linked for all partitions. The birth-death model (Gernhard 2008) was used for the tree prior. Preliminary runs using the same models and partitions selected by PartitionFinder 2 in section 2.5, examined with Tracer 1.6 (Rambaut et al. 2014), gave unacceptably low effective sample size (ESS) values below 100 for both the posterior and prior distributions. This appeared to be due to certain base substitution rates tending towards zero and giving low ESS values, which we interpreted as over-parameterization (Surina et al. 2014). To mitigate this, the models for partitions 3, 6, 7, 9, 10, and 11 were relaxed from the GTR to the HKY model in the final analysis, which yielded acceptable ESS values. In the final analysis two separate MCMC runs of 25,000,000 generations were performed with pre-burnins of 150,000 and their output combined with LogCombiner 2.4.7. The combined output was analyzed with Tracer v. 1.6, resulting in all ESS values above 200 and convergence in the prior, likelihood, and posterior values. The tree files from both runs were combined and reduced to 10,000 trees with LogCombiner 2.4.7, then a maximum clade credibility tree was created and annotated with TreeAnnotator v. 2.4.7 with 15% burnin and mean height nodes then visualized with FigTree 1.4.0.

Generated fungal divergence dates were compared to the origins of their associated mycangia using the crown dates estimated by Gohli et al. (2017) and/or

Pistone et al. (2018). Specifically, the mesonotal pouch mycangium clade included the *Xylosandrus* complex (i.e., Xyleborini genera with mesonotal mycangia: *Anisandrus, Cnestus, Eccoptopterus, Hadrodemius,* and *Xylosandrus*); the prothoracic coil mycangium clade included *Corthylus* (subtribe Corthylina)*;* the prothoracic pleural mycangium clade included *Trypodendron* (tribe Xyloterini); and the pronotal disk mycangium clade included *Remansus* and *Scolytoplatypus* (tribe Scolytoplatypodini). Both studies used four relevant fossils and BEAST to estimate divergence times for the subfamily Scolytinae. Gohli et al. (2017) used 5 genes from 305 species and Pistone et al. (2017) used 18 genes from 182 species.

Results

Fungus-beetle associations

We successfully identified putative species of Ceratocystidaceae from nine Scolytoplatypodini species representing the three recognized lineages of the tribe (Jordal 2013), including three species from the African/Malagasy lineage of *Scolytoplatypus* (*S. fasciatus, S. permirus,* and *S. rugosus*), six species from the Asian lineage of *Scolytoplatypus* (*S. daimio, S. eutomoides, S. mikado*, *S. pubescens, S. shogun*, and *S. tycon*), and a species from *Remansus* (*R. mutabilis*) (Table 1). We also obtained new ITS sequences of *Ambrosiella* symbionts from 5 Asian Xyleborini beetle species, including *Anisandrus apicalis* and four *Xylosandrus* species (*X. brevis, X. crassiusculus, X. germanus,* and *X.* aff. *germanus*). Individual Xyleborini species were in some cases associated with more than one mycangial symbiont, as reported by Lin et al. (2017), and some Ceratocystidaceae mycangial symbionts were associated with more than one ambrosia beetle species. One fungus species from a *Scolytoplatypus* was found to have a

sexual state, only the second discovered among mycangial symbionts of ambrosia beetles (Mayers et al. 2017).

African and Malagasy *Scolytoplatypus*

Galleries of *S. permirus* from two different locations in Madagascar (Table 1) had thick, homogeneous, white-grey ambrosia growth, and the larval cradles were sealed from the main tunnels by frass and fragmented mycelium. The material was desiccated, and isolation attempts from the galleries and the females inside were unsuccessful. The DNA extracted from the ambrosia growth in both galleries yielded the same ITS sequence, which grouped within the Ceratocystidaceae and was more similar to sequences of *Phialophoropsis* than of *Ambrosiella* (Fig. 1). Deep-seated phialides were not seen at the tip of the conidiophores, but the ambrosia growth contained monilioid branches of disarticulating, irregular to globose, thallic-arthric propagules (Figs. 2N–R). Fungal propagules in the mycangium of a *S. permirus* female from a third location

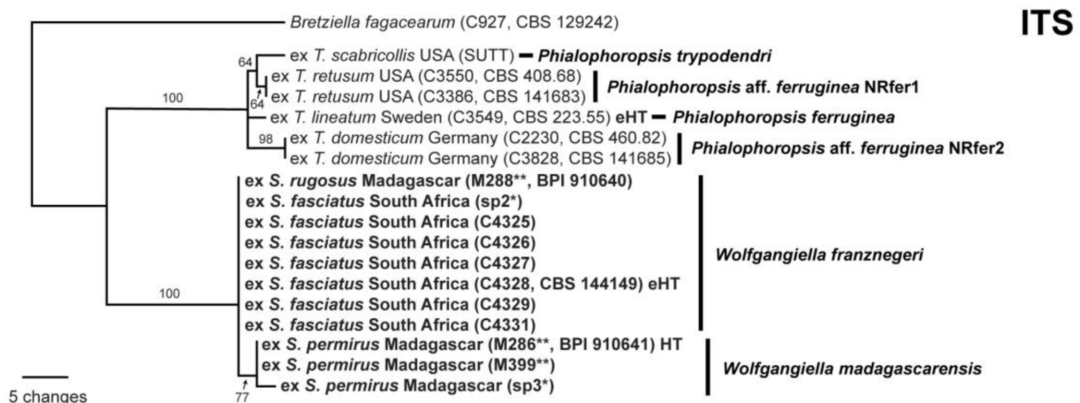

Figure 1. One of four unrooted maximum parsimony trees of ITS rDNA sequences of *Phialophoropsis* and close relatives. Sequences in bold are new to this study. Isolate or specimen numbers from the Iowa State University collection (C or M), Westerdijk Fungal Biodiversity Institute (CBS), or U.S. National Fungus Collections (BPI). Sequences in bold were obtained in this study. Single asterisks indicate sequences obtained from DNA extracted from whole beetles or mycangial spore masses; double asterisks indicate sequences from DNA extracted from gallery growth. Sequences without asterisks are from DNA extracted from cultures. Country of origin of the beetle, gallery, or culture is indicated. Bootstrap support values (> 50%) from 1000-replicate maximum parsimony analysis are indicated on branch labels. *Ceratocystis fagacearum* was used as an outgroup with midpoint rooting. Bar = 5 changes (bp). An '**e**' indicates an ex-type culture; **HT** = holotype; **IT** = isotype; and **PT** = paratype.

in Madagascar (Table 1) formed a homogeneous mass of thallic-arthric fungal propagules that were solitary or in short chains (Fig. 2T), and the DNA from this spore mass yielded an ITS sequence nearly identical (Fig. 1) to that obtained from the two galleries of *S. permirus* (but with a repeated “TC” in the first variable region of ITS1).

A gallery of Malagasy *S. rugosus* had nearly-identical ambrosia growth to that in galleries of *S. permirus*, and DNA extracted from the *S. rugosus* gallery yielded an ITS sequence nearly identical to sequences of the fungus associated with *S. permirus* (Fig. 1), though lacking a repeated “CC” in the first variable region of ITS1. Isolations from the desiccated gallery and the single female inside were not successful. Conidiophores in the gallery of *S. rugosus* (Fig. 2G) were similar to those in galleries of *S. permirus,* but

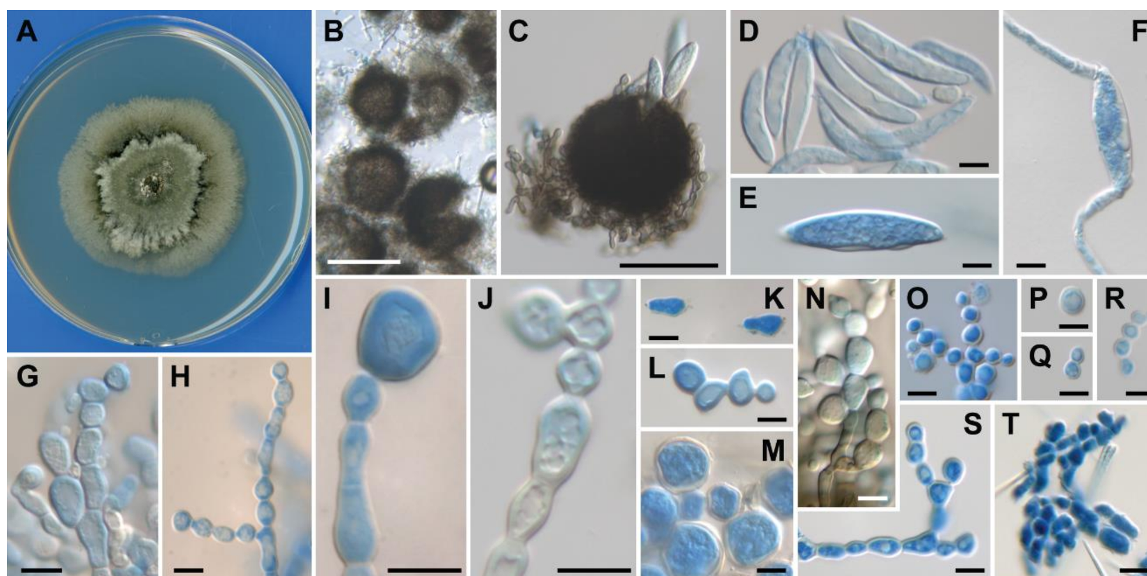

Figure 2. *Wolfganiella franznegeri* (A–J) and *W. madagascarensis* (K–T). (A). Culture morphology at 15d on MYEA. (B). Ascomata. (C). Ascospores emerging from ascocarp. (D, E). Ascospores. (F). Germinating ascospore. (G-J). Conidiophores. (K, L). Detached thallic-arthric conidia. (M). Larger, thick-walled propagules in culture. (N–S). Conidiophores and detached thallic-arthric conidia. (T). Propagules in mycangium of *S. permirus.* (A–C, E, F, H–M). ex-holotype isolate CBS 144149 from *S. fasciatus.* (D, G) from gallery BPI 910640 of *S. rugosus*. (N–S). Holotype BPI 910641 from gallery of *S. permirus*. All photos except A by Nomarski interference microscopy of material stained with cotton blue. A imaged with Epson 10000XL. Bar = 10 µm.

galleries of the former had spherical ascomata in the homogenous mat of ambrosia growth. The ascomata lacked necks or ostioles and contained large, banana-shaped spores (Fig. 2D).

The DNA extracted from the mycangium contents of a South African *S. fasciatus* female preserved in ethanol (Table 1) yielded an ITS sequence identical to that of the gallery of the Malagasy *S. rugosus* (Fig. 1). Isolations from six fresh South African *S. fasciatus* females yielded isolates of a green-grey, slow-growing fungus (Fig. 2A), and the ITS sequences derived from those cultures were identical to that obtained from the preserved *S. fasciatus* specimen (Fig. 1). The six cultures produced branching, disarticulating, thallic-arthric propagules (Figs. 2H–M), identical to those observed in galleries of the Malagasy *S. fasciatus*, and the six cultures also produced the spherical, immature ascomata found in the *S. rugosus* gallery. In two of the isolates (C4325 and C4328), the ascomata matured and produced large, boat-shaped ascospores (Figs. 2C, E) identical to those produced by the ascomata in the *S. rugosus* gallery. Single ascospores isolated on sterile MYEA from fertile ascomata using a sterile needle (Mayers et al. 2017) from C4328 successfully germinated from both ends (Fig. 2F). The colonies produced looked similar to the parent culture and yielded the same ITS sequence, but these colonies did not produce ascomata and appeared debilitated in that they produced less surface growth, had a slower growth rate, attained smaller maximum colony diameters.

Asian *Scolytoplatypus*

Isolates from *Scolytoplatypus* spp. in Japan and Taiwan (Table 1) had ITS sequences that were unique among the Ceratocystidaceae but were most similar to those of *Ambrosiella* (Fig. 3). Five Japanese *S. shogun* females yielded green-grey,

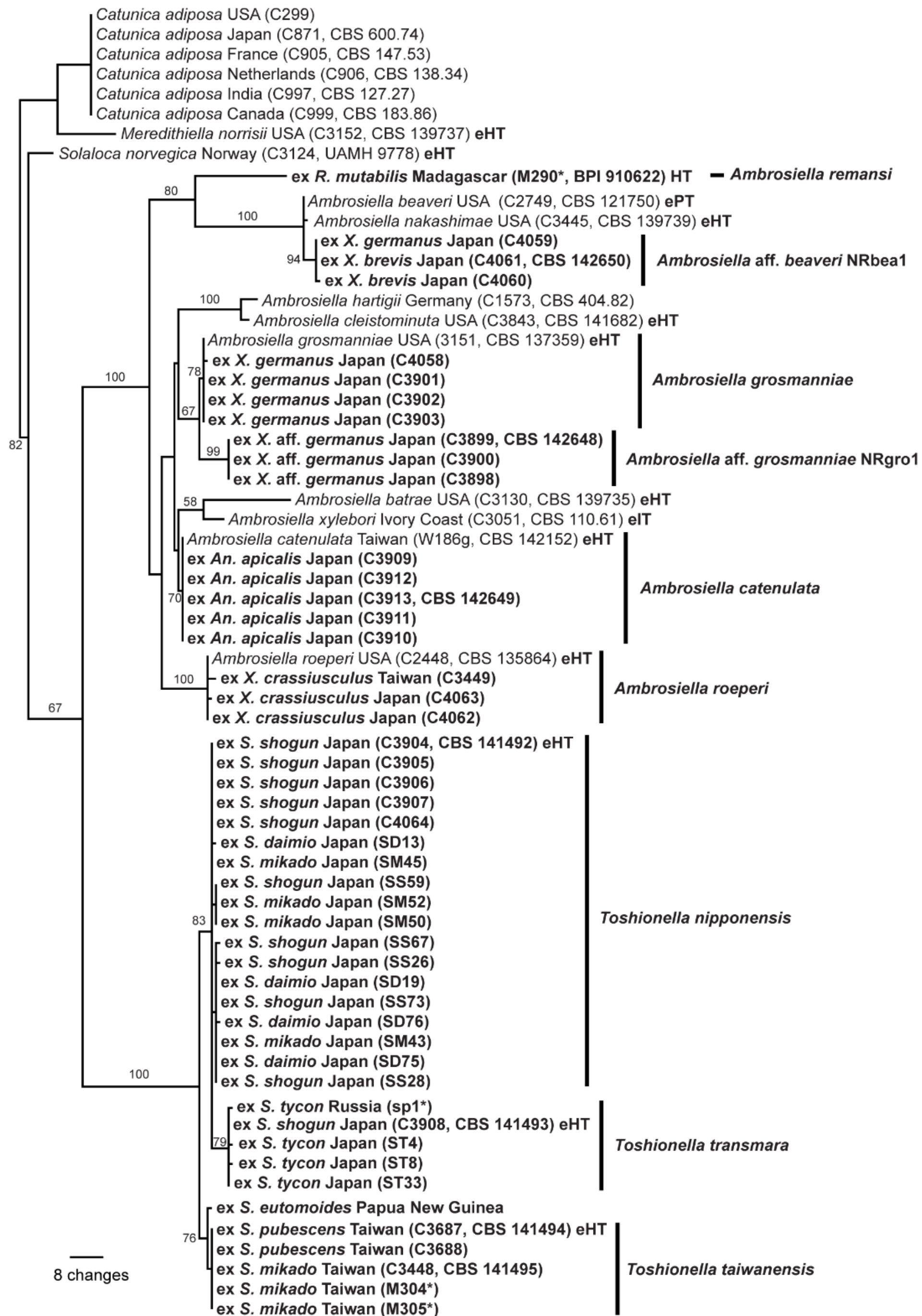

Figure 3. One of twelve unrooted maximum parsimony (MP) trees of ITS rDNA sequences of Ambrosiella and close relatives. Sequences in bold are new to this study. Isolate numbers in the Iowa State University collection (C or M), Westerdijk Fungal Biodiversity Institute (CBS), U.S. National Fungus Collections (BPI), the UAMH Centre for Global Microfungal Biodiversity (UAMH), or other designations provided in parentheses where available. Single asterisks indicate sequences obtained from DNA extracted from whole beetles or mycangial spore masses; double asterisks from DNA extracted from gallery growth. Sequences without asterisks from DNA extracted from cultures. Country of origin of the beetle, gallery, or culture is indicated. Bootstrap support values > 50% indicated on branches. Catunica adiposa and Meredithiella norrisii were used as outgroups. Bar = 5 changes (bp). An ‘e’ indicates an ex-type culture; HT = holotype, IT = isotype, and PT = paratype

slow-growing cultures (Figs. 4A, G, Q) with an identical ITS sequence. All five isolates produced terminal, globose, thick-walled aleurioconidia (Figs. B–E) that were much larger than those observed in *Ambrosiella* (Mayers et al. 2015, 2017; Lin et al. 2017). The individual cells of the branching conidiophores occasionally became globose and thick-walled (Fig. 4C) and were difficult to differentiate from aleurioconidia.

A sixth isolate from *S. shogun* (C3908) had conidiophores and branching, disarticulating, thallic-arthric propagules (Figs. 4H–M) similar to those of the other *S. shogun* isolates, but C3908 had an atypical colony morphology (Fig. 4G) and a different ITS sequence. The DNA extracted from the mycangium of a Russian *S. tycon* (Table 1) yielded an ITS sequence that differed from C3908 at only one base position. Propagules in the *S. tycon* mycangium appeared to be thallic-arthric propagules (Figs. 4O, P), and in contrast to the propagules observed in mycangia of the Malagasy/African *Scolytoplatypus* spp., the propagules in the *S. tycon* mycangium appeared to sometimes branch, as illustrated in mycangia of *S. shogun* (Nakashima et al. 1987).

Two *S. pubescens* females collected from different locations in Taiwan (Table 1) yielded isolates of a dark, olive-green fungus (Fig. 4Q) with an ITS sequence that was most similar to those from Japanese *S. shogun* (Fig. 3). Chains of aleurioconidia formed in culture generally were longer and the conidiophores less branched compared to those in *S. shogun* isolates (Figs. R–V), and the *S. pubescens* isolates produced more aerial hyphae and unique vertical hyphae with branching, dark brown chlamydospores (Figs. 4X, Y). The DNA extracted from two mycangia and one isolate from a Taiwanese *S. mikado* female yielded identical ITS sequences to the isolates from *S. pubescens* (Fig. 3).

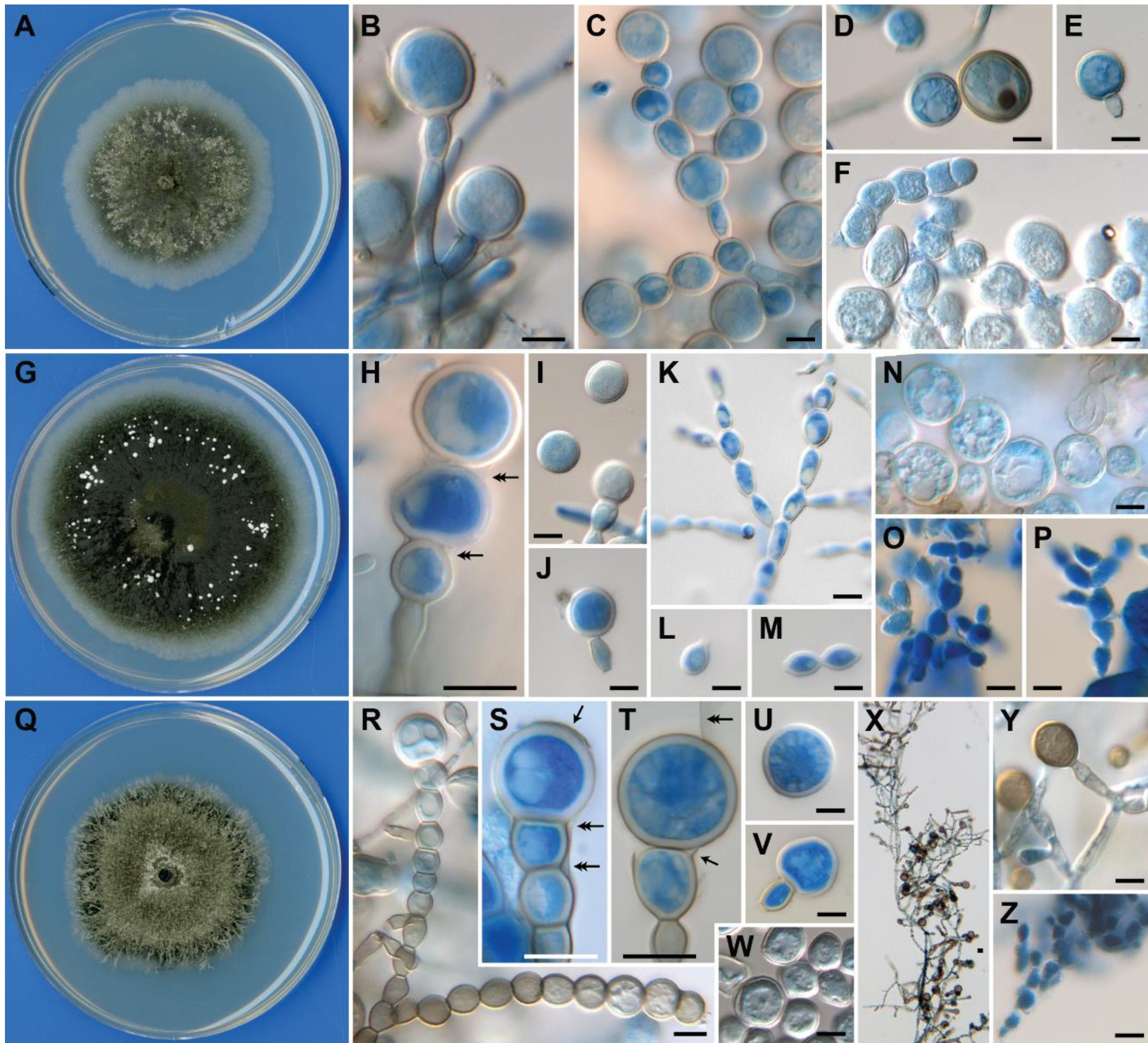

Figure 4. *Toshionella nipponensis* (A–F), *T. transmara* (G–P), and *T. taiwanensis* (Q–Z). (A, G, Q). Culture morphology at 8.5d on MYEA. (B). Conidiophore bearing aleurioconidia. (C). Conidiophore with globose, thick-walled cells. (D). Detached, single aleurioconidia. (E). Detached aleurioconidium with penultimate conidiophore cell attached. (F). Globose, thick-walled propagules forming wet mounds in culture (B). Conidiophore bearing chain of aleurioconidia. Arrows indicate membranous sheath. (I). Detached solitary aleurioconidia. (J). Detached aleurioconidium with penultimate conidiophore cell attached. (K). Branched chains of disarticulating thallic-arthric conidia. (L, M). Detached thallic-athric conidia. (N). Globose, thick-walled propagules forming wet mounds in culture. (O, P). Propagules in mycangium of *Scolytoplatypus tycon.* (R). Long, branched conidiophore bearing aleurioconidia. (S). Young conidiophore with terminal aleurioconidium subtended by developing aleurioconidia. Single arrow indicates remnant of conidiogenous cell. Double arrows indicate membranous sheath. (T). Aleurioconidium breaking free of conidiophore. Single arrow indicates collarette. Double arrow indicates sloughing membranous sheath. (U). Solitary aleurioconidium. (V). Aleurioconidium with penultimate conidiophore cell attached. (W). Globose, thick-walled propagules forming wet mounds in culture. (X). Vertical hyphal tower bearing pigmented chlamydospores. (Y). Pigmented chlamydospores. (Z). Propagules in mycangium of *S. mikado.* (A–F). Ex-holotype isolate CBS 141492 from *S. shogun*. (G–N). Ex-holotype isolate CBS 141493 from *S. shogun*. (Q–Y). Ex-holotype isolate CBS 141494 from *S. pubescens.* All photos except A, G, Q by Nomarski interference microscopy of material stained with cotton blue. A, G, Q imaged with Epson 10000XL. Bar = 10 µm.

Isolate C3448 from *S. mikado* initially had colony morphology similar to that of *S. pubescens* isolates, but C3448 later differentiated into a slower-growing form with a reddish tint. Propagules in the mycangia of *S. mikado* were single or chained, sometimes-branching and thallic-arthric (Fig. 4Z).

Cultures had been obtained in 2002 from *S. daimio, S. mikado, S. shogun,* and *S. tycon* collected in Japan (Table 1). The cultures have since been lost, but partial to full ITS sequences from 2002 were available. The ITS sequences of the *S. daimio, S. mikado,* and *S. shogun* cultures grouped with sequences obtained from the fresher isolates from *S. shogun* (Fig. 3). Small one- or two-bp differences among the 2002 ITS sequences may be due to ambiguities or errors in older sequencing technology. The sequences from *S. tycon* isolates grouped with that of one of the S. shogun isolates (C3908) and the DNA extracted from the Russian *S. tycon* mycangium (Fig. 3).

An ITS sequence provided by J. Skelton (U. Florida) from the spore mass oozing from the mycangium of a female *S. eutomoides* caught in Mu village, Chimbu Province, Papua New Guinea by J. Hulcr (U. Florida) was similar to the sequences of the Taiwan *Scolytoplatypus* spp., differing only in a two-bp deletion in the variable region of ITS1 (Fig. 3).

Remansus

Galleries of *R. mutabilis* collected in Madagascar (Table 1) appeared to be recently-initiated and devoid of ambrosia growth. The DNA extracted from the mycangium contents of a female taken from one of the galleries yielded an ITS sequence that closely matched sequences of *Ambrosiella* (Fig. 3). This mycangium was full of arthric-thallic propagules that were solitary or in short, linear chains (Fig. 5C, D) and

were very similar to mycangium propagules of *Ambrosiella* (Harrington et al. 2014; Mayers et al. 2015, 2017). Isolations were unsuccessful from the desiccated galleries and from the mycangia of two other females.

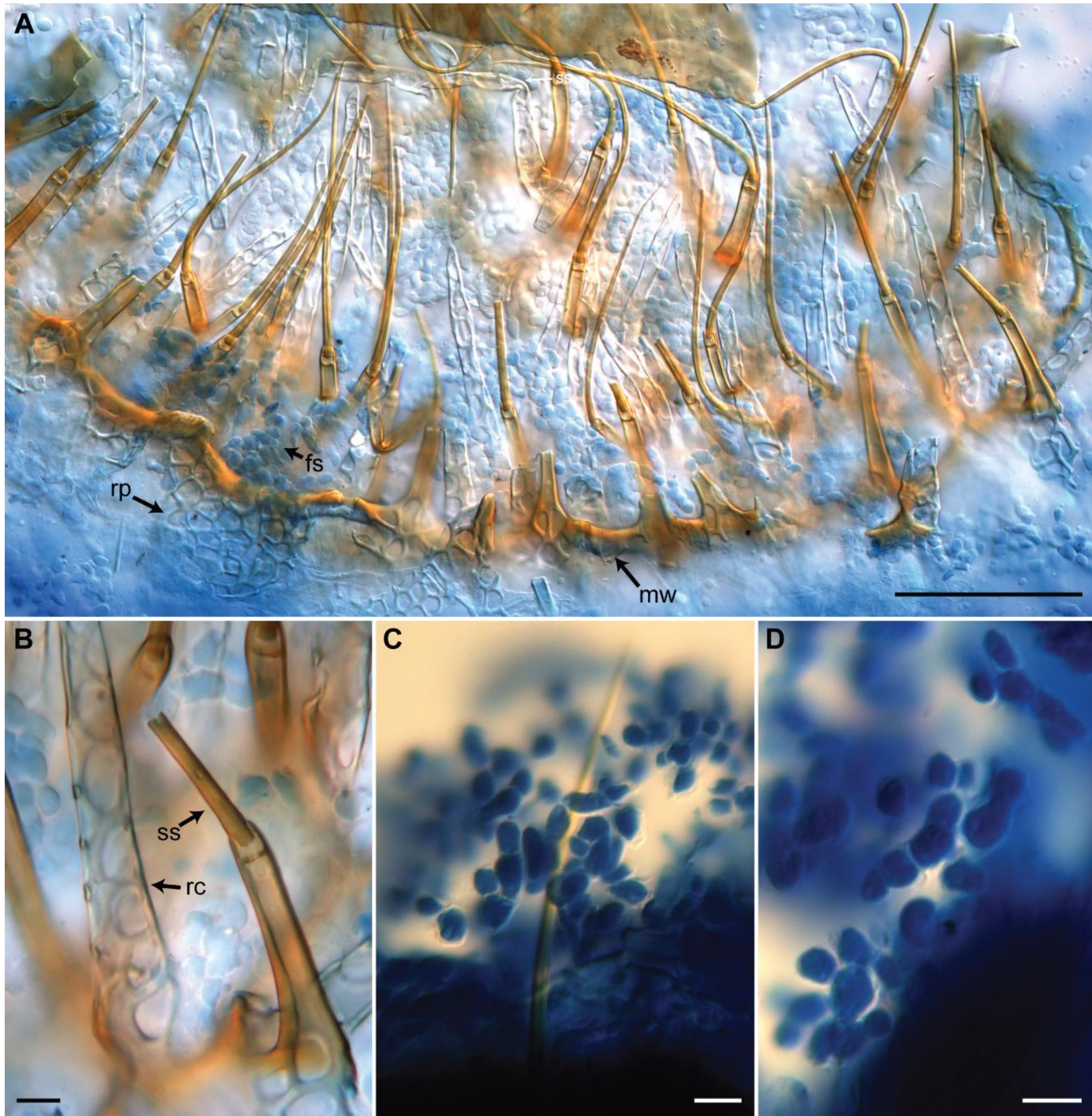

Figure 5. Mycangium of *Remansus mutabilis* and mycangium propagules of *Ambrosiella remansi.* (A). Cross-section of pronotal disk mycangium, showing mycangium wall (mw), reticulated pouches (rp), and fungal spores (fs). (B). Detail of mycangium wall, showing socketed seta (ss) on hollow cylindrical pedestal and reticulated cone (rc). (C). Propagules of *A. remansi* surrounding seta. (D). Propagules of *A. remansi.* All photos by Nomarski interference microscopy of BPI 910622, stained with cotton blue. (A). Bar = 100µm. (B-D) Bar = 10µm

Xyleborine beetles

Isolations were made from several Xyleborini species in the *Xylosandrus* complex that were sympatric with the studied Asian *Scolytoplatypus* species. All of these Xyleborini had *Ambrosiella* symbionts, as expected (Mayers et al. 2015, 2017; Lin et al. 2017). Two Japanese and one Taiwanese *X. crassiusculus* yielded isolates of *A. roeperi* (Fig. 3) (Harrington et al. 2014; Lin et al. 2017), and four Japanese *X. germanus* yielded isolates of *A. grosmanniae* (Fig. 3) (Mayers et al. 2015; Lin et al. 2017). Three unidentified Japanese females identified as *Xylosandrus* aff. *germanus* yielded isolates of a putative undescribed species (*Ambrosiella* aff. *grosmanniae* NRgro1) that was closely related to *A. grosmanniae* (Fig. 3), but the undescribed species had distinct culture morphology, formed concentric rings of mounded hyphae in culture, and it sporulated much more densely than *A. grosmanniae*. This may coincide with the cryptic diversity in *X. germanus* and its *A. grosmanniae* symbionts reported by Ito and Kajimura (2017). Two Japanese *X. brevis,* as well as one Japanese *X. germanus,* yielded isolates of another putative undescribed species (*Ambrosiella* aff. *beaveri* NRbea1) in the *A. beaveri* complex (Fig. 3). Members of this complex had been reported from *X. brevis* and several other beetle species in the *Xylosandrus* complex (Lin et al. 2017). Five Japanese *Anisandrus apicalis* yielded isolates with ITS sequences and morphology identical to *A. catenulata* (Fig. 3)*,* which had been isolated from multiple species in the *Xylosandrus* complex (Lin et al. 2017), but this is the first report of its association with *An. apicalis*.

Scolytoplatypodini mycangia

Consistent with Schedl's (1962) illustrations, all observed mycangia of Scolytoplatypodini were disc-shaped cavities just under the cuticle on the dorsal side of the pronotum that emptied through a central, circular pore on the dorsal side. Waxy

masses of fungal propagules in an unidentified matrix generally plugged the pores and oozed out when pressure was applied to the dissected mycangium. As seen in Schedl's (1962) illustrations, the interior of *Scolytoplatypus* mycangia were lined with long setae that led towards the pore and presumably helped guide fungal propagules to exit. The morphology of these internal setae differed between African and Asian *Scolytoplatypus* species. The setae of African *Scolytoplatypus* species (*S. fasciatus, S. rugosus, S. permirus*) were of two types: shorter, thinner setae that grew directly from the sclerotized plates of the mycangium wall, and longer, thicker setae that grew from short, cone-shaped sockets on the mycangium wall, as illustrated in the African species *S. acuminatus* (Schedl 1962). In contrast, Asian *Scolytoplatypus* species (*S. pubescens, S. mikado, S. tycon*) had only one type of setae, which were socketed on the apexes of tall, hollow, reticulated, conical towers that were extensions of the reticulated network of the mycangium wall, as illustrated in multiple Asian *Scolytoplatypus* species (Berger and Cholodkovsky 1916; Schedl 1962; Nakashima et al. 1987; Beaver and Gebhardt 2006).

Mycangia of the rare and recently-described (Jordal 2013) genus *Remansus* had not been illustrated previously. The mycangium of the female *R. mutabilis* collected in Madagascar was a pronotal disk similar to that of *Scolytoplatypus*. However, its interior setae were of only one type, which were socketed on the apexes of tall, hollow, elongated, non-reticulated, cylindrical, and pigmented pedestals (Figs. 5A, B). Interspersed with these pedestals were tall, hollow, reticulated, conical towers (Fig. 5B) that did not support terminal setae but were otherwise similar to the reticulated cones supporting the interior setae of Asian *Scolytoplatypus*. The mycangium wall of *R. mutabilis* was composed of a large, reticulated network, between which hung more

finely-reticulated, semi-spherical pouches (Fig. 5A). This reticulated network was more similar to the reticulated mycangium walls of Asian *Scolytoplatypus* than the sclerotized plates of African *Scolytoplatypus* (Schedl 1962). Both the setal pedestals and reticulated cones of *R. mutabilis* appeared to be contiguous with this reticulated network. Fungal propagules were found throughout the mycangium lumen as well as in the semi-spherical pouches (Fig. 5A).

Phylogenetic analyses

We hypothesized that the mycangial fungi of the three Scolytoplatypodini lineages would form a novel monophyletic group within the Ceratocystidaceae. The recovered symbionts were all members of the Ceratocystidaceae, but they did not form a single monophyletic group. The ITS sequences of African *Scolytoplatypus* symbionts were related to *Trypodendron* (Xyloterini) symbionts (*Phialophoropsis*). Parsimony analysis of the *Phialophoropsis*-affiliated alignment produced four trees, including Fig. 1, with a consistency index of 0.9494 and differing only in branch lengths within *Phialophoropsis*. The African *Scolytoplatypus* symbionts formed a distinct sister group to *Phialophoropsis,* with good bootstrap support (Fig. 1). The ITS sequences of the mycangial symbionts of Asian *Scolytoplatypus* and *R. mutabilis* were more similar to *Ambrosiella,* and parsimony analysis of the *Ambrosiella*-affiliated alignment produced 84 trees, including Fig. 3, with a consistency index of 0.7823 and differing only in the topology among the 2002 sequences from Japanese *Scolytoplatypus* symbionts. The Asian *Scolytoplatypus* symbionts formed a distinct sister group to *Ambrosiella* with good bootstrap support, but the symbiont of *R. mutabilis* grouped within *Ambrosiella sensu stricto* with good bootstrap support and near the *A. beaveri* complex with moderate

support (Fig. 3). New ITS sequences of Xyleborini symbionts were placed within *Ambrosiella sensu stricto*, as expected (Lin et al. 2017; Mayers et al. 2015, 2017).

Multi-gene Bayesian analysis confirmed the sister relationships of African *Scolytoplatypus* symbionts with *Phialophoropsis* and Asian *Scolytoplatypus* symbionts with *Ambrosiella*, as well as the placement of the *R. mutabilis* symbiont with *Ambrosiella sensu stricto,* where it was placed as the first-diverging taxon (Fig. 6). Including *Meredithiella*, there were five distinct, well-supported clades of ambrosia fungi with high posterior probability, RAxML bootstrap, and parsimony bootstrap support values. The inferred relationships among genera in the Ceratocystidaceae were similar to those of previous analyses, with the ambrosia beetle symbionts, *Huntiella*, and three poorly placed species grouping separately from the other half of the family (de Beer et al. 2014, 2017). The placement of *Huntiella* within the ambrosia half of the family was somewhat ambiguous. The genus was positioned under a short internode with 0.98 posterior probability support, but this branch was observed to collapse to a polytomy when certain *Huntiella* taxa were added to or removed from analysis. The ambiguous phylogenetic placements of *Bretziella fagacearum, Ceratocystis adiposa,* and *C. norvegica* with respect to the ambrosia beetle symbionts were better resolved than in earlier studies (de Beer et al. 2014, 2017; Mayers et al. 2015, 2018). *Ceratocystis adiposa* was placed as sister taxon to *Meredithiella* with 1.0 posterior probability support, *B. fagacearum* formed a sister taxon to the *Phialophoropsis/*African *Scolytoplatypus* symbiont clade with moderate support, and *C. norvegica* was placed as sister taxon to the *Ambrosiella/*Asian *Scolytoplatypus* symbiont clade with moderate support.

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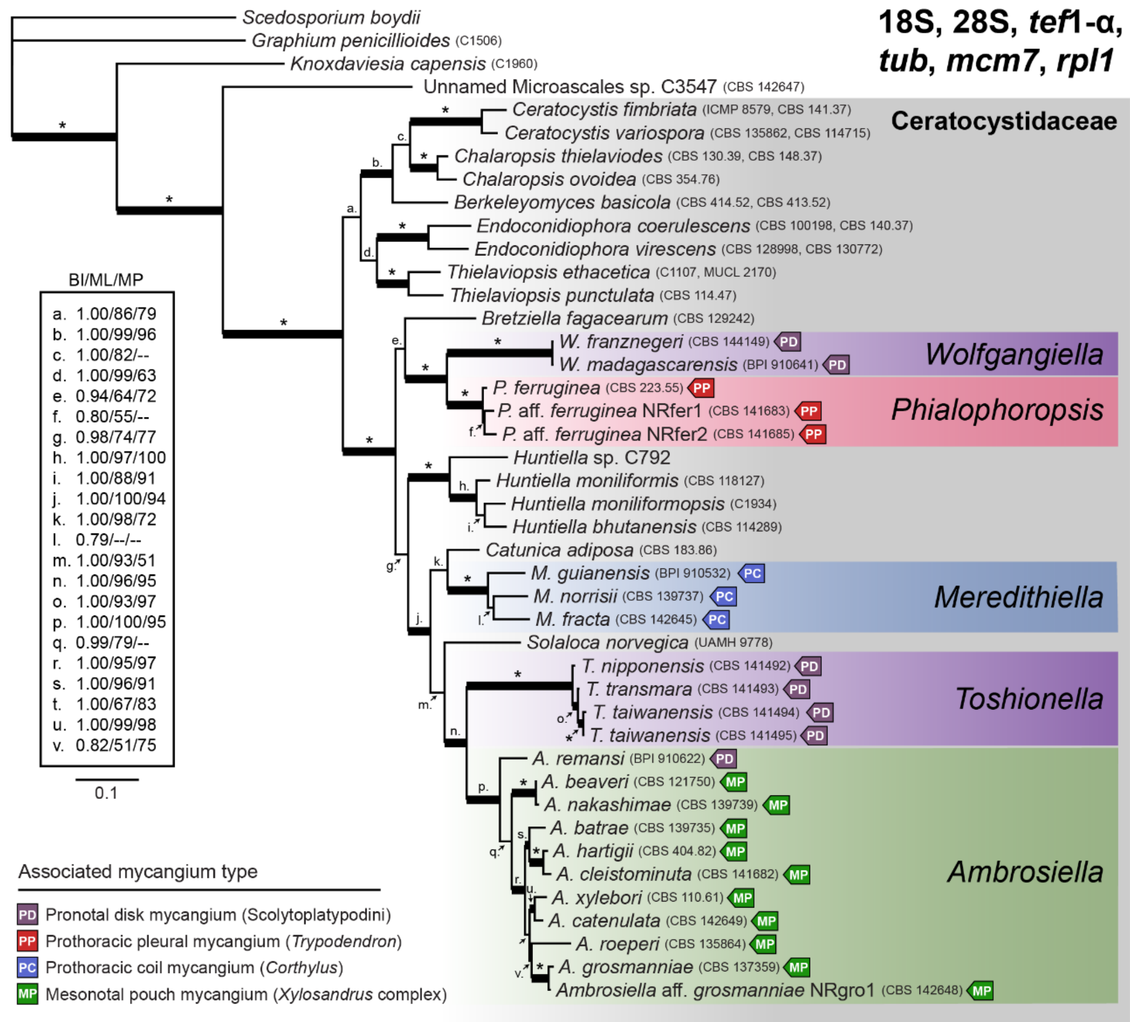

Figure 6. Phylogenetic tree from Bayesian analysis of a six-gene dataset (18S rDNA, 28S rDNA, tef1-α, tub, mcm7, and rpl1) of ambrosia fungi and other representative of the Microascales. The family Ceratocystidaceae is indicated by a grey-shaded rectangle. Colored tags indicate the association of each ambrosial species with the mycangium types in the key. Isolate or specimen numbers indicate the Iowa State University collection (C or M), Westerdijk Fungal Biodiversity Institute (CBS), U.S. National Fungus Collections (BPI), the Belgian Co-ordinated Collections of Micro-organisms (MUCL), the International Collection of Microorganisms from Plants (ICMP), or the UAMH Centre for Global Microfungal Biodiversity (UAMH). Posterior probabilities from Bayesian inference (BI), bootstrap support values from 1000-replicate maximum likelihood analysis in RAxML (ML), and bootstrap support values from 1000-replicate maximum parsimony analysis in PAUP (MP) are indicated in box at left margin with lowercase letters indicating the associated branch. Branches with BI = 1.0, ML = 100, and MP = 100 support are labelled with an asterisk, and branches with BI = 1.0, ML ≥ 95, and MP ≥ 95 are indicated with thicker line weights. Posterior probability values ≥ 0.995 and < 1.0 are represented as “0.99,” bootstrap support values ≥ 99.5 and < 100 are represented as “99,” and bootstrap support values < 50 are indicated with two hyphens (‘--‘). Bar = 0.1 estimated substitutions per site.

Both *C. adiposa* and *C. norvegica* were clearly placed outside of *Ceratocystis sensu stricto* but not accommodated by any currently-defined genera (de Beer et al. 2014, 2017; Mayers et al. 2015; Nel et al. 2017). Each of these species have no known close relatives, and they are biologically and morphologically distinct from each other and the ambrosia beetle symbionts.

Taxonomy

In recognition of their distinct morphological characteristics, and supported by clear phylogenetic separation from known taxa, two new genera are proposed for the symbionts of African and Asian *Scolytoplatypus.* The symbiont of *Remansus mutabilis* is described as a new species of *Ambrosiella.* Two new monotypic genera are created to accommodate the problematic non-ambrosial taxa *C. adiposa* and *C. norvegica*.

Disclaimer: The novel taxa proposed in this chapter of the dissertation are not intended to represent validly published names under the International Code of Nomenclature for algae, fungi, and plants.

WOLFGANGIELLA C. Mayers & T.C. Harr., **gen. nov.**

MycoBank MB 824930.

Etymology. Feminine. After entomologist Wolfgang Dietrich Schedl, who characterized the mycangia of *Scolytoplatypus* among other significant contributions to mycangium diversity.

Irregular to spherical thallic-arthric conidia on branching hyphae that disarticulate singly or in chains. Ascocarps when present spherical, pigmented, non-ostiloate, containing fusiform to falcate ascospores. Associated with ambrosia beetles, including *Scolytoplatypus* in Africa and Madagascar.

Type species: Wolfgangiella franznegeri C. Mayers, T.C. Harr., & F. Roets, sp. nov.

Wolfgangiella is most closely related to *Phialophoropsis* (Fig. 6), and its arthric-thallic conidia are somewhat similar to the disarticulating cells observed in *Phialophoropsis* (Mathiesen-Käärik 1953). However, all studied *Phialophoropsis* spp. produce chained conidia from deep-seated phialides (Batra 1967; Mayers et al. 2015), which were not observed in *Wolfgangiella*. Cultures of the type species for *Wolfgangiella* also produce significantly less pigment than *Phialophoropsis* spp.

Wolfgangiella franznegeri C. Mayers, T.C. Harr., & F. Roets, **sp. nov.** (Figs. 2A–M)

MycoBank MB 824932.

Etymology. After the German botanist Franz Wilhelm Neger (1868–1923), an early pioneer in the study of the ambrosia beetle symbiosis.

Typus. **South Africa:** Western Cape Province, Betty's Bay, Harold Porter National Botanical Garden, 34°21'1.58"S, 18°55'37.03"E, from mycangium of *Scolytoplatypus fasciatus* caught in flight, 21 Jan 2017, *F. Roets,* holotype (dried culture, BPI 910639); ex-type living culture C4328 (CBS 144149).

Colonies on malt yeast extract agar 5 – 25 mm after 7 days at 25° C, odor sweet, margin submerged, hyaline to olivaceous buff with olivaceous clumps, reverse buff to isabelline to pale mouse grey, surface growth aerial, white to pale olivaceous grey, with superficial white spherical sporodochia in clusters bearing tufts of conidiophores. *Ascomata* embedded in mounded mycelium, cleistothecious, spherical, brown, texture intricata, 28 – 142 µm in diameter at maturity, lacking necks or ostioles. *Asci* not observed. *Ascospores* 7 – 19 × 33 – 60 µm, thick-walled, fusiform to falcate.

Conidiophores 14 – 66 µm long, composed of branching, disarticulating chains of thallic-arthric conidia. *Conidia* irregular to globose, 4.5 – 13 (32) × 6 – 16 (32) µm, detaching singly or in linear or branching chains.

Other specimens examined. **Madagascar:** Andasibe-Mantadia National Park, ambrosia growth in gallery of *Scolytoplatypus rugosus* in *Ocotea* sp., May 2015, *B. Jordal*, BPI 910640. **South Africa:** Western Cape, Diepwalle Forest Station, propagules in mycangium of *S. fasciatus* in unidentified tree, November 2007, *B. Jordal,* sp2.

Other cultures examined. **South Africa:** Western Cape Province, Betty's Bay, Harold Porter National Botanical Garden, from mycangium of *Scolytoplatypus fasciatus* caught in flight, 21 Jan 2017, *F. Roets,* C4325. Western Cape Province, Betty's Bay, Harold Porter National Botanical Garden, from mycangium of *S. fasciatus* caught in flight, 21 Jan 2017, *F. Roets,* C4326. Western Cape Province, Betty's Bay, Harold Porter National Botanical Garden, from mycangium of *S. fasciatus* caught in flight, 21 Jan 2017, *F. Roets,* C4327. Western Cape Province, Betty's Bay, Harold Porter National Botanical Garden, from mycangium of *S. fasciatus* caught in flight, 21 Jan 2017, *F. Roets,* C4329. Western Cape Province, Betty's Bay, Harold Porter National Botanical Garden, from mycangium of *S. fasciatus* caught in flight, 21 Jan 2017, *F. Roets,* C4331.

Notes. Wolfgangiella franznegeri was isolated from six of six females of *S. fasciatus* trapped in flight in South Africa, and it dominated a gallery of *S. rugosus* in Madagascar. *S. fasciatus* and *S. rugosus* are close relatives, but the former is only known from South Africa and the latter from a small area in Madagascar (Jordal 2013). ITS sequences from all sources were identical (Fig. 1).

Wolfgangiella madagascarensis C. Mayers, McNew, & T.C. Harr., **sp. nov.** (Figs.2N–T)

MycoBank MB 824933.

Etymology. After the country of origin, Madagascar.

Typus. **Madagascar:** Ambohitantely Forest Reserve, ambrosia growth in gallery of *Scolytoplatypus permirus* in *Ocotea* sp. or *Macaranga* sp., May 2015, *B. Jordal*, holotype (BPI 910641).

Conidiophores composed of branching, disarticulating chains of thallic-arthric conidia. *Conidia* irregular or globose to subglobose, 4 – 13.5 (17) µm in diameter, detaching singly or in chains. *Growth in mycangium* composed of irregular cells 3.5 – 7 × 4.5 – 12.5 µm in size, solitary or in branched or unbranched chains.

Other specimens examined: **Madagascar:** Ranomafana National Park, propagules in mycangium of *Scolytoplatypus permirus* caught in flight, 2012, *B. Jordal,* sp3. Andasibe-Mantadia National Park, ambrosia growth in gallery of *S. permirus* in *Ocotea* or *Macaranga* sp., May 2015, *B. Jordal*, M399.

Notes. Wolfgangiella madagascarensis was observed in galleries from two locations in Madagascar and detected by ITS sequence analysis at another location, all associated with *S. permirus*, which is only known from Madagascar (Jordal 2013). The ITS sequence obtained from the mycangium contents differed by a two-base repeat in the first variable region compared to the sequences from galleries (Fig. 1). The DNA sequences of *W. madagascarensis* and *W. franznegeri* differed slightly in the ITS region (Fig. 1) and in the multi-gene alignment (one insertion in 18S rDNA, three substitutions in *tef*1-a, one insertion in the *tub* intron, and one substitution in *mcm7*). Aside from the

presence of ascomata and ascospores in *W. franznegeri* cultures, and two species of *Wolfgangiella* are morphologically similar.

TOSHIONELLA C. Mayers & T.C. Harr., **gen. nov.**

MycoBank MB 824934.

Etymology. Feminine. After Toshio Nakashima, who contributed the majority of our previous knowledge on Asian *Scolytoplatypus* symbionts.

Aleurioconidia terminal, thick-walled, globose, single or in basipetal chains, sometimes surrounded by a membranous sheath, on branching monilioid or simple conidiophores. Associated with ambrosia beetles, including *Scolytoplatypus* spp. in Asia. Sexual state unknown.

Type species: Toshionella nipponensis C. Mayers, T.C. Harr, & H. Masuya, sp. nov.

One or more members of this genus were previously reported from Asian *Scolytoplatypus* and treated as putative species of *Ambrosiella* (Nakashima et al. 1987, 1992; Nakashima 1989; Kinuura et al. 1991; Kinuura 1995; Beaver and Gebhardt 2006). *Toshionella* is closely related to *Ambrosiella* (Fig. 6), and its conidiophores are similar, but its aleurioconidia are much larger. The phialidic conidiophores reported in *Ambrosiella* (Lin et al. 2017; Mayers et al. 2015, 2017) were not observed in *Toshionella*. The thin membranous sheath observed around chains of aleurioconidia (Figs. 4H, S, T) has been reported in *A. cleistominuta* (Mayers et al. 2017) and *C. adiposa* (Hutchinson 1939; Bhat 1972; Hawes and Beckett 1977a, 1977b). Though rarely, *Toshionella* aleurioconidia sometimes disarticulate with the penultimate conidiophore cell attached

(Figs. 4E, J, V), as is common in *Ambrosiella* and *Meredithiella* (Harrington et al. 2014; Mayers et al. 2015, 2018). All *Toshionella* cultures produced wet and gooey mounds of thick-walled, globose to irregular, hyaline cells (Figs. 4F, N W), which may be the "sprout cells" previously reported forming a "slimy layer" in galleries of *Scolytoplatypus* spp. (Nakashima 1989, Beaver and Gebhardt 2006).

Toshionella nipponensis C. Mayers, T.C. Harr, & H. Masuya, **sp. nov.** (Figs. 4A–F)

MycoBank MB 824935.

Etymology. After the country of origin, Japan.

Typus. **Japan:** Akita prefecture, Tazawako, from mycangium of emerging *Scolytoplatypus shogun*, July 2014, *H. Masuya*, holotype (dried culture, BPI 910635); ex-type living culture C3904 (CBS 141492).

Colonies on malt extract yeast agar 32 – 55 mm after 7 days at 25° C, surface growth white, fluffy in patches, later covering surface and becoming chalky, pale olivaceous to olivaceous grey, reverse grey olivaceous to olivaceous black, becoming dark slate blue, margin hyaline, becoming greenish black. In older cultures, irregular buff sporodochia on surface, sometimes bearing red liquid drops, later center growth thick, dense, raised, and red-brown. *Conidiophores* 8.5 – 35 µm long, single- or multiple-celled, hyaline becoming red-brown, branched, composed of thick-walled monilioid cells, bearing terminal aleurioconidia, conidiophore cells later becoming thick-walled and disarticulating into single cells. *Aleurioconidia* hyaline becoming faintly red-brown, globose to subglobose, ovoid, 17.5 – 25.5 × 18 – 26 µm, generally wider than tall, often flat on bottom, borne singly or in chains, terminally, from monilioid conidiophores, terminally or intercalary on simple hyphae, or rarely directly from the side of

conidiophore. In older cultures, thick-walled cells presumed to be aleurioconidia accumulate in moist mounds on the culture surface, globose to ellipsoidal, thick-walled, 7 – 25 µm in diameter.

Other cultures examined: **Japan:** Akita prefecture, Tazawako, from mycangium of *Scolytoplatypus shogun*, July 2014, *H. Masuya*, C3905. Akita prefecture, Tazawako, from mycangium of *S. shogun*, July 2014, *H. Masuya*, C3906. Akita prefecture, Tazawako, from mycangium of *S. shogun*, July 2014, *H. Masuya,* C3907. Iwate prefecture, Hachimantai, from mycangium of *S. shogun*, 7 July 2014, *H. Masuya*, C4064.

Notes. T. nipponensis was isolated from five *S. shogun* females in Japan, and the cultures had an identical ITS sequence that was nearly identical to those obtained from Japanese *S. daimio, S. mikado,* and *S. shogun* isolates in 2002 (Fig. 3). The fungus illustrated in galleries of Japanese *S. daimio, S. mikado,* and *S. shogun* (Nakashima et al. 1987, 1992; Nakashima 1989; Kinuura et al. 1991; Kinuura 1995) and informally described by Nakashima et al. (1987) is probably *T. nipponensis.* An atypical isolate from *S. shogun* that differed in phenotype, DNA sequences, and culture morphology is described below as a separate species.

Toshionella transmara C. Mayers, T.C. Harr., & H. Masuya, **sp. nov.** (Figs. 4G–P)

MycoBank MB 824936.

Etymology: After Latin transmara (adj) ‘sea-crossing’, as it was detected from both sides of the Sea of Japan.

Typus. **Japan:** Iwate prefecture, Hachimantai, from mycangium of *Scolytoplatypus shogun*, June 2015, *H. Masuya*, holotype (dried culture, BPI 910638); ex-type living culture C3908 (CBS 141493).

Colonies on malt extract yeast agar 40 – 63 mm after 7 days at 25° C, surface growth superficial, greyish sepia to olivaceous grey, covered in randomly dispersed superficial, spherical, white to off-white sporodochia 1 – 1.2 mm in diameter, distributed randomly, in concentric rings, or clustered around site of transfer, reverse greenish black, margin greenish black, aerial hyphae absent. *Conidiophores* 8 –40 um long, single- or multi-cellular, hyaline, branched, composed of thick-walled monilioid cells, bearing terminal aleurioconidia. Conidiophore cells later becoming thick-walled, disarticulating into single cells. *Aleurioconidia* hyaline, thick-walled, globose to subglobose, ovoid, (8.3) 14 – 18 × (8.5) 14 – 19 µm, generally wider than tall, often flat on bottom, borne singly or in chains, terminal on monilioid conidiophores or on single-celled side branches, or rarely directly from the side of conidiophores. Thick-walled cells presumed to be aleurioconidia occasionally accumulate in moist mounds on the culture surface, globose to ellipsoidal, thick-walled, 10.5 – 30 µm in diameter. *Thallic-arthric conidia* thick walled, 9 – 13.5 (16) × 11 – 17 (19.5) µm, usually ellipsoidal to globular, borne in branching monilioid chains on the surface of mature sporodochia, disarticulating singly or in chains. *Mycangial growth* of branching, thallic-arthric cells, 4.5 – 10 × 5 – 14 µm, solitary or in chains.

Other specimens examined: **Russia:** Vladivostok, propagules in mycangium of *Scolytoplatypus tycon* caught in flight, July 2008, *B. Jordal,* sp1.

Notes. This species is closely related to *T. nipponensis* (Fig. 3) but can be differentiated by its faster rate of growth, scattered spherical sporodochia, and lack of chalky white surface growth. The single living representative (C3908 = CBS 141493) was isolated from *S. shogun* in Japan, but nearly-identical ITS sequences were obtained

from the mycangium of a female *S. tycon* caught in Vladivostok, Russia (sp1) and three cultures from Japanese *S. tycon* sequenced in 2002 (Fig. 3). Nakashima et al. (1992) may have illustrated *T. transmara* in galleries of Japanese *S. tycon*.

Toshionella taiwanensis C. Mayers, T.C. Harr., & H.H. Shih. **sp. nov.** (Figs. 4Q–Z)

MycoBank MB 824937.

Etymology. After the country of origin, Taiwan.

Typus. **Taiwan:** Kaohsiung municipality, Douna, from *Scolytoplatypus pubescens* caught in flight, February 2014, *H. Shih,* holotype (dried culture, BPI 910637); ex-type living culture C3687 (CBS 141494).

Colonies on malt extract yeast agar (22) 40 – 53 mm after 7 days at 25° C, reverse greenish black, margins hyaline to buff, submerged, becoming white, dense, raised, fruity smell at 5 days, somewhat fading by 14 days, covered with a dense white to olivaceous grey carpet of aerial mycelium and conidiophores. *Subsurface hyphae* visible beneath the dendroid margin of surface growth, deeply pigmented with diffusible pigment rarely extending beyond surface growth, deep brown to greenish black. *Conidiophores* 15 – 115 µm long, hyaline or red-brown, branched, composed of thick-walled monilioid cells or thin-walled irregular cells, produced densely across the entire surface of the culture, bearing terminal aleurioconidia, conidiophore cells developing into thick-walled chlamydospores. *Aleurioconidia* hyaline, thick-walled, globose to subglobose, ovoid, 13.5 – 23 × 12.5 –22 µm, generally wider than tall, often flat on bottom, produced terminally, singly or in chains, on long monilioid conidiophores or single-celled side branches or directly on simple hyphae. *Chlamydospores* of two types, borne terminally on branching, aerial, erect towers of simple hyphae, thick-walled, globose to subprolate,

obovoid, deeply pigmented, 15 – 16 × 15.5 – 18 µm, generally taller than wide; or ellipsoidal to globular, lightly red-brown, thick-walled, disarticulating singly or in chains, borne in branching monilioid chains. *Thick-walled cells* (aleurioconidia) in moist mounds on the surface of old cultures, globose to ellipsoidal, thick-walled, 10.5 – 26.5 µm in diameter. *Mycangial growth* branching, thallic-arthric, of thick-walled cells, 3.5 – 8 × 4.5 – 11.5 µm, solitary or in chains.

Other cultures examined: **Taiwan:** Kaohsiung municipality, Jingdashan, from *Scolytoplatypus pubescens* caught in flight, February 2014, *H. Shih,* C3688. Lienhuachih, from *S. mikado* caught in flight, Aug 2014, *C. Wuest*, C3448 (dried culture BPI 910636; living culture CBS 141495).

Other specimens examined: **Taiwan:** Kaohsiung municipality, Lienhuachih, propagules in mycangium of *Scolytoplatypus mikado* caught in flight, June 2013, *H. Shih,* M304. Lienhuachih, propagules in mycangium of *S. mikado* in *Cinnamomum* sp., June 2013, *H. Shih,* M305.

Notes. This new species is associated with both *S. pubescens* and *S. mikado* in Taiwan and is related to the Japanese *Toshionella* species (Fig. 3). Sequences obtained in 2002 from Japanese *S. mikado* were of *T. nipponensis* rather than *T. taiwanensis,* implying geographic rather than species-specific distribution of the two species (Fig. 3). *Toshionella taiwanensis* can be differentiated from the Japanese *Toshionella* by its dense, fluffy surface growth and the presence of upright, branching aerial towers bearing terminal, pigmented chlamydospores (Figs. 4X, Y), which are unique among the studied ambrosia fungi but similar to the aleurioconidia produced by species of *Ceratocystis sensu lato* (Paulin-Mahady et al. 2002). The fungus isolated from *S. pubescens* in Taiwan

by Lin (2016) and given the provisional name '*Ambrosiopsis globosa'* is probably *T. taiwanensis.* The isolate from *S. mikado* (C3448 = CBS 141495) initially resembled the two isolates from *S. pubescens*, but C3448 later grew more slowly (32 –36 mm after 7 days at 25° C with a maximum growth diameter of 63 – 67 mm) and produced a diffusible red-brown pigment that was not observed in the other isolates. The changed culture also lacked fluffy surface growth, instead forming conidiophores in loose tufts across the surface and below the surface of the agar.

AMBROSIELLA Brader ex Arx & Hennebert emend, T.C. Harr., Mycotaxon 111: 354. 2010.

Type species: *Ambrosiella xylebori* Brader ex Arx & Hennebert.

Ambrosiella remansi C. Mayers & T.C. Harr., **sp. nov.** (Figs. 5C, D)

MycoBank MB 824938.

Etymology. After *Remansus,* the genus from which the only known specimen was associated.

Typus. **Madagascar:** Andasibe-Mantadia National Park, propagules in mycangium of female *Remansus mutabilis*, May 2015, *B. Jordal*, holotype (BPI 910622).

Growth in mycangium composed of thallic-arthric cells 4 – 10 × 6 – 13 μm in size, globose to irregular in shape, single or in chains of two or more cells.

Notes. This species was found in the mycangium of *R. mutabilis,* the type species for the genus *Remansus*, which is only known from Madagascar (Jordal 2013). The ITS sequence obtained from this single specimen places it with *Ambrosiella* (Fig. 3), and the multigene phylogeny places the fungus as an early-diverging member of *Ambrosiella*

(Fig. 6). Though its conidial state is unknown, the mycangial growth of *R. remansa* is identical to the non-branched, thallic-arthric mycangial propagules of *Ambrosiella* (Harrington et al. 2014; Mayers et al. 2015, 2017) and unlike the sometimes-branching mycangial growth of *Toshionella.* The rarity of *Remansus* specimens (Jordal 2013) precludes further study at this time, but the novelty of the discovery as the first *Ambrosiella* sp. not associated with a host in the *Xylosandrus* complex warrants the naming of *A. remansi* as a new species.

CATUNICA C. Mayers & T.C. Harr., **gen. nov.**

MycoBank MB 824939.

Etymology. Feminine. An arbitrary construction based on the Latin catena (n), chain; and tunica (n), tunic/membrane.

Perithecial bases globose, black, superficial. *Perithecial necks* long, black. *Ostiolar hyphae* hyaline to brown, divergent. *Asci* not observed. *Ascospores* hyaline, half-moon-shaped, sometimes with a sheath. *Conidiophores* phialidic, hyaline to brown, simple, producing terminal conidia. *Conidia* variable: hyaline to brown-pigmented, ovoid, truncate, thin and smooth-walled to verrucose, becoming dark brown to black, globose, thick-walled, ornamented with conspicuous papillae, in long chains, often enveloped by a brown membranous sheath, detaching in chains, with or without sheath.

Type species: Catunica adiposa (Butler) C. Mayers & T.C. Harr.

The only known species of the genus, *C. adiposa* is a causal agent of sugar cane rot (Butler 1906, Sartoris 1927), has been implicated in human illness (Agarwal et al. 2014), and has been isolated from *Prunus* (Paulin-Mahady et al. 2002), *Pinus* (Talbot

1956), air (e.g. CBS 138.34), books (e.g. IMI 21285), and other substrates. *Catunica* has distinctive variation in conidium morphology ranging from typical barrel-shaped conidia from deep-seated phialides (endoconidia) to thick-walled, dark, papillae-ornamented conidia (aleurioconidia). These conidial types were referred to as phialoconidia and chlamydospores by Nag Raj and Kendrick (1975). We agree with other authors (Butler 1906; Sartoris 1927; Davidson 1935; Moreau 1952; Hunt 1956; Talbot 1956; Uphadhyay 1981) that both types of spores are produced from common conidiophores and change over time. It may produce the longest perithecium necks in the family (Malloch and Blackwell 1993) and also produces distinctive, half-moon-shaped ascospores with gelatinous sheaths. The phylogenetic position of *C. adiposa* is unique and distinct (Fig. 6), and its relationship to *Meredithiela* spp., which are obligate symbionts of ambrosia beetles, is difficult to reconcile.

Catunica adiposa (E.J. Butler) C. Mayers & T.C. Harr., **comb. nov.** (Fig. S1)

MycoBank MB 824940.

Etymology: After the "fatty substance" in which the ascospores are suspended (Butler 1906).

Basionym: Sphaeronema adiposum E.J. Butler, Mem. Dept. Agric. India, Bot. Ser. 1: 40 (1906).

≡ *Ceratostomella adiposa* (E.J. Butler) Sartoris, J. Agric. Res. 35: 585 (1927).

≡ *Ophiostoma adiposum* (E.J. Butler) Nannf., *In* Melin & Nannf., Svenska Skogsv.-Fören. Tidskr. 32: 408 (1934).

≡ *Endoconidiophora adiposa* (E.J. Butler) R.W. Davidson, J. Agric. Res. 50: 802 (1935).

≡ *Ceratocystis adiposa* (E.J. Butler) C. Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22 (1952).

= *Ceratostomella major* J.F.H. Beyma, Zentrabl. Bakteriol., 2. Abt. 91: 348 (1935).

≡ *Ophiostoma majus* (J.F.H. Beyma) Goid., Boll. Staz. Patol. Veg. Roma 15: 158 (1935).

≡ *Ceratocystis major* (J.F.H. Beyma) C. Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22 (1952).

Typus: **India:** Bihar, Champaran, Seeraha, on *Saccharum officinarum,* 30 October 1903, *E.J. Butler* lectotype (HCIO 3531).

Descriptions: Butler (1906); Sartoris (1927); Davidson (1935); Moreau (1952); Hunt (1956); Talbot (1956); Nag Raj and Kendrick (1975); Uphadhyay (1981).

Notes. Butler (1906) did not designate a type specimen when describing *Sphaeronema adiposum*, but he founded Herbarium Cryptogamae Indiae Orientalis (HCIO) in 1905, and into it he deposited a specimen under the name *S. adiposum*, HCIO 3531. This specimen consists of an infected section of *S. officinarum* with red-brown streaks and dark black fungal growth consistent with his 1906 descriptions, and its collection info of 1903 in Bihar matches his report of his first discovery of the fungus (personal communication, HCIO). The specimen is available in HCIO, which is maintained by the Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi, India. As this is the only known specimen to be directly identified by Butler as *S. adiposum,* we have designated it as lectotype for the species. The only specimen previously cited as holotype for *C. adiposa* is IMI 21355, by Nag Raj and

Kendrick (1975). This specimen, which consists of dried cultures isolated from sugarcane in India on an uncertain date and prepared slides, was deposited in 1927 by W. McRae, who was then the Imperial Mycologist and colleague of Butler. A letter by McRae accompanying the specimen, and presumably explaining its origin, is lost (personal communications, CABI and Royal Botanic Gardens, Kew). Another specimen, IMI 21285, includes envelopes labelled “type” and “extype”, but this specimen was never published as type material and was collected in England decades after the species description (personal communications, CABI and Royal Botanic Gardens, Kew). We did not examine the holotype, but six isolates on hand of *C. adiposa* (Table S1) displayed the characters of *C. adiposa* (Fig. S1) as illustrated by Butler (1906) from his specimens, as well as those illustrated by Hunt (1956) and Nag Raj and Kendrick (1975), who both examined IMI 21355. The ITS sequences of the six isolates, from six different countries, were identical (Fig. 3). A draft genome is available for *C. adiposa* CBS 136.34 (Wingfield et al. 2016a).

SOLALOCA T.C. Harr., **gen. nov.**

MycoBank MB 824941.

Etymology. Feminine. An arbitrary construction based on the Latin sola (adj), ‘alone/by oneself’, and locus (n), ‘place/specific location’.

Perithecial bases and necks black. *Ostiolar hyphae* hyaline, convergent. *Asci* not observed. *Ascospores* hyaline, thick-walled, cylindrical to rarely curved. *Conidiophores* not observed.

Type species: Solaloca norvegica (J. Reid & Hausner) T.C. Harr.

Solaloca norvegica was described from seven isolates from galleries of *Ips typographus* in *Picea abies* in Norway in 1974, but the new species was not described until 2010 (Reid et al. 2010). Conidia were not described, and we failed to find conidia in the ex-type strain C3124 (UAMH 9778, =UAMH 11187). Phylogenetically, *Solaloca* is placed within the family Ceratocystidaceae (Fig. 6) but with no obvious relatives. In morphology (perithecia and ascospores) and habitat, it resembles the conifer-inhabiting members of *Endoconidiophora* (Reid et al. 2010), and it shares no obvious characters with ambrosia beetle symbionts.

Solaloca norvegica (J. Reid & Hausner) T.C. Harr., **comb. nov.**

MycoBank MB 824942.

Etymology: After the country of origin, Norway (Reid et al. 2010).

Typus: **Norway:** Ostfold, near Sandem, from gallery of *Ips typographus* in *Picea abies*, September 1973, *J. Reid,* holotype (dried culture and ex-type living culture under same accession, UAMH 11187).

Basionym: Ceratocystis norvegica J. Reid & Hausner, Botany 88: 977 (2010).

Description: See Reid et al. (2010).

Notes. We examined culture UAMH 9778 (a duplicate of the holotype/ex-type UAMH 11187) and found its characters to be consistent with the original description.

Divergence date estimates

The topology of the Ceratocystidaceae tree from the BEAST analysis (Fig. S2) was identical to that of the MrBayes tree (Fig. 6). The estimated crown age for the Ceratocystidaceae at 81.2 Ma was somewhat older than the estimate of 61.9 Ma by van der Nest et al. (2015), who used similar secondary analysis and calibration points but fewer taxa. The clade accommodating all ambrosia fungi in the family (Fig. 7, node 1)

had a mean crown age of 63.2 Ma, older than estimates of the origin of the pronotal disk mycangium at 34.4 Ma (Gohli et al. 2017) and 52 Ma (Pistone et al. 2018). The estimated age of the split between *Wolfgangiella* and *Phialophoropsis* (Fig. 7, node 2) was 31.8 Ma. There was insufficient divergence to obtain a reasonable crown age for *Wolfgangiella*. The mean crown age of *Phialophoropsis* was estimated at 5.3 Ma,

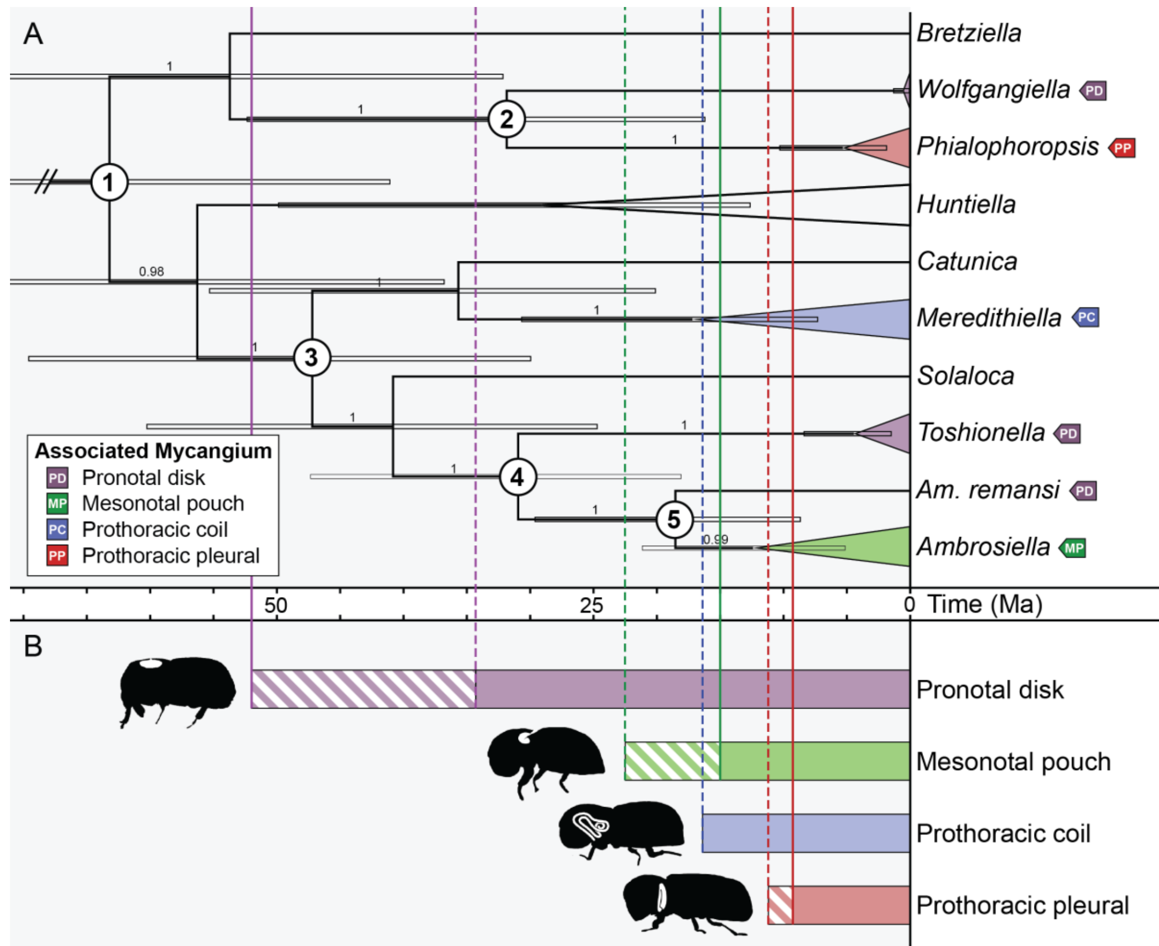

Figure 7. Estimated divergence dates of ambrosia fungi in the Ceratocystidaceae compared to the origins of the mycangia of their associated ambrosia beetle hosts. (A). A portion of the dated phylogenetic tree in Figure S2. Genera with more than one species representative are indicated by triangles, with the point of the triangle at the crown age of the genus. Colored triangles and tags indicate that the clade is associated with the indicated mycangium type in the key. Horizontal node bars indicate 95% HPD (highest posterior density) range. (B). Bars below the tree represent estimated divergence dates for each of the indicated mycangium types. Silhouettes showing the location of each mycangium (white) inside the beetles' bodies (black) are included to the left of each bar. For the paired mycangia (prothoracic coil and prothoracic pleural) only one of the paired mycangia are shown. Dotted vertical lines indicate mean crown divergence estimates by Gohli et al. (2017), solid vertical lines indicate estimates by Pistone et al. (2017). The section of the bar between estimates from the two studies, where both available, is hatched. Horizontal scale is in millions of years (Ma).

younger than the estimated origin of the prothoracic pleural mycangium at 11.2 ma (Gohli et al. 2017) and 9.0 ma (Pistone et al. 2017) (Fig. 7).

The mean estimated age of the common clade containing ambrosia fungi with aleurioconidia (Fig. 7, node 3) was 47.2 Ma, in between the estimates for origin of the pronotal disk mycangium at 34.4 Ma and 52 Ma (Gohli et al. 2017, Pistone et al. 2018). The mean crown age of *Meredithiella* was 17.2 Ma, which closely matched an estimated divergence date of 16.4 Ma for the prothoracic coil mycangium of *Corthylus* (Gohli et al. 2017). The estimated age of the split between *Toshionella* and *Ambrosiella* (Fig. 7, node 4) was 30.9 Ma. The mean crown age of *Ambrosiella,* including *A. remansi* (Fig. 7, node 5)*,* was 18.5 Ma. The mean crown age of *Ambrosiella* species associated with ambrosia beetles in the *Xylosandrus* complex was 12.4 Ma, younger than estimates for the *Xylosandrus* complex at 22.5 Ma (Gohli et al. 2017) and for all of tribe Xyleborini at 15 Ma (Pistone et al. 2017) (Fig. 7).

Discussion

This study is the first to fully characterize the mycangia and mycangial symbionts of the ambrosia beetle tribe Scolytoplatypodini. It is also the most comprehensive molecular phylogeny of ambrosia fungi in the Ceratocystidaceae and the first to compare the evolutionary history and biogeography of ambrosia fungi in the Ceratocystidaceae to the mycangia they inhabit. Two novel genera, *Wolfgangiella* and *Toshionella,* were needed to accommodate the symbionts of *Scolytoplaypus*, bringing the number of symbiotic genera in the Ceratocystidaceae to five: *Ambrosiella, Meredithiella, Toshionella, Phialophoropsis,* and *Wolfgangiella.*

Scolytoplatypodini mycangia and setae

Seven Asian *Scolytoplatypus* species reportedly do not have pronotal mycangium openings (Beaver and Gebhardt 2006), and Bright (1994) noted that in one of these species (*S. reticulatus*) the females instead have a smooth, circular spot in place of the mycangium opening. These species are unusual and should be examined further to determine if they represent a monophyletic group with a single loss of the mycangium, if they utilize alternative methods of transporting their symbionts or carry alternative primary symbionts, and if some vestigial remnant of the mycangium still exists in the pronotum under the smooth circular spot. If confirmed, the absence of mycagia in these species would represent the only known losses of a mycangium in an ambrosia beetle lineage, except for a possible analogous case in a subclade of *Camptocerus* that reverted to phloem feeding (Smith 2013).

All other females of the Scolytoplatypodini share a common mycangium type that may be the oldest among Scolytine ambrosia beetles, perhaps emerging as early as 52 Ma (Pistone et al. 2018). The large, conspicuous, disc-shaped mycangium and associated internal setae were first illustrated by Berger and Cholodkovsky (1916), but Nunberg (1951) was the first to propose that the organ might be used to grow and transmit an ambrosia fungus. The mycangium is filled with fungal propagules, surrounded with abundant secretory gland cells, and lined with interior setae that help guide the propagules to exit through a circular pore on the dorsal pronotum (Schedl 1962; Beaver and Gebhardt 2006). The gross mycangium morphology of the three lineages in the tribe (African *Scolytoplatypus,* Asian *Scolytoplatypus*, and *Remansus*) is similar, though the pore is located more anteriorly in *Remansus* (Jordal 2013) and the mycangia differ in their walls and interior setae. The inner mycangium walls of Asian *Scolytoplatypus*

(Beaver and Gebhardt 2006) and *Remansus* are heavily reticulated, but the mycangium wall of African *Scolytoplatypus* is simpler and composed of sclerotized cuticular plates (Schedl 1962). In African species such as *S. acuminatus* (Schedl 1962), and confirmed by this study in *S. fasciatus, S. permirus*, and *S. rugosus*, the setae are of two types: most are short, thin, and grow directly from the sclerotized mycangium wall, but some of the setae are longer, thicker, and socketed into squat cones in the wall of the mycangium and reach through the mycangia pore. In contrast, Asian species such as *S. shogun* (Schedl 1962, Nakashima et al. 1987), *S. daimio* and *S. mikado,* (Schedl 1962), *S. blandfordi* (Beaver and Gebhardt 2006), and *S. tycon* (Berger and Cholodkovsky 1916) have setae that are socketed on the apices of tall, reticulated cones rising from the reticulated mycangium wall, and the setae reach the mycangial pore. Interestingly, the mycangium of *R. mutabilis* had a third form of mycangial setae that are socketed terminally on hollow cylindrical pedestals, which may be homologous with the squat conical sockets that support the longer setae in African *Scolytoplatypus*. In the *Remansus* mycangium, reticulated finger-like cones also project into the mycangium and appear similar to the reticulated cones of Asian *Scolytoplatypus,* but these cones lack terminal setae in *Remansus*. In both *Remansus* and Asian *Scolytoplatypus,* the reticulated cones appear to contain active gland cells; the cones stain deeply with cotton blue, implying active cytoplasm, and Berger and Cholodkovsky (1916) illustrated cells with nuclei filling the spaces between the reticulations. Alternatively, the cones could serve as channels for glandular secretions from the mass of gland cells outside the mycangium wall (Schedl 1962). In either case, the cones may help penetration of secreted nutrients into the dense mass of fungal propagules in the mycangium. Additionally, the reticulated cones may fill

space in the mycangium so that a smaller fungal mass is required to fill the mycangium and, thus, the spore mass would be more easily squeezed out of the mycangium opening. A simple movement of the beetle's head and thorax muscles push the spore mass out of the opening (Schedl 1962).

The disc-shaped mycangia of the three Scolytoplatypodini lineages clearly have a single evolutionary origin. The similarly of mycangium walls and the highly developed reticulated cones in *Remansus* and Asian *Scolytoplatypus*, and their closely-related symbionts (*Ambrosiella* and *Toshionella*), might be explained by African *Scolytoplatypus* being a sister group to the other two lineages as implied by K. Schedl (1975). However, phylogenetic analyses (Jordal 2013, 2018; Gohli et al. 2017; Pistone et al. 2018) place *Remansus* sister to a monophyletic *Scolytoplatypus*. Under this assumption, Asian *Scolytoplatypus* and *Remansus* either: (1) independently evolved the complex reticulation of their mycangium walls and their tall, reticulated cones; or (2) the African lineage of *Scolytoplatypus* underwent a reduction in complexity of the mycangium wall and lost the reticulated cone structures. Further, the *Toshionella* and *Ambrosiella* symbionts associated with the mycangia with reticulated cones are closely related and form large, lipid-filled aleurioconidia and conidiophore cells, which are believed to be superior adaptations for insect grazing (Harrington et al. 2014; Mayers et al. 2015). In contrast, the *Wolfgangiella* ambrosia growth in the galleries of African *Scolytoplatypus* is less luxuriant and provides smaller propagules for insenct grazing. Species of the African lineage of *Scolytoplatypus* are the only extant members of Scolytoplatypodini in the purported Afro-tropical ancestral origin of the tribe (Pistone et al. 2017), while the Asian species are strictly Asian and *Remansus* is known only from Madagascar. Thus, the

relatedness of the fungal symbionts of *Remansus* and Asian *Scolytoplatypus*, the derived nature of their ambrosia growth and mycangia, and their proposed migrations out of Africa appear to be in conflict with phylogenetic analyses of the tribe.

Mycangium morphology is often underappreciated in taxonomic treatments of ambrosia beetles. The mycangium setae in *Scolytoplatypus* are one of several clear morphological traits separating the Asian and African clades (Schedl 1975; Beaver and Gebhardt 2006; Jordal 2013) and support a separate genus for the Asian clade (the African *S. permirus* is the type species of *Scolytoplatypus*). The subgenera *Spongocerus* and *Taeniocerus* were previously proposed for Japanese *Scolytoplatypus* spp. and are available, but they were not generally accepted (Beaver and Gebhardt 2006) and were synonymized with *Scolytoplatypus* by Wood (1983).

Fungal farming represents a major evolutionary feature in the Scolytinae (Gohli et al. 2017), and mycangia play an essential role in the upkeep, survival, and transmission of vital symbiotic fungi, which are themselves likely under appreciable evolutionary pressure. Mycangium-associated gland cells secrete nutrients that support the active growth of mycangium propagules (Schneider 1975), and mycangia represent a significant energy investment for the long-term maintenance of domesticated fungal cultivars. Reticulated mycangium walls such as those in some Scolytoplatypodini are a common feature of large and complex mycangia that harbor Ceratocystidaceae. Reticulated walls have been observed in the mycangia of *Trypodendron* (Francke-Grosmann 1956; Schneider and Rudinsky 1969) and *Corthylus* (Giese 1967; Mayers et al. 2018), and in *Cnestus mutilatus* the spaces between the reticulations of the mesonotal mycangium are filled with glandular cells (Stone et al. 2007). In *Trypodendron* the mycangium is

surrounded by gland cells that presumably secrete through the openings of the reticulated walls (Schneider and Rudinsky 1969). Thus far, all genera of ambrosia beetles with reticulated mycangial walls have been associated with symbionts in the Ceratocystidaceae.

Evolution of ambrosia fungi in the Ceratocystidaceae

Vanderpool et al. (2017) proposed that *Raffaelea* (Ophiostomatales) symbionts were first domesticated by the Platypodid tribe Tesserocerini at about 86 Ma, and *Raffaelea* spp. were then horizontally acquired by multiple ambrosia beetle lineages in the Scolytinae beginning around 48–60 Ma. These Scolytid beetles presumably developed mycangia from nooks and crannies of the beetles' exoskeleton that collected fungal spores, especially in parts of the body near secretory glands (such as those providing lubrication for moveable body parts) that could produce nutrients for the fungi to grow in the mycangia (Francke-Grosmann 1967; Schneider 1975). New Scolytid fungus-farming lineages and novel mycangia were likely facilitated by the availability of *Raffaelea* cultivars, and their mycangia could have led to the domestication of other superior ambrosia fungi, such as those in the Ceratocystidaceae.

Species of *Raffaelea* generally freely associate with different types of mycangia in unrelated ambrosia beetle species in different tribes or subfamilies (Harrington et al. 2010, 2014; Vanderpool et al. 2017; Skelton et al. 2018), and there can be several *Raffaelea* spp. within the mycangia of a single beetle (Harrington and Fraedrich 2010; Harrington et al. 2011). This does not appear to be the case for Ceratocystidaceae symbionts, which co-evolved with the mycangia of their ambrosia beetle farmers. The fungus-host beetle relationships in *Ambrosiella* were hypothesized to be species-specific (Mayers et al. 2015), but this is probably not strictly the case (Lin et al. 2017; Mayers et

al. 2018). Instead, the genera of Ceratocystidaceae associated with ambrosia beetles appear to be associated with specific mycangium types, but species can be traded among beetle species with the same mycangium type. Also, specific ambrosia beetles are not necessarily associated with particular species of ambrosia fungi, since *S. mikado* carries *T. nipponensis* in Japan but *T. taiwanensis* in Taiwan. As pointed out by Bateman et al. (2015), Hulcr et al. (2017), and Skelton et al. (2018), most studies have been with introduced populations of Xyleborini, and the introduced populations appear to have specific mycangial symbionts. However, the sampled species in our study were all from their native ranges, and species-specific associations were less apparent.

Several points of evidence suggest that there were multiple origins of ambrosia beetle symbiosis in the Ceratocystidaceae. The Scolytoplatypodini must have acquired Ceratocystidaceae in at least two separate evolutionary events, and other ambrosial genera in the family may or may not have been novel domestications.

Phylogenetic analyses show that the ambrosial genera are interspersed with non-ambrosial genera *Huntiella, Bretziella, Catunica,* and *Solaloca*. These taxa include wound colonizers (*Huntiella*), plant pathogens (*B. fagacearum* and *C. adiposa*), and a bark beetle associate with an unknown ecological role (*S. norvegica*) (Reid et al. 2010; de Beer et al. 2014). The five ambrosial genera share highly-derived and apparently convergent traits conducive to their role as obligate ambrosia beetle symbionts, including large, lipid-filled conidia that are sensitive to desiccation, easily-fragmented conidiophores, dense ambrosia growth in galleries, and dimorphic mycangial forms. Multiple reversions from such derived traits back to a free-living lifestyle would appear highly unlikely (Farrell et al. 2001), and such reversions have not been observed in

Raffaelea (Vanderpool et al. 2017). The non-ambrosial taxa all form the long-necked perithecia and stick ascospore masses that are found throughout the family, which facilitate dispersal on insect exoskeletons (Malloch and Blackwell 1993). The two ambrosial genera with known sexual states (*Ambrosiella* and *Wolfgangiella*) are unique in forming cleistothecious ascomata without sticky ascospore drops. Rather than four reversions from cleistothecia back to necked perithecia, it seems more likely that the common ambrosial ancestor had long-necked perithecia and the cleistothecious ascomata arose convergently in the absence of an evolutionary pressure on cuticular dispersal of ascospores.

There are also significant morphological differences among the ambrosial genera, most notably the presence of aleurioconidia in the ambrosia growth of *Toshionella, Ambrosiella,* and *Meredithiella*, whereas *Phialophoropsis* and *Wolfgangiella* lack aleurioconidia (as does their nearest neighbor, the non-ambrosial *B. fagacearum*). *Phialophoropsis* produces relatively unremarkable phialoconidia and *Wolfgangiella* only produces fragmenting arthroconidia, neither of which were observed to contain conspicuous lipid bodies. The ascospores of *Ambrosiella* and *Wolfgangiella*, despite being produced in similarly cleistothecious ascomata, also differ in that the *Wolfgangiella* ascospores are much larger and more curved. Thus, as does the phyplogenetic data, morphology suggests at least two origins for the symbiosis.

Pistone et al. (2017) estimated an Afrotropical origin for tribe Scolytoplatypodini. If an *Ambrosiella/Toshionella*-like symbiont was the first ambrosia fungus cultivated by the tribe at the origin of the mycangium at 30–50 Ma, the descendants of that fungal symbiont may have accompanied *Remansus* to Madagascar (Jordal 2013) and the Asian

Scolytoplatypus to Asia. However, it is difficult to explain how this hypothetical early fungus symbiont was then replaced in mainland Africa by *Wolfgangiella* symbionts, which appear to produce inferior ambrosia growth with smaller conidiophores and lipid bodies when compared to *Ambrosiella* and *Toshionella*. Perhaps the common Scolytoplatypodini ancestor had a poorly-developed pronotal mycangium and carried a mixed assemblage of fungi, and lineage sorting in the beetle tribe resulted in a different fungal lineage to co-adapt with each geographic *Scolytoplatypus* lineage. Morphology suggests that the African *Scolytoplatypus* mycangium is the least developed in its lack of wall reticulation and poorly developed conical structures, and it may not have been as highly selective for fungal symbionts in its earlier evolutionary history. However, only three African *Scolytoplatypus* spp. were sampled in Madagascar and South Africa, and more extensive surveys may find a wider diversity of symbionts in African Scolytoplatypodini.

Incongruence between fungal divergence dates and mycangium origins also suggest that there was more than one origin of the symbiosis in the family Ceratocystidaceae. Although there were significant 95% HPD ranges in both our analysis and the estimates for origins of mycangium types (Gohli et al. 2017; Pistone et al. 2017), the mean crown of the ambrosial common ancestor at 63.2 Ma is much older than the Gohli et al. (2017) estimate for the crown of tribe Scolytoplatypodini at 34.3 Ma and somewhat older than the Pistone et al. (2018) estimate at 52 Ma. Accepting these dates, a single origin of ambrosia symbiosis in the fungal family would have predated the origin of the first large Scolytine mycangium.

Ambrosiella* and *Toshionella

Toshionella and *Ambrosiella* are closely related and likely derived from a single domestication, perhaps at 31 to 47 Ma based on our phylogenetic analyses. The genera have similar condiophores and they form similar aleurioconidia with large lipid bodies, which may be particularly nutritious for grazing beetles (Harrington et al. 2014). *Toshionella* are carried by Asian *Scolytoplatypus,* but *Remansus* (represented in this study only by *R. mutabilis*) is found only in Madagascar (Jordal 2013) and carries a symbiont aligned with *Ambrosiella sensu stricto*. *Ambrosiella* have otherwise been found associated only with species of Xyleborini with mesonotal mycangia, i.e., the *Xylosandrus* complex (Lin et al. 2017; Mayers et al. 2015, 2017). With *A. remansi* as the sole representative of extant *Remansus* symbionts, it could be speculated that the split between the *Remansus* symbionts (*Ambrosiella*) and the Asian *Scolytoplatypus* symbionts (*Toshionella*) was at 31 Ma, which would have followed the estimated *Remansus*/*Scolytoplatypus* split using either the estimates of Pistone et al. (2018) or Gohli et al. (2017). The origin of the mesonotal mycangium early in the evolution of the Xyleborini may have corresponded with the acquisition of *Ambrosiella* from *Remansus*. The Gohli et al. (2017) estimate for the divergence of the *Xylosandrus* complex (and therefore the mesonotal mycangium) is at 22.5 Ma. However, Pistone et al. (2017) estimated the origin of the entire tribe Xyleborini at 15 Ma, and the crown age of the *Xylosandrus* complex would have to be slightly younger than 15 Ma (Cognato et al. 2011, Gohli et al. 2017), which predates our estimated crown age of *Ambrosiella* symbionts of the Xyleborini at 12.4 Ma. The origin of tribe Xyleborini has been proposed to be Afro-Asian (Cognato et al. 2011), and perhaps the *Xylosandrus* complex and its mesonotal mycangium originated in Africa. A single transfer of *Ambrosiella* to the

Xyleborini may have prompted the diversification of the monophyletic *Xylosandrus* complex (Hulcr and Cognato 2010; Hulcr and Stelinski 2017) and their *Ambrosiella* symbionts (Mayers et al. 2015). It is possible that as more *Scolytoplatypus* symbionts are discovered, it will become apparent that *Ambrosiella* and *Toshionella* are part of a spectrum of symbionts rather than two clearly-distinct groups. However, the studied species of *Toshionella* produce much larger, lipoid cells in the ambrosia layer than do *Ambrosiella* spp.

Meredithiella

The clade containing the ambrosia fungi with aleurioconidia (*Meredithiella, Ambrosiella,* and *Toshionella*) has an estimated crown divergence date of 47 M. *Corthylus* could have acquired an early Scolytoplatypodid symbiont, and *Meredithiella* has aleurioconidia similar to those found in *Ambrosiella* and *Toshionella* (Mayers et al. 2018). However, this clade contains two non-ambrosial taxa (*C. adiposa* and *S. norvegica*), and a single domestication for *Meredithiella* and *Toshionella/Ambrosiella* is problematic because *Meredithiella* is associated with the New World genus *Corthylus* (Mayers et al. 2018), and the Asian/African Scolytoplatypodini are not thought to have ever been sympatric with *Corthylus*. Further, the estimated crown divergence of *Meredithiella* and *Ambrosiella* at 47 Ma would have been post-Gondwanan. The estimated ages of *Mereditiella* and *Corthylus* were quite similar, and a independent origin for this symbiosis seems likely (Mayers et al. 2015).

It is not clear if other members of the Corthylina have mycangia homologous to those of *Corthylus*. *Amphicranus* is sister to *Corthylus* (Pistone et al. 2017), but neither the symbionts nor mycangia of *Amphicranus* are known. *Microcorthylus* may have mycangia similar to *Corthylus* (Schedl 1962), and a species of *Geosmithia* was observed

in galleries of an unnamed *Microcorthylus* sp. (Kolařík and Kirkendall 2010), but its mycangial symbionts have not been confirmed.

Wolfgangiella* and *Phialophoropsis

Two genera of ambrosia fungi in the Ceratocystidaceae lack aleurioconidia and appear distantly related to other ambrosia beetle symbionts in the family and to each other. The split of *Phialophoropsis* and *Wolfgangiella* was estimated at 32 Ma, but both taxa were placed on long branches. *Trypodendron* has a prothoracic pleural mycangium, and its primary symbionts are *Phialophoropsis* spp. (Mayers et al. 2015), which show limited diversity and an estimated crown age of only 5.3 Ma. Both the Gohli et al. (2017) estimate of 11.2 Ma and the Pistone et al. (2018) estimate of 9.0 Ma for the crown of *Trypodendron* spp. predate the crown age of *Phialophoropsis*, but the tribe Xyloterini is much older.

Wolfgangiella and *Phialophoropsis* are only distantly related to each other and their sister taxon *Bretziella fagacearum,* and the three genera have no known relatives. Each of the genera show very little genetic diversity and share few significant common morphological characters, other than the absence of aleurioconidia. *Wolfgangiella* and *Phialophoropsis* may represent two separate and relatively recent ambrosia domestications. The limited sampling to date indicates that only *Trypodendron* carry *Phialophoropsis* symbionts (Mayers et al. 2015), and *Phialophoropsis* shows limited genetic diversity. *Wolfgangiella* spp. associated with *Scolytoplatypus* on the west coast of South Africa and in Madagascar barely differed in DNA sequence. This may be because the *Scolytoplatypus* in Madagascar came from Africa as recently as 7 Ma (Jordal 2013), and the fungal symbionts of *Scolytoplatypus* in central and northern Africa may be more diverse.

Diffuse coevolution between mycangia and Ceratocystidaceae fungi

The association of large and complex mycangium types with specific fungal genera is likely the result of co-adaptations, but species-level associations appear to have followed a pattern of diffuse co-evolution. A single common origin of a mycangium type would have been followed by progressive adaptations in their carried fungi, perhaps with a succession of early cultivars replaced with better alternatives and horizontal transfer among beetle species with the same mycangium type. Fungal adaptations for superior ambrosia growth may have been matched by reciprocal adaptations in the mycangium, leading to the tightly linked fungal lineage-mycangium associations observed today. Under this scenario, the crown age of these mycangia should closely match or predate the crown age of their fungal cultivars. This appears to have been the case with the *Xylosandrus* complex and its *Ambrosiella* symbionts, *Corthylus* and its *Meredithiella* symbionts, and *Trypodendron* with its *Phialophoropsis* symbionts. Species-level coevolution is not apparent, but a more diffuse coevolution is seen within *Ambrosiella* (Lin et al. 2017), *Meredithiella* (Mayers et al. 2018), *Phialophoropsis* (Chapter 6), *Toshionella* and *Wolfgangiella*, which appear to be delineated by geography more than species of host beetle.

The Scolytoplatypodini may have been the first to acquire Ceratocystidaceae as fungal cultivars, but taxon sampling remains limited for this tribe. African *Scolytoplatypus* were only studied in southern Africa and Madagascar, but related species are found in central and western Africa (Browne 1971; Schedl 1975; Jordal 2013). The African *S. congonus* and *S. unipilus* represent interesting phylogenetic intermediaries between the studied African/Malagasy and Asian species of the genus (Jordal 2013, 2018). Only one species of *Remansus* was available for study, and the fungal symbionts

of Asian *Scolytoplatypus* were only identified from eastern Asia, though they are found in South Asia as well (Beaver and Browne 1975; Schedl 1975; Beaver and Gebhardt 2006). Study of extant species, such as *S. kunala* (Mandelshtam and Petrov 2010), along the purported migration route out of Africa would help confirm whether or not the *Scolytoplatypus*-*Wolfgangiella* and *Scolytoplatypus-Toshionella* associations show evolutionary fidelity or are merely geographically delineated.

This study serves as a first look at Scolytoplatypodini symbionts and a foundation for greater examination of the most specialized symbioses among ambrosia beetles. Symbionts for the three genera in tribe Xyloterini also need to be studied to determine whether or not the tribe may represent an alternative origin for the Ceratocystidaceae associated with the Scolytoplatypodini, and relatives of *Corthylus* such as *Amphicranus* need to be studied to ascertain if other Corthylini have Ceratocystidaceae symbionts.

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Tables

Table 1. Collection information for selected studied fungal material, including ITS accession numbers.

Ambrosia fungus	Associated ambrosia beetle	Specimen Type	Specimen ID (Collection ID)[1]	Location	Year collected	Collected by	ITS GenBank
Wolfgangiella							
W. franznegeri	*Scolytoplatypus fasciatus*	Propagules in mycangium of female in unidentified tree	sp2	Diepwalle Forest Station, Western Cape, South Africa	November 2007	B. Jordal	same as MG950180
W. franznegeri	*S. fasciatus*	Culture from female (ethanol trap)	C4325	Near Betty's Bay, Western Cape, South Africa	January 2017	F. Roets	same as MG950180
W. franznegeri	*S. fasciatus*	Culture from female (ethanol trap)	C4326	Near Betty's Bay, Western Cape, South Africa	January 2017	F. Roets	same as MG950180
W. franznegeri	*S. fasciatus*	Culture from female (ethanol trap)	C4327	Near Betty's Bay, Western Cape, South Africa	January 2017	F. Roets	same as MG950180
W. franznegeri	*S. fasciatus*	Culture from female (ethanol trap)	C4328 (CBS 144149) **eHT**	Near Betty's Bay, Western Cape, South Africa	January 2017	F. Roets	MG950180
W. franznegeri	*S. fasciatus*	Culture from female (ethanol trap)	C4329	Near Betty's Bay, Western Cape, South Africa	January 2017	F. Roets	same as MG950180
W. franznegeri	*S. fasciatus*	Culture from female (ethanol trap)	C4331	Near Betty's Bay, Western Cape, South Africa	January 2017	F. Roets	same as MG950180
W. franznegeri	*S. rugosus*	Ambrosia growth in *Ocotea* sp.	M288 (BPI 910640)	Andasibe-Mantadia NP, Madagascar	May 2015	B. Jordal	KX342063
W. madagascarensis	*S. permirus*	Ambrosia growth in unidentified tree	M286 (BPI 910641) **HT**	Ambohitantely FR, Madagascar	May 2015	B. Jordal	KX342062
W. madagascarensis	*S. permirus*	Ambrosia growth in unidentified tree	M399	Andasibe-Mantadia NP, Madagascar	May 2015	B. Jordal	same as KX342062
W. madagascarensis	*S. permirus*	Propagules in mycangium of female (ethanol trap)	sp3	Ranomafana NP, Madagascar	2012	B. Jordal	KX342061
Toshionella							
T. nipponensis	*S. shogun*	Culture from female (on unidentified tree)	C3904 (CBS 141492) **eHT**	Tazawako, Akita prefecture, Japan	July 2014	H. Masuya	KX342064
T. nipponensis	*S. shogun*	Culture from female (on unidentified tree)	C3905	Tazawako, Akita prefecture, Japan	July 2014	H. Masuya	same as KX342064
T. nipponensis	*S. shogun*	Culture from female (on unidentified tree)	C3906	Tazawako, Akita prefecture, Japan	July 2014	H. Masuya	same as KX342064
T. nipponensis	*S. shogun*	Culture from female (on unidentified tree)	C4064	Hachimantai, Iwate prefecture, Japan	July 2014	H. Masuya	same as KX342064
T. transmara	*S. shogun*	Culture from female (on unidentified tree)	C3908 (CBS 141493) **eHT**	Hachimantai, Iwate prefecture, Japan	June 2015	H. Masuya	KX342065

Table 1 continued

T. transmara	*S. tycon*	Propagules in mycangium of female (ethanol trap)	sp1	Vladivostok, Russia	July 2008	B. Jordal	KX342066
T. taiwanensis	*S. pubescens*	Culture from female (ethanol trap)	C3687 (CBS 141494) **eHT**	Douna, Kaohsiung, Taiwan	February 2014	H. Shih	KX342067
T. taiwanensis	*S. pubescens*	Culture from female (ethanol trap)	C3688	Jingdashan, Kaohsiung, Taiwan	February 2014	H. Shih	same as KX342066
T. taiwanensis	*S. mikado*	Culture from female (ethanol trap)	C3448 (CBS 141495)	Lienhuachih, Taiwan	August 2014	C. Wuest	same as KX342066
T. taiwanensis	*S. mikado*	Propagules in mycangium of female (ethanol trap)	M304	Lienhuachih, Taiwan	June 2013	H. Shih	same as KX342066
T. taiwanensis	*S. mikado*	Propagules in mycangium of female (in *Cinnamomum* sp.)	M305	Lienhuachih, Taiwan	June 2013	H. Shih	same as KX342066
Ambrosiella							
Ambrosiella aff. *beaveri* NRbea1	*Xylosandrus germanus*	Culture from female (in *Lindera triloba*)	C4059	Aichi, Toyota, Inabu, Japan	July 2014	H. Kajimura	same as MG950182
Ambrosiella aff. *beaveri* NRbea1	*X. brevis*	Culture from female (in *Lindera triloba*)	C4060	Aichi, Toyota, Inabu, Japan	July 2014	H. Kajimura	MG950181
Ambrosiella aff. *beaveri* NRbea1	*X. brevis*	Culture from female (in *Lindera triloba*)	C4061 (CBS 142650)	Aichi, Toyota, Inabu, Japan	July 2015	H. Kajimura	MG950182
A. catenulata	*Anisandrus apicalis*	Culture from female	C3909	Morioka, Iwate Prefecture, Japan	April 2015	H. Masuya	same as MG950184
A. catenulata	*An. apicalis*	Culture from female	C3910	Morioka, Iwate Prefecture, Japan	April 2015	H. Masuya	same as MG950184
A. catenulata	*An. apicalis*	Culture from female	C3911	Morioka, Iwate Prefecture, Japan	April 2015	H. Masuya	same as MG950184
A. catenulata	*An. apicalis*	Culture from female	C3912	Morioka, Iwate Prefecture, Japan	April 2015	H. Masuya	same as MG950184
A. catenulata	*An. apicalis*	Culture from female	C3913 (CBS 142649)	Morioka, Iwate Prefecture, Japan	April 2015	H. Masuya	MG950184
A. grosmanniae	*X. germanus*	Culture from female	C3901	Hachimantai, Iwate prefecture, Japan	June 2015	H. Masuya	same as KR611324
A. grosmanniae	*X. germanus*	Culture from female	C3902	Hachimantai, Iwate prefecture, Japan	June 2015	H. Masuya	same as KR611324
A. grosmanniae	*X. germanus*	Culture from female	C3903	Hachimantai, Iwate prefecture, Japan	June 2015	H. Masuya	same as KR611324
A. grosmanniae	*X. germanus*	Culture from female (in *Lindera triloba*)	C4058	Aichi, Toyota, Inabu, Japan	July 2010	H. Kajimura	MG950185
Ambrosiella aff. *grosmanniae* NRgro1	*Xylosandrus* aff. *germanus*	Culture from female	C3898	Iwate Prefecture, Morioka, Japan	April 2015	H. Masuya	same as MG950186
Ambrosiella aff. *grosmanniae* NRgro1	*Xylosandrus* aff. *germanus*	Culture from female	C3899 (CBS 142648)	Iwate Prefecture, Morioka, Japan	April 2015	H. Masuya	MG950186
Ambrosiella aff. *grosmanniae* NRgro1	*Xylosandrus* aff. *germanus*	Culture from female	C3900	Iwate Prefecture, Morioka, Japan	April 2015	H. Masuya	same as MG950186
A. remansi	*Remansus mutabilis*	Propagules in mycangium of female in unidentified tree	M290 (BPI 910622) **HT**	Andasibe-Mantadia NP, Madagascar	May 2015	B. Jordal	KX342068

Table 1 continued

A. roeperi	*X. crassiusculus*	Culture from female (ethanol trap)	C3449	Fushan, Taiwan	July 2014	H. Shih	MG950187
A. roeperi	*X. crassiusculus*	Culture from female (in *Quercus crispula*)	C4062	Aichi, Toyota, Inabu, Japan	September 2013	H. Kajimura	same as KF669871
A. roeperi	*X. crassiusculus*	Culture from female (in *Quercus crispula*)	C4063	Aichi, Toyota, Inabu, Japan	September 2013	H. Kajimura	MG950183

[1]Includes isolate or specimen numbers from the culture collection of T.C. Harrington, Iowa State University (C or M), Westerdijk Fungal Biodiversity Institute (CBS), and U.S. National Fungus Collections (BPI). Holotype specimens indicated by bold 'HT'; ex-holotype cultures indicated by bold 'eHT'.

Supplemental Tables

Table S1. GenBank accesions for other ITS sequences used in parsimony analyses.

Fungal species and authority	Specimen ID (Collection ID)	Location	ITS GenBank	Source
Ambrosiella batrae C. Mayers, McNew & T.C. Harr.	C3130 (CBS 139735)	USA	KR611322	Mayers et al. (2015)
Ambrosiella beaveri Six, de Beer & W.D. Stone	C2749 (CBS 121750)	USA	KF669875	Harrington et al. (2014)
Ambrosiella catenulata Y.T. Lin & H.H. Shih	W186g (CBS 142152)	Taiwan	LC175301	Lin et al. (2017)
Ambrosiella cleistominuta C. Mayers & T.C. Harr.	C3843 (CBS 141682)	USA	KX909940	Mayers et al. (2016)
Ambrosiella grosmanniae C. Mayers, McNew & T.C. Harr.	C3151 (CBS 137359)	USA	KR611324	Mayers et al. (2015)
Ambrosiella hartigii L.R. Batra	C1573 (CBS 404.82)	Germany	KF669873	Harrington et al. (2014)
Ambrosiella nakashimae McNew, C. Mayers & T.C. Harr.	C3445 (CBS 139739)	USA	KR611323	Mayers et al. (2015)
Ambrosiella roeperi T.C. Harr. & McNew	C2448 (CBS 135864)	USA	KF669871	Harrington et al. (2014)
Ambrosiella xylebori Brader ex Arx & Hennebert	C3051 (CBS 110.61)	Ivory Coast	KF669874	Harrington et al. (2014)
Catunica adiposa (E.J. Butler) C. Mayers & T.C. Harr.	C299	USA	same as DQ318195	This study
Catunica adiposa	C871 (CBS 600.74)	Japan	same as DQ318195	This study
Catunica adiposa	C905 (CBS 147.53)	France	same as DQ318195	This study
Catunica adiposa	C906 (CBS 138.34)	Netherlands	DQ318195	Reid et al. (2010)
Catunica adiposa	C997 (CBS 127.27)	India	same as DQ318195	This study
Catunica adiposa	C999 (CBS 183.86)	Canada	same as DQ318195	Mayers et al. (2015)
Bretziella fagacearum (Bretz) Z.W.deBeer, Marinc., T.A. Duong & M.J.Wingf.	C927 (CBS 129242)	USA	same as KC305152	Mayers et al. (2015)
Meredithiella norrisii McNew, C. Mayers & T.C. Harr.	C3152 (CBS 139737)	USA	KR611326	Mayers et al. (2015)
Phialophoropsis trypodendri L.R. Batra	SUTT	USA	KR611329	Mayers et al. (2015)
Phialophoropsis aff. *ferruginea* NRfer1	C3550 (CBS 408.68)	USA	KC305145	Hamelin et al. (Unpublished)
Phialophoropsis aff. *ferruginea* NRfer1	C3386 (CBS 141683)	USA	MF399190	Mayers et al. (Unpublished)
Phialophoropsis aff. *ferruginea* NRfer2	C2230 (CBS 460.82)	Germany	KC305146	Hamelin et al. (Unpublished)
Phialophoropsis aff. *ferruginea* NRfer2	C3828 (CBS 141685)	Germany	MF399188	Mayers et al. (Unpublished)
Phialophoropsis ferruginea (Math.-Käärik) T.C. Harr.	C3549 (CBS 223.55, MUCL 9940)	Sweden	MF399187	Mayers et al. (Unpublished)
Solaloca norvegica (J. Reid & Hausner) T.C. Harr.	C3124 (UAMH 9778)	Norway	DQ318194	Reid et al. (2010)

Table S2. GenBank accession numbers for sequences used in multi-locus analyses.

Species	Strain/specimen and Collection IDs[1, 2]	Country	Associated ambrosia beetle	GenBank Accession No.[3] 28S rDNA	18S rDNA	*tef*1-α	*tub*	*mcm7*	*rpl1*
***Wolfgangiella* C. Mayers & T.C. Harr.**									
W. franznegeri C. Mayers, T.C. Harr., & F. Roets	C4328 (CBS 144149) **eHT**	South Africa	*Scolytoplatypus fasciatus*	MG269974	MG950188	MG944393	MG269951	MG270167	MG272461
W. madagascarensis C. Mayers, McNew, & T.C. Harr.	M286 (BPI 910641) **HT**	Madagascar	*Scolytoplatypus permirus*	MG269975	KX342069	KX354417	MG269950	MG270168	MG272462
***Toshionella* C. Mayers & T.C. Harr.**									
T. nipponensis C. Mayers, T.C. Harr., & H. Masuya	C3904 (CBS 141492) **eHT**	Japan	*Scolytoplatypus shogun*	MG269978	KX342070	KX354420	MG269942	MG270160	MG272454
T. transmara C. Mayers, T.C. Harr., & H. Masuya	C3908 (CBS 141493) **eHT**	Japan	*Scolytoplatypus shogun*	MG269979	KX342071	KX354422	MG269943	MG270159	MG272453
T. taiwanensis C. Mayers, T.C. Harr., & H.H. Shih	C3687 (CBS 141494) **eHT**	Taiwan	*Scolytoplatypus pubescens*	MG269980	=KX342071	KX354425	MG269940	MG270161	MG272451
T. taiwanensis	C3448 (CBS 141495)	Taiwan	*Scolytoplatypus mikado*	MG269981	=KX342071	KX354423	MG269941	—	MG272452
***Ambrosiella* Brader ex Arx & Hennebert emend, T.C. Harr.**									
A. batrae C. Mayers, McNew & T.C. Harr.	C3130 (CBS 139735) **eHT**	USA	*Anisandrus sayi*	KY744584	KR673881	KT290320	MG269932	MG270152	MG272445
A. beaveri Six, de Beer & W.D. Stone	C2749 (CBS 121750) **ePT**	USA	*Cnestus mutilatus*	KF646765	KR673882	KT318380	MG269938	MG270156	MG272448
A. catenulata Y.T. Lin & H.H. Shih	C3913 (CBS 142649)	Japan	*Anisandrus apicalis*	MG269982	MG950189	MG944394	MG269937	MG270154	MG272446
A. cleistominuta C. Mayers & T.C. Harr.	C3843 (CBS 141682) **eHT**	USA	*Anisandrus maiche*	KY744585	KX925304	KX925309	MG269936	MG270153	MG272443
A. nakashimae McNew, C. Mayers & T.C. Harr.	C3445 (CBS 139739) **eHT**	USA	*Xylosandrus amputatus*	KY744586	KR673883	KT318381	MG269939	MG270158	MG272450
A. grosmanniae C. Mayers, McNew & T.C. Harr.	C3151 (CBS 137359) **eHT**	USA	*Xylosandrus germanus*	KY744587	KR673884	KT318382	MG269933	MG270150	MG272444
A. hartigii L.R. Batra	C1573 (CBS 404.82)	Germany	*Anisandrus dispar*	KY744588	KR673885	KT318383	MG269931	MG270157	MG272442
A. remansi C. Mayers & T.C. Harr.	M290 (BPI 910622) **HT**	Madagascar	*Remansus mutabilis*	—	KX342072	KX354426	—	—	—
A. roeperi T.C. Harr. & McNew	C2448 (CBS 135864) **eHT**	USA	*Xylosandrus crassiusculus*	KF646767	KR673886	KT318384	MG269935	MG270151	MG272449
A. xylebori Brader ex Arx & Hennebert	C3051 (CBS 110.61) **eIT**	Ivory Coast	*Xylosandrus compactus*	KM495318	KR673887	KT318385	MG269930	KM495407	KM495495
A. aff *grosmanniae* NRgro1	C3899 (CBS 142648)	Japan	*Xylosandrus* aff. *germanus*	MG269983	MG950190	MG944395	MG269934	MG270155	MG272447

Table S2 continued

***Phialophoropsis* L.R. Batra emend. T.C. Harr.**									
P. ferruginea (Math.-Käärik) T.C. Harr.	C3549 (CBS 223.55) **eHT**	Sweden	*Trypodendron lineatum*	MF399166	MF398168	MF375458	MG269947	MG270166	MG272458
Phialophoropsis sp. NRfer1	C3828 (CBS 141685)	Germany	*Trypodendron domesticum*	MF399167	MF398169	MF375459	MG269948	MG270164	MG272460
Phialophoropsis sp. NRfer2	C3386 (CBS 141683)	USA	*Trypodendron retusum*	MF399169	MF398171	MF375461	MG269949	MG270165	MG272459
***Meredithiella* McNew, C. Mayers & T.C. Harr.**									
M. norrisii McNew, C. Mayers & T.C. Harr.	C3152 (CBS 139737) **eHT**	USA	*Corthylus punctatissimus*	KY744589	KR673888	KT318386	MG269944	MG270162	MG272456
M. fracta C. Mayers, C. Bateman & T.C. Harr.	C4171 (CBS 142645) **eHT**	USA	*Corthylus papulans*	KY744590	KY744594	KY773179	MG269945	MG270163	MG272457
M. guianensis C. Mayers, C. Bateman & T.C. Harr.	M552 (BPI 910532) **HT**	French Guiana	*Corthylus crassus*	KY744223	KY744227	KY773180	MG269946	—	—
Other *Ceratocystidaceae*									
Berkeleyomyces basicola (Berk. & Broome) W.J. Nel, Z.W. de Beer, T.A. Duong, & M.J. Wingf.	C1372 (CBS 414.52, MUCL 8363)	Netherlands	—	AF222458	KX925307	HM569628	MG269963	—	—
	CMW7068 (CBS 413.52)	Netherlands	—	—	—	—	—	KM495484	KM495574
Bretziella.fagacearum (Bretz) Z.W.deBeer, Marinc., T.A.Duong & M.J.Wingf.	C927 (CBS 129242)	USA	—	=AF222483	KR673892	KT318389	MG269953	MG270170	MG953416
Catunica adiposa (E.J. Butler) C. Mayers & T.C. Harr.	C999 (CBS 183.86)	Canada	—	=KM495320	KR673891	HM569644	MG269952	MG270169	MG953415
Ceratocystis fimbriata Ellis & Halst	C1099 (ICMP 8579)	Papua New Guinea	—	=KR347445	KR673893	HM569615	MG269954	—	—
	CMW 15049 (CBS 141.37)	USA	—	—	—	—	—	KM495432	KM495520
Ceratocystis variospora (R.W. Davidson) C. Moreau emend. J.A. Johnson & T.C. Harr.	C1963 (CBS 135862)	USA	—	KF646773	KX925305	KR347450	MG269956	—	—
	CMW 20935, C1843 (CBS 114715)	USA	—	—	—	—	—	KM495471	KM495561
Chalaropsis ovoidea (Nag Raj & W.B. Kendr.) A.E. Paulin, T.C. Harr. & McNew	C1375, CMW 22733 (CBS 354.76)	Netherlands	—	AF275502	KY744595	HM569625	MG269957	KM495487	KM495577

Table S2 continued

Chalaropsis thielavioides (Peyronel) A.E. Paulin, T.C. Harr. & McNew	C1378 (CBS 130.39)	USA	—	AF222480	MF398184	HM569627	MG269958	—	—
	CMW 22736 (CBS 148.37, MUCL 6235)	Italy	—	—	—	—	—	KM495489	KM495579
Endoconidiophora coerulescens Münch.	C301 (CBS 100198)	USA	—	AF275510	KR673895	HM569653	MG269960	—	—
	CMW26365, C313, C695 (CBS 140.37, MUCL 9511)	Germany	—	—	—	—	—	KM495418	KM495506
Endoconidiophora virescens R.W. Davidson	C252 (CBS 128998)	USA	—	=KM495385	KX925306	HM569645	MG269959	—	—
	CMW17339, C261 (CBS 130772)	USA	—	—	—	—	—	KM495472	KM495562
Huntiella bhutanensis (M. van Wyk, M.J. Wingf. & Kirisits) Z.W. de Beer, T.A. Duong & M.J. Wingf.	CMW8217 (CBS 114289)	Bhutan	—	All six genes were extracted from genome assembly MJMS00000000.					
Huntiella moniliformis (Hedgc.) Z.W. de Beer, T.A. Duong & M.J. Wingf	CMW10134 (CBS 118127)	India	—	All six genes were extracted from genome assembly JMSH00000000.					
Huntiella moniliformopsis (Yuan & Mohammed) Z.W. de Beer, T.A. Duong & M.J. Wingf.	C1934 (DAR 74609)	Australia	—	KF646769	KR673898	HM569638	MG269962	—	—
Huntiella sp. C792	C792	USA	—	KY744592	KR673897	KT318392	MG269961	MG270172	MG272465
Solaloca norvegica (J. Reid & Hausner) T.C. Harr.	C3124 (UAMH 9778)	Norway	—	KY744591	KR673894	KT318390	MG269955	MG270171	MG272455
Thielaviopsis ethacetica Went	C1107	South America	—	KY744593	KR673899	HM569632	MG269964	—	—
	CMW 37775 (IMI 50560, MUCL 2170)	Malaysia	—	—	—	—	—	KM495426	KM495514
Thielaviopsis punctulata (Hennebert) A.E. Paulin, T.C. Harr. and McNew	C869, CMW 1032 (CBS 114.47, 146. MUCL 9526)	USA	—	AF275513	KX925308	KX925310	=MG269965	KM495459	KM495548
Outgroups									
Unnamed Microascales sp. C3547	C3547 (CBS 142647)	USA	—	MF399171	MF398173	MF375463	MG269966	MG270148	MG272463
Knoxdaviesia capensis M.J. Wingf. & P.S. van Wyk	C1960, CMW997	South Africa	—	MG269985	FJ176834	HM569657	MG269967	KM495478	KM495568
Graphium penicillioides Corda	C1506	Czech Republic	—	MG269984	MG950191	MG944396	MG269968	MG270149	MG272464

Table S2 continued

Scedosporium boydii (Shear) Gilgado, Gené, Cano & Guarro	(IHEM 23826)	France	—	All six genes were extracted from genome assembly NJFT00000000.
Xylaria sp. JS573	—	—	—	All six genes were extracted from genome assembly JWIU00000000.
Sclerotinia sclerotiorum (Lib.) de Bary	—	—	—	All six genes were extracted from genome assembly AAGT01000000.
Aspergillus niger Tiegh.	(CBS 513.88)	—	—	All six genes were extracted from genome assembly ASM285v2.

[1]Includes isolate or specimen numbers from the culture collection of T.C. Harrington, Iowa State University (C or M), Westerdijk Fungal Biodiversity Institute (CBS), U.S. National Fungus Collections (BPI), New South Wales Plant Pathology Herbarium (DAR), UAMH Centre for Global Microfungal Diversity (UAMH), International Collection of Microorganisms from Plants (ICMP), Belgian Co-ordinated Collections of Micro-organisms (IHEM), Royal Botanic Gardens Kew HerbIMI (IMI), and and the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria (CMW). [2]Isolates of ambrosia fungi are followed by a bolded abbreviation representing their type status: 'HT', holotype; 'IT', isotype; 'ET', epitype; 'PT', paratype; preceded by 'e', ex-type culture. [3]GenBank accession numbers preceded by ‘'=’ represent an identical (100% identity) match with that accession.

Supplemental Figures

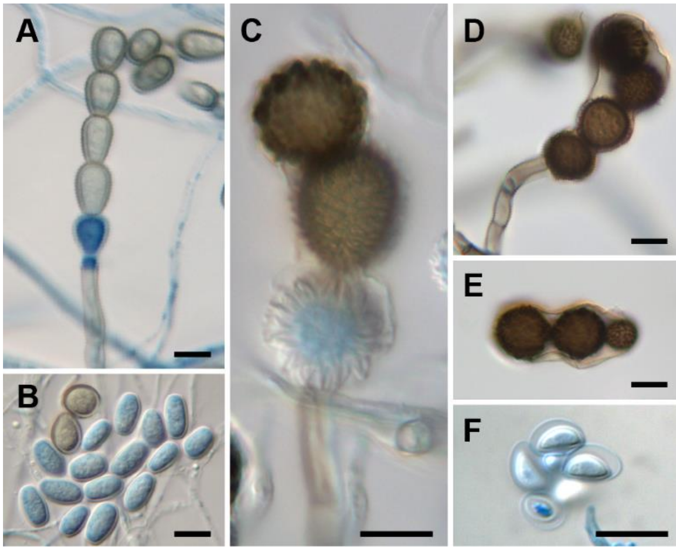

Figure S1. *Catunica adiposa.* (A). Chain of conidia produced from phialidic conidiophore. (B). Detached, pigmented and unpigmented conidia. (C). Two mature, darkly-pigmented aleurioconidia with basal developing aleurioconidium, membranous sheath visible. (D). Chain of mature aleurioconidia with membranous sheath visible. (E). Detached aleurioconidia in membranous sheath. (F). Ascospores. (A, B). Isolate C871 (CBS 600.74) from Japan. (C). Isolate C906 (CBS 138.34) from The Netherlands. (E–F) Isolate C299 from USA. Bar = 10 µm.

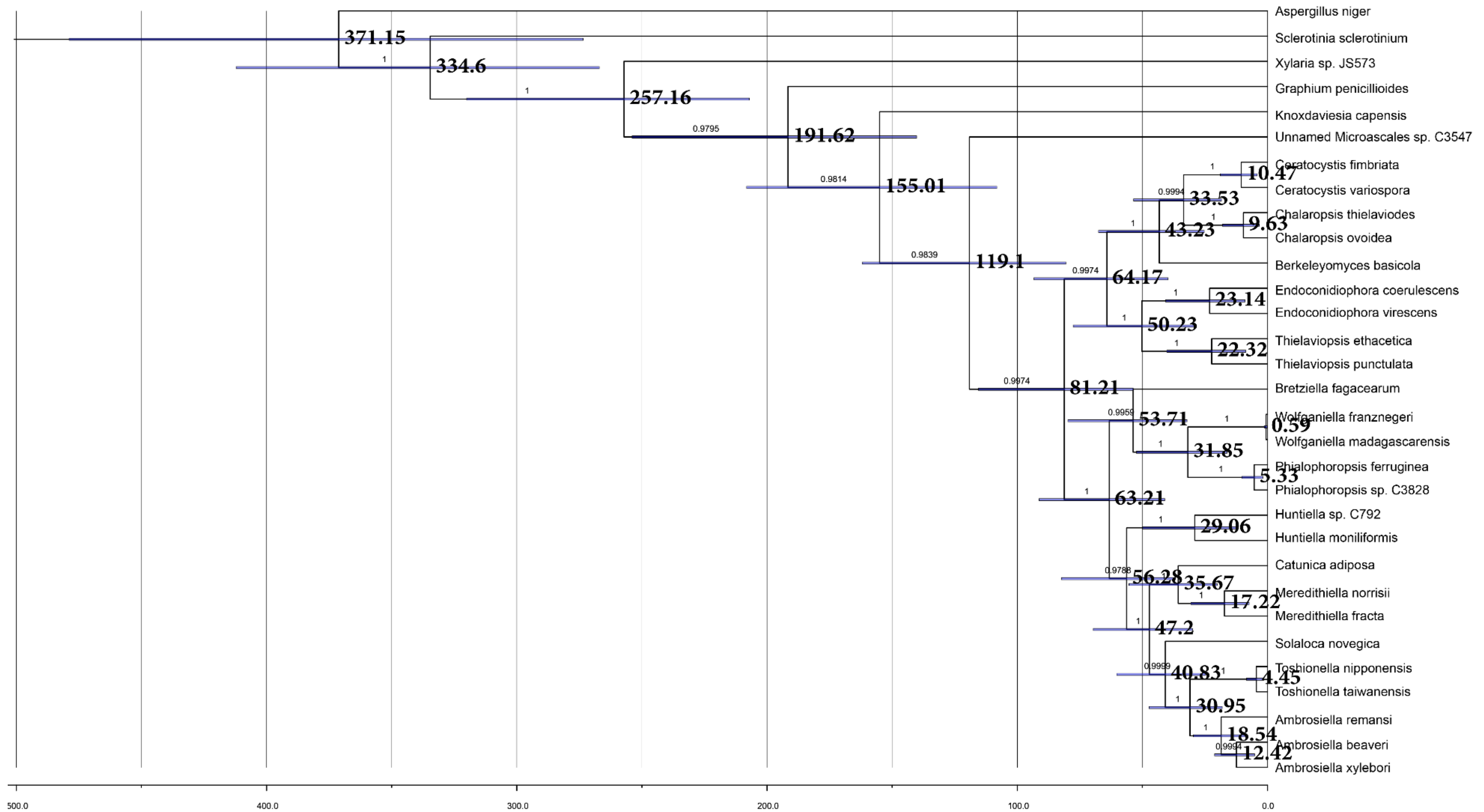

Figure S2. Raw output produced by secondary-calibrated analysis in BEAST of the six-gene tree dataset (18S rDNA, 28S rDNA, *tef*1-α, *tub*, *mcm7*, and *rpl1*). Horizontal bars are 95% Highest Posterior Density (HPD). Branch support values are Bayesian posterior probability from BEAST analysis. Nodes labelled with node ages; scale in millions of years ago (Ma). *Aspergillus niger* was used as the outgroup.

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CHAPTER 6. MYCANGIAL SYMBIONTS OF THE XYLOTERINI: *PHIALOPHOROPSIS* FROM *TRYPODENDRON*, *TOSHIONELLA* FROM *INDOCRYPHALUS*, AND THE UNUSUAL MYCANGIA AND SYMBIONTS FROM *XYLOTERINUS POLITUS*

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Abstract

Ambrosia beetles carry co-adapted fungal cultivars in a variety of storage organs called mycangia, which occur in various body parts and vary greatly in complexity. The understudied fungi associated with mycangia of ambrosia beetles in tribe Xyloterini (*Trypodendron, Indocryphalus,* and *Xyloterinus politus*) were morphologically and phylogenetically characterized. The association of *Phialophoropsis* (Ceratocystidaceae), including three new species, with *Trypodendron* was confirmed. Isolations of fungi and extraction of DNA from the prothoracic mycangia and galleries of seven *Trypodendron* spp. consistently yielded the same *Phialophoropsis* symbiont from each beetle species, though some *Phialophoropsis* spp. were shared among beetle species. This supports

earlier observations that fungal species in the Ceratocystidaceae are adapted to a mycangium type rather than to individual beetle species. Using rDNA sequencing, the Asian *I. pubipennis* was unexpectedly associated with an undescribed *Toshionella*, which are otherwise mycangial symbionts of Asian species of *Scolytoplatypus* (Scolytoplatypodini). The *I. pubipennis* mycangium was also prothoracic, but was found to be substantially smaller than that of *Trypodendron*. *Xyloterinus politus* has two different mycangia and carries a different symbiont in each: *Raffaelea* cf. *canadensis* RNC5 (Ophiostomatales) in paired oral mycangia and *Kaarikia abrahamsonii* gen. et sp. nov. in reduced prothoracic mycangia. These findings further support the concept that developments of new mycangium types are critical events in the evolution of ambrosia beetles and their co-adapted fungal genera. In addition to their highly adapted mycangial symbionts, both *Trypodendron* and *Xyloterinus* harbor a surprising diversity of non-mutualist gallery commensals, including several *Raffaelea* spp.

Introduction

Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae) cultivate and feed on fungal gardens along the walls of their galleries in sapwood. The adults carry propagules of their fungal symbionts in a variety of specialized structures called mycangia (Francke-Grosmann 1956, 1967; Hulcr and Stelinski 2017). Ambrosia beetles are associated with and may feed upon a wide variety of commensal fungi (Batra 1967, Francke-Grosmann 1967), but mycangial fungi (sometimes called primary ambrosia fungi) are obligate symbionts adapted to serve as a food source and to be carried in the mycangia (Batra 1985). Mycangial fungi are generally dimorphic, forming a palisade of ambrosia growth in the gallery and adopting a yeast- or athrospore-like growth in the mycangium (Francke-Grosmann 1956, 1963, 1967), where they actively

grow and proliferate, nourished by glandular secretions of the beetle (Abrahamson 1969; Schneider and Rudinsky 1969; Schneider 1975).

Both ambrosia beetles (Hulcr and Stelinsky 2017) and mycangial fungi (Harrington et al. 2010; Mayers et al. 2015; Bateman et al. 2016) are polyphyletic groups that evolved multiple times. In the beetles, each lineage appears to be marked by the development of a different type of mycangium. Some lineages of ambrosia beetles have elaborate and relatively large mycangia (Mayers et al. 2015, 2018). Genera of ambrosia fungi in the Ceratocystidaceae (Microascales) appear to have co-adapted with such elaborate mycangium types: *Ambrosiella* with the mesonotal mycangia of the *Xylosandrus* complex in tribe Xyleborini (Harrington et al. 2014; Mayers et al. 2015, 2017; Lin et al. 2017), *Meredithiella* with the coiled tubular mycangia of *Corthylus* in subtribe Corthylina (Mayers et al. 2015, 2018), *Phialophoropsis* with the prothoracic mycangia of *Trypodendron* in tribe Xyloterini (Mayers et al. 2015), and *Ambrosiella, Toshionella,* and *Wolfgangiella* with the pronotal disc mycangia of tribe Scolytoplatypodini (Chapter 5).

There are three genera of ambrosia beetles in tribe Xyloterini (Kirkendall et al. 2015). The genus *Trypodendron* have paired mycangia that are two large, folded cavities on the inside of the prothorax that exit laterally on both sides of the prothorax (Francke-Grosmann 1956, 1967). *Phialophoropsis trypodendri* and *P. ferruginea* have been reported as mycangial symbionts of *Trypodendron* spp. (Mayers et al. 2015), but molecular evidence suggests there may be cryptic diversity in *Phialophoropsis* and that different *Phialophoropsis* spp. are associated with different *Trypodendron* spp. (Cassar 1993; Blackwell & Jones 1997; Mayers et al. 2015). *Indocryphalus* has prothoracic

mycangia with openings that appear to differ from those of *Trypodendron* (Wood 1957; Beaver 2000; Cognato et al. 2015), but the interior morphology of its mycangium has not been characterized, and its fungal symbionts are unknown. The monotypic *Xyloterinus* is unique in being the only known Scolytine ambrosia beetle species to have two different types of mycangia: oral mycangia in male and female adults, and shallow prothoracic basins on the ventral posterior prothorax in females (Abrahamson and Norris 1966; Francke-Grosmann 1967). Abraham and Norris (1969) reported that the two mycangia of *X. politus* appear to carry different, unnamed mycangial fungi. The oral symbiont was tentatively identified as a *Raffaelea* sp., but the prothoracic symbiont was unidentified.

The primary aim of this study was to isolate and characterize the primary mycangial symbionts of species in the three genera of tribe Xyloterini, and we hoped to illustrate the mycangium of *Indocryphalus.* We expected that that the symbiont in the oral mycangium of *X. politus* would prove to be a *Raffaelea*, as proposed by Abraham and Norris (1969), and we hypothesized that the prothoracic mycangia of *Trypodendron, Indocryphalus,* and *Xyletorinus* were homologous and associated with primary symbionts in *Phialophoropsis* or related genera in the Ceartocystidaceae.

Materials and methods

Specimen collection and fungal isolation

In the USA, beetles were collected from active galleries in infested wood or captured in flight with lineatin lure. In Europe, beetles were collected from active galleries in infested wood or caught in flight with ethanol-baited traps. Galleries were chopped from wood bolts using chisels and dissected with sterile tools. In Japan beetles were collected as they landed on the trunks of unidentified trees.

Fungal isolations focused on beetle mycangia or ambrosia growth in galleries using sterile needles to directly plate fungal material onto malt extract agar with streptomycin (SMA; 1% malt extract, Difco Laboratories, Detroit, Michigan, USA; 1.5% agar, Sigma-Aldrich, St. Louis, Missouri, USA; and 100 ppm streptomycin sulfate added after autoclaving). Direct plating of dissected mycangia was found to be more effective than grinding whole beetles and dilution plating (Mayers et al. 2015). Beetles were first surface-sterilized for 10 s in 75% ethanol, followed by two washes in sterile water and drying on paper towels. Parts of the beetle containing the mycangia were dissected with sterile forceps and plated directly on SMA. Plates were incubated at room temperature and lighting, and filamentous colonies growing from the pieces of mycangium were subcultured and maintained on malt yeast extract agar (MYEA; 2% Difco malt extract, 0.2% Difco yeast extract, 1.5% agar). Some live beetles (some surface-sterilized, some not) were transferred to dry SMA (dried overnight at ambient temperature) then allowed to walk around freely for one or more days (‘walkabout plates’). These beetles were transferred to new SMA plates daily, and filamentous colonies growing on the SMA plates were subcultured.

Microscopic observation and descriptions

Fungal material was mounted in cotton blue for light microscopy using Normarski interference contrast (Olympus BH-2). Some images taken at different focus levels were combined into focus-stacked composites using CombineZP (Alan Hadley). Contrast, brightness, and tonal range of some images were adjusted using Leica Application Suite V3.6 (Leica Camera Inc., Allendale, NJ) or Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA). *Indocryphalus pubipennis* mycangia and contents were

similarly imaged. Images of *X. politus* mycangia and contents were unstained or stained with Trypan blue and photographed with bright field microscopy.

For species descriptions, agar plugs were cut from the margin of colonies on MYEA with a #1 cork borer (3mm diam.), transferred to fresh MYEA, and grown at 25 C in the dark for 7 d. Color designations are per Rayner (1970). Representative cultures were deposited in the Westerdijk Fungal Biodiversity Institute (CBS), and dried cultures and gallery specimens were deposited in the U.S. National Fungus Collections (BPI).

DNA sequencing and phylogenetic analyses

Most of the template DNA for ITS sequencing was extracted from galleries, whole beetles, dissected mycangia, or cultures using PrepMan Ultra (Applied Biosystems, Foster City, CA), and the DNA was concentrated, if needed, using Amicon® ultra-0.5 Centrifugal Filter Devices (EMD Millipore, Billerica, CA). DNA was also extracted from cultures with the ProMega Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) or, for cultures with excessive pigment, the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, GA). Amplification and sequencing of the nuc rDNA ITS1-5.8S-ITS2 (ITS barcode) used the general primer pair ITS1F/ITS4 or ITS4 and the *Ceratocystidaceae*-specific primers Cerato1F, ITSCer3.7R, and ITSCer3.1 (Mayers et al. 2015). Amplification and sequencing of nuc 28S rDNA (28S), nuc 18S rDNA (18S), translation elongation factor 1-alpha (*tef*1-a), beta tubulin (*tub*), DNA replication licensing factor MCM7 (*mcm7*), and 60S ribosomal protein (*rpl1*) used the primers and protocols described in Chapter 5. Complementary and overlapping DNA reads were checked and assembled using Sequence Navigator v 1.0.1 or AutoAssembler v 1.3.0 (Applied Biosystems, Foster City, CA). The ITS barcode was used to identify unknown fungal isolates, except for members of the Ophiostomatales such as *Raffaelea,*

for which 28S rDNA was used instead due to ITS sequencing difficulties (Harrington et al. 2010).

Where possible, insect identifications were confirmed by sequencing mitochondrial cytochrome c oxidase subunit I (COI barcode). This was especially important for *T. lineatum,* for which coloration can vary considerably and is not diagnostic (Wood 1957). One leg was removed, placed in 50 µl of Prepman Ultra, crushed with the tip of a sterile needle, and extracted per the recommended protocol at 100° C for ten minutes. Amplification and sequencing was performed with the primers LepF1 and LepR1 (Hebert et al. 2004), except with *X. politus,* for which these primers were not effective and S1718 and A2411 (Simon et al. 1994) were used instead (Jordal et al. 2011). PCR conditions for both primers sets were as follows: 2 m at 94° C; five cycles of 30 s at 94° C, 40 s at 45° C, and 1 m at 72° C; 35 cycles of 30 s at 94° C, 40 s at 51° C, and 1 m at 72° C; and 10 m at 72° C.

Barcode sequences (ITS, 28S, and COI) were trimmed and compared against the NCBI GenBank database using the NCBI BLASTn suite (National Center for Biotechnology Information, Bethesda, MD), and COI sequences were also compared to sequences in the dataset of Gohli et al. (2017) provided by J. Gohli. All unique sequences were deposited in GenBank. To illustrate sequence variation among the fungi, ITS sequences were manually aligned in two datasets to accommodate ambiguity in variable regions. The first aligned dataset contained the ITS sequences of *Phialophoropsis* and relatives; this dataset had 506 characters, including gaps (treated as a fifth state), of which 46 were parsimony-informative, 31 were variable but parsimony-uninformative, and 429 were constant. The second alignment contained *Ambrosiella* and relatives; this second

dataset had 552 characters, including gaps (treated as a fifth state), of which 117 were parsimony-informative, 57 were variable but parsimony-uninformative, and 378 were constant. A maximum parsimony (MP) tree was produced for each alignment with PAUP 4.0b10 (Swofford 2003). The *Phialophoropsis*-aligned dataset used *Bretziella fagacearum* as the outgroup with midpoint rooting, and the *Ambrosiella*-aligned dataset used *Catunica adiposa* and *Meredithiella norrisii* as outgroup taxa, which were forced into a monophyletic sister group to the ingroup. Branch support values were obtained via 1000-replicate bootstrap analysis of maximum parsimony in PAUP.

A six-gene (18S rDNA, 28S rDNA, *tef*-1a, *tub, mcm7,* and *rpl1*) tree of the Ceratocystidaceae was produced with MrBayes 3.2.2 (Ronquist et al. 2012) using the same alignment and methods in Chapter 5 with the addition of sequences obtained in this study (Table 1, Table S1). The resulting alignment is available at TreeBASE (URL: http://purl.org/phylo/treebase/phylows/study/TB2:S22561). The alignment had 5276 characters after exclusion of 618 characters found in introns and other ambiguously-aligned regions (Chapter 5). In addition to posterior probability branch support from Bayesian analysis, additional branch support was produced via 1000-replicate bootstrap analysis of maximum parsimony in PAUP. Of the analyzed 4658 characters, 1065 were parsimony-informative, 271 were parsimony-uninformative, and 3322 were constant.

The two unidentified symbionts of *X. politus* were phylogenetically placed using a two-gene (28S and 18S rDNA) analysis of the Ophiostomatales and other representatives of the Sordariomycetes. The alignments of Réblová et al. (2015, 2016) and Senanayake et al. (2016) were combined, supplemented with sequences of isolates obtained in this study, pruned to relevant representative taxa after preliminary analyses, and further

supplemented with sequences of close relatives available on GenBank (Table S2). Excluded from the alignment (TreeBASE URL: http://purl.org/phylo/treebase/phylows/study/TB2:S22562) were four ambiguously-aligned segments of the 28S gene (20 bp, 9 bp, 12 bp, and 13 bp, respectively), leaving an unpartitioned alignment of 2683 characters, of which 383 were parsimony-informative, 213 were variable but parsimony-uninformative, and 2087 were constant. *Xylaria hypoxylon* was used as an outgroup taxon. A Bayesian tree was produced (GTR+I+G model, 3,000,000 generations, 150,000-generation burnin), and a consensus tree and branch support values were generated as described above.

Results

Beetle collection and fungal associations

Using ITS barcoding and morphological characters, we identified five putative species of *Phialophoropsis* associated with seven species of Xyloterini ambrosia beetles: *Trypodendron scabricollis*, *T. lineatum, T. domesticum, T. signatum, T. retusum, T. betulae,* and *T.* aff. *proximum* (Table 1). Although it appeared that some *Phialophoropsis* spp. were associated with more than one *Trypodendron* species, each of the seven *Trypodendron* species were associated with a single putative *Phialophoropsis* sp. (Fig. 1A). A *Toshionella* sp. was identified in the mycangium of *Indocryphalus pubipennis* (Fig. 1B). We also identified two fungi from the mycangia of *Xyloterinus politus*: *Raffaelea* aff. *canadensis* RNC5 from the oral mycangia and a novel fungus apparently related to the Ophiostomatales from the prothoracic mycangia. Several genera in the

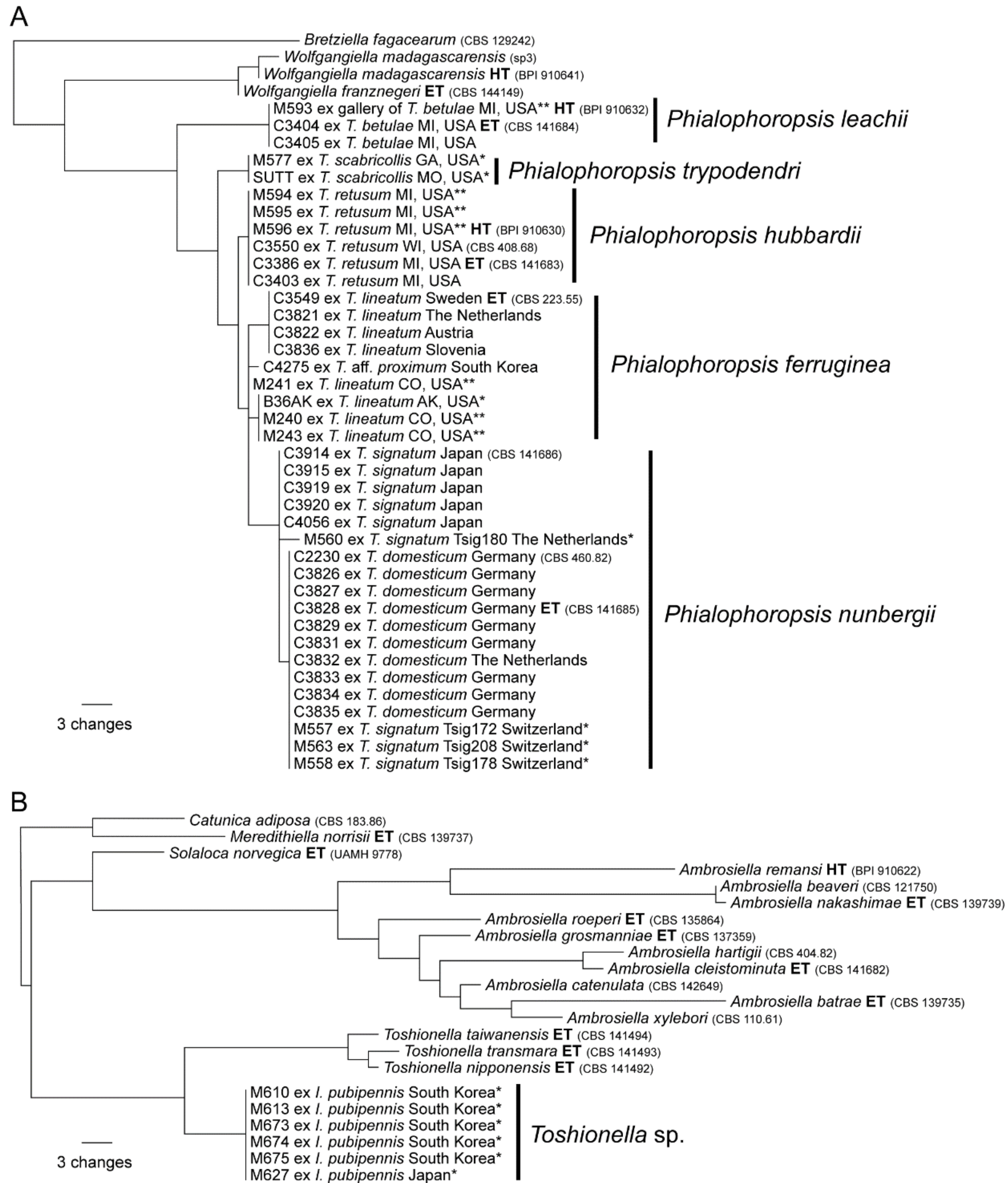

Figure 1. Maximum parsimony (MP) trees from alignments of ITS rDNA sequences of *Phialophoropsis* and relatives (A) and *Ambrosiella* and relatives (B) in the Ceratocystidaceae. (A). One of four most parsimonious trees produced by heuristic search in PAUP, including symbionts of *Trypodendron,* rooted to *Ceratocystis fagacearum*. Vertical bars denote *Phialophoropsis* species as defined in this study. (B). One of eight most parsimonious trees produced by heuristic search in PAUP, with the putative *Toshionella* symbiont of *Indocryphalus pubipennis* denoted with vertical line. Isolate or specimen numbers in the Iowa State University collection precede insect name; accession numbers for the Westerdijk Biodiversity Institute (CBS) or U.S. National Fungus Collections (BPI) are provided in parentheses where available. Single asterisks indicate sequences obtained from DNA extracted from whole beetles or mycangia; double asterisks indicate sequences from gallery growth; sequences without asterisks are from pure culture. Country or state (two letter abbreviation) of origin of the beetle or gallery material is indicated. Sequences from holotype followed by “HT”; from ex-type cultures followed by “ET”.

Ophiostomatales, including *Raffaelea* and *Leptographium*, were recovered as gallery commensals and superficial associates of several *Trypodendron* spp. and *X. politus*.

Trypodendron scabricollis

The type species for *Phialophoropsis* is *P. trypodendri*, the primary symbiont of *T. scabricollis* (Batra 1967). No living culture of *P. trypodendri* was available. Batra's (1967) holotype material (KANU 394426) from the University of Kansas Ronald L. McGregor Herbarium (KANU) contained galleries of *T. scabricollis* collected in Newton County, Arkansas and dried cultures of *P. trypodendri* from the holotype. Batra (1963, 1967) and Seifert et al. (2011) illustrated phialidic conidiophores in the type material of *P. trypodendri* (see taxonomy section for more details). Phialidic conidiophores could not be found in larval cradles in the holotype, but we did observe phialoconidia and potential phialides in material associated with isotypes in BPI. The dark, red-brown mycelia, stained media, and superficial sterile surface growth of the dried culture of the holotype were typical for the culture morphology described in other species of *Phialophoropsis* (Leach et al. 1940; Funk 1965; Batra 1967; Cassar 1993; Kühnholz 2004). However, our attempts to obtain an ITS sequence from DNA extracted from a larval cradle and a dried culture were unsuccessful.

In an earlier study (Mayers et al. 2015), DNA was extracted from ethanol-preserved *T. scabricollis* females trapped in flight at nine locations in Missouri, each of which yielded an identical ITS sequence (**KR611329**) of a *Phialophoropsis* sp. using *Ceratocystis*-specific primers. Two additional female *T. scabricollis* were caught in flight in Georgia using lineatin lures in spring 2017. The beetles were dry, and isolation of fungi was unsuccessful, but an ITS sequence identical to that from the Missouri *T. scabricollis* was obtained from the DNA extracted from the mycangium of one of the

beetles (Fig. 1). The identity of the beetle was confirmed by COI sequence (**MF373741**), which matched a *T. scabricollis* sequence of Gohli et al. (2017). The Georgia specimens had pleural-prothoraxic mycangia opening on the lateral sides of the prothorax, with interior morphology typical of other *Trypodendron* spp.

Trypodendron lineatum

Phialophoropsis ferruginea was described by Mathiesen-Käärik (1953) from galleries of *T. lineatum* in Sweden. We obtained the ex-type culture (MUCL 9940, =CBS 223.55, =C3549) from the Belgian Coordinated Collections of Micro-organisms (BCCM). The culture was isolated in 1952 from a gallery of *T. lineatum* in *Picea abies* in Regna, Sweden by Mathiesen-Käärik. The mycelium of C3549 was red-brown and produced a red-brown diffusible pigment and sparse surface growth on MYEA, consistent with reports of *P. ferruginea* (Mathiesen-Käärik 1953; Funk 1965; Batra 1967). Three additional isolates were obtained from mycangia of *T. lineatum* females caught in-flight at three different locations in June 2015 (C3821, Wageningen, The Netherlands; C3822, Glanz, Austria; C3836, Slovenia). The four isolates were nearly identical in culture morphology, and ITS sequences obtained from them were identical (e.g. **MF399187**) (Fig. 1A). ITS sequences obtained by Mayers et al. (2015) from ambrosia growth in a *T. lineatum* gallery in Colorado and from female beetles trapped in-flight in Alaska lacked a repeated "GT" in the ITS1 region compared to the European sequences. In addition, the sequences from gallery M240 from Colorado (**KR611328**) and B36 from Alaska have three Ts at the end of ITS2, whereas gallery M241 from Colorado (**MF399197**) and the European isolates have four Ts. The COI sequences of the three European beetles (e.g., **MF373743**) closely matched sequences of European *T. lineatum*, and a COI sequence from an Alaska beetle (**MF373742**) matched closely to

other North American specimens of *T. lineatum* (e.g., **MF373744**). North American and most European *T. lineatum* COI sequences group separately (Jordal and Kambestad 2014).

Mathiesen-Käärik's isolate (C3549) produced swollen, beaded hyphae (Fig. 2F), which she considered to be the sole means of propagation (Mathiesen-Käärik 1953). However, we also observed conidiophores in C3549 that were rarely produced on one or few small isolated tufts on MYEA. The conidiophores had deep-seated phialides (Fig. 2A–C), as expected in *Phialophoropsis* (Batra 1967, Mayers et al. 2015), with solitary or long chains of phialoconidia (Fig. 2D, E). These matched the conidiophores observed in USA galleries of *T. lineatum* (Mayers et al. 2015).

Though no *Phialophoropsis* culture was isolated from *T. lineatum* collected in Alaska or Colorado, several other fungi were occasionally isolated on media selective for Ophiostomatales (CSMA, 1% malt extract, Difco; 1.5% agar, Sigma-Aldrich; 200 ppm cycloheximide and 100 ppm streptomycin sulfate added after autoclaving) or SMA.

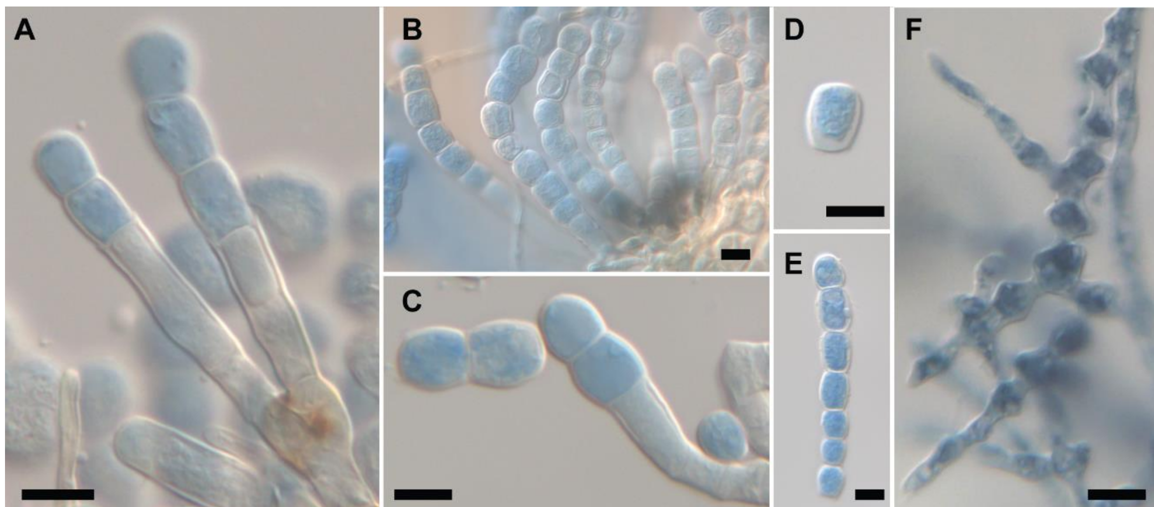

Figure 2. *Phialophoropsis ferruginea* from *Trypodendron lineatum.* (A). Branched phialoconidiophores. (B). Long chains of conidia from phialoconidiophores. (C). Shallow phialide with detached conidia. (D). Single detached conidium. (E). Chained, detached conidia. (F). Beaded hyphae. (A–E). Isolate C3549 (CBS 223.55; ex-type). (F). From gallery in Colorado. Photos by Nomarski interference microscopy of material stained with cotton blue. Bars = 10 μm.

Three isolates of a *Raffaelea* sp. ('*Raffaelea* cf. *brunnea* RNB3') were isolated from a non-surface-sterilized Alaska beetle and two Colorado galleries. The isolates had a unique 28S sequence (e.g. C3493; **MF399176**), which was most similar to the 28S sequence of *R. brunnea* (526/531 bp matching, 99%, with **EU177457**) and identical to the sequence of the unnamed TR25 (533/533 bp, 100% match, with **EU984281**) isolated from *T. rufitarsis* in *Pinus cortorta* in western Canada (Kühnholz 2004; Alamouti et al. 2009). Other fungi isolated from surfaces of USA *T. lineatum* and its galleries included *Leptographium abietinum* and unidentified *Ophiostoma* spp. with 28S sequences similar to those of *O. montium* (C3501; **MF399181**) and *O. piceae* (C3492; **MF399182**).

Trypodendron* aff. *proximum

A single female of an undescribed *Trypodendron* sp. with affinity to *T. proximum* was collected in the Korea National Arboretum, Gyeonggi-do, Pocheon-si, South Korea. It yielded an isolate (C4275 = CBS 144148) with culture characteristics similar to those of *P. ferruginea,* and its ITS sequence (**MF399198**) was similar to that of *P. ferruginea* isolate C3549, differing from it by the deletion of a 'GT' in ITS1 and a C-to-T substitution in ITS2 (Fig. 1A). The isolate sporulated abundantly and produced many tufts of phialidic conidiophores that appeared identical to those of C3549.

Trypodendron domesticum

Culture C2230 (= CBS 460.82), isolated from a *T. domesticum* gallery in *Fagus sylvatica* in Germany (Zimmerman 1973), has been used as a representative of *P. ferruginea* (Six et al. 2009; de Beer et al. 2014; Harrington et al. 2014). However, its ITS sequence (**KC305146**) was distinct from the ex-type culture (C3549) of *P. ferruginea* (Fig. 1A). Our isolate C2230 appeared to be debilitated, as it grew slowly, was hyaline rather than pigmented, and did not sporulate. Nine fresh isolates (including C3828 = CBS

141685) were recovered from nine *T. domesticum* females in Spring 2015; eight were trapped in Hainich, Germany, and one in Wageningen, Netherlands. The identity of one of the German specimens was confirmed by its COI sequence (**MF373734**). The nine isolates had an ITS sequence identical to that of C2230 (Fig. 1A) but appeared healthy, with growth rates and red-brown pigment expected of *Phialophoropsis* (Fig. 3M, N).

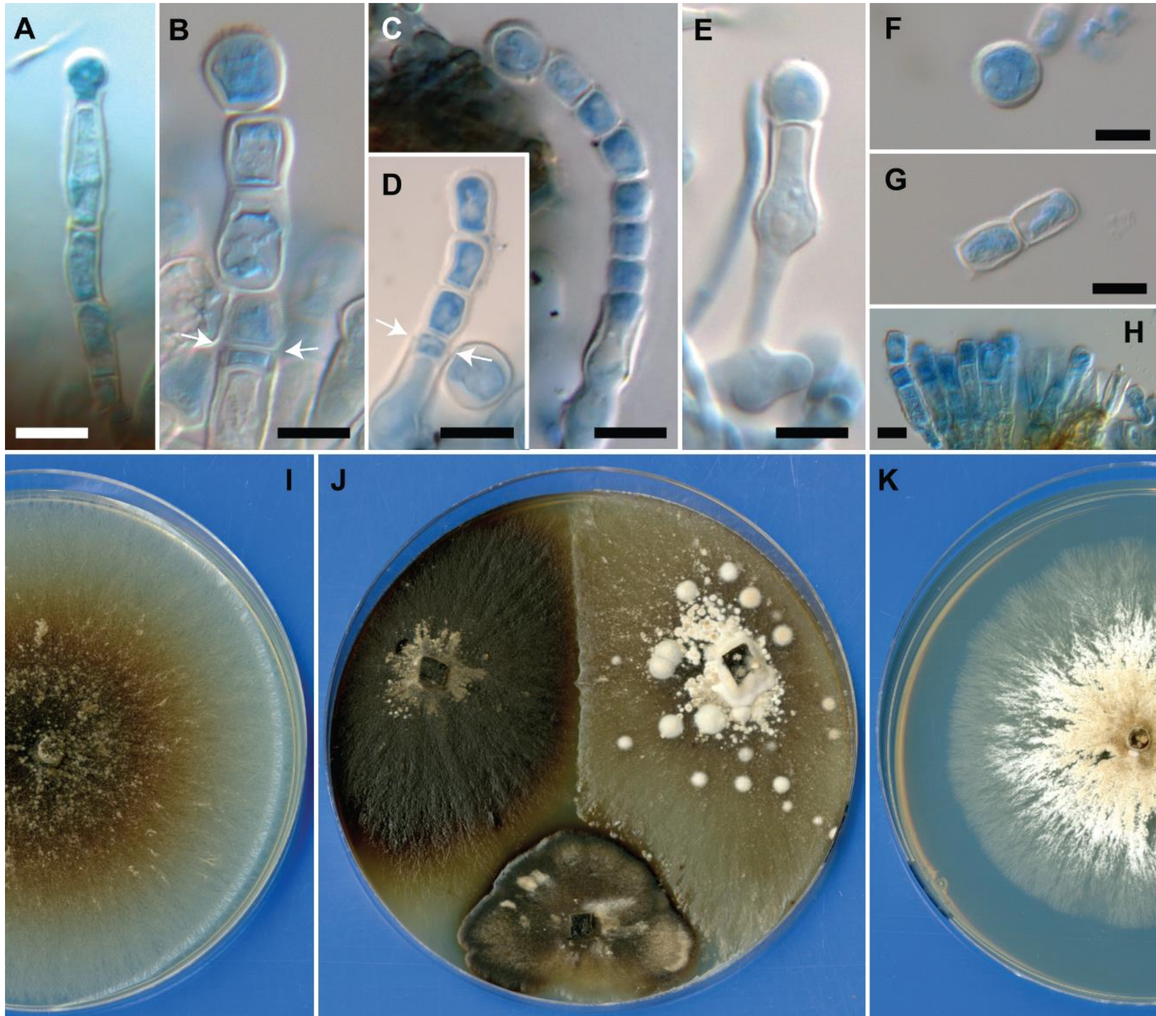

Figure 3. *Phialophoropsis nunbergii* from *Trypodendron domesticum* and *T. signatum*. (A–E). Phialoconidiophores. (A). With single terminal conidium. (B, D). Chained phialoconidia from shallow phialide, with phialide collar indicated by arrows. (C). Long chain of phialoconidia, with first conidium larger and more globose. (E). Phialide supported by subtending stalk. (F, G) Detached phialoconidia. (H). Palisade of phialoconidiophores in gallery. (I, K). Growth at 7d on MYEA. (J). Growth at 14d on MYEA of three transferred subcultures from the same isolate. (A, I, J). Isolate C3828 (CBS 141685; ex-type) from *T. domesticum*. (B, F, G). Isolate C3826 from *T. domesticum.* (C–E, K) Isolate C3914 (CBS 141686) from *T. signatum*. (H). In gallery of *T. signatum* in *Fagus sylvatica.* (A–H) imaged by Nomarski interference microscopy of material stained with cotton blue, Bars = 10 µm. (I–K) imaged with Epson 10000XL scanner, plate diam. 90mm.

Two isolates produced conidiophores with deep-seated phialides (Fig. 3A–D) on small, isolated tufts in culture and solitary or chained phialoconidia (Fig. 3E, F), similar to cultures of *P. ferruginea,* but with somewhat shallower phialides. The nine isolates from *T. domesticum* tended to spontaneously sector into one of four mycelial phenotypes, and the phenotypes persisted when transferred (Fig. 3N).

Trypodendron signatum

Galleries of *T. signatum* in *Fagus sylvatica* were collected in Bern, Switzerland in July 2012. The galleries contained ambrosia growth with deep-seated phialides (Fig. 3G), but ITS amplification was not successful from this material. Three females from the same collection stored in ethanol yielded ITS sequences identical to sequences of *Phialophoropsis* from *T. domesticum* (Fig. 1A). COI barcoding was not successful for these beetles, but their antennae clubs were as expected for *T. signatum* (Grüne 1979), which is otherwise easily confused with *T. lineatum*. The DNA extracted from a single *T. signatum* female caught in-flight in the Netherlands and stored in ethanol yielded a *Phialophoropsis* ITS sequence (**MF399196**) that differed from the other *T. domesticum* and *T. signatum* sequences by a repeated 'TA' near the beginning of ITS1 that was unique among *Phialophoropsis* spp. (Fig. 1A). COI sequencing was successful on this beetle (**MF373737**), which confirmed its identity as *T. signatum*.

Several additional *T. signatum* females were collected in Morioka, Iwate Prefecture, Japan in April 2015. Isolations from six of the beetles yielded isolates of a dark brown fungus with an ITS sequence (**MF399189**) nearly identical to the common sequence from *T. domesticum* and *T. signatum*, differing by a G-to-A substitution in ITS1 region that is unique among *Phialophoropsis* spp. (Fig. 1A). The culture morphology of these six *T. signatum* isolates was similar to isolates from *T. domesticum,* but the isolates

from *T. signatum* tended to have a dense, contiguous carpet of white surface growth and produced less pigment (Fig. 3O), and one isolate (C3914, = CBS 141686) sporulated densely in culture. The conidiophores of C3914 were uniquely flask-shaped and subtended by a thin stalk (Fig. 3H, I). No other isolates from *T. signatum* sporulated, and gallery growth was not observed. COI sequencing was not successful from a female from this collection, but it had morphological characters consistent with *T. signatum* (Grüne 1979).

Trypodendron retusum

Isolate C3550 (= CBS 408.68 = MUCL 14520, isolated by D.M. Norris in 1968 from a *T. retusum* female infesting *Populus* sp. in Wisconsin, USA) has also been used as a representative of *P. ferruginea* (Paulin-Mahady et al. 2002; Alamouti et al. 2009; Six et al. 2009; Harrington et al. 2014). Its ITS sequence (**KC305145**) was distinct from *P. ferruginea* C3449 (Fig. 1A), as was the culture, which was sterile and fast-growing, with fluffy, red-brown aerial hyphae.

Galleries of *T. retusum* in *Populus grandidentata* were collected in Michigan on four occasions: in Benzie County in July 2013, and in Montcalm County in May 2014, July 2014 and May 2015. COI sequences obtained from two *T. retusum* females, Tret213 from 2013 (**MF373738**) and Tret216 from July 2014 (**MF373739**), were most similar to the sequence of T. retusum in the dataset of Gohli et al. (2017). Larval cradles in all four collections had moist, translucent, beige to red-brown carpets of ambrosia growth (Fig. 4A) composed of palisades of phialoconidiophores (Fig. 4B, C). The ambrosia growth in the cradles yielded ITS sequences (e.g. **MF399194**) identical to that of C3550 (Fig. 1A). Isolations of *Phialophoropsis* were unsuccessful from the 2013, July 2014, and 2015 collections, but ITS sequences from cradles of the three collections were identical to that

of C3550 (Fig. 1A). Isolations from the mycangium of a female and from the ambrosia growth in an egg niche from the May 2014 collection yielded two cultures with characteristics expected of *Phialophoropsis*, including the production of brown pigment, though these cultures often shifted to very slow growth rates with excessive pigment production. The two isolates yielded ITS sequences (e.g. **MF399190**) identical to that of C3550 and the three galleries (Fig. 1A), but the faster, fluffier growth of C3550 was not observed in these field isolates. Sporulation was not observed in the *Phialophoropsis* isolates from *T. retusum*.

A female taken from the May 2014 collection had a spore mass attached to one of its coxa (Fig. 4H, black arrow), which presumably came from the nearby mycangium opening on the proepimeron, from which another small mass of fungal cells was observed (Fig. 4H, white arrow). The coxal spore mass was composed of thick-walled propagules, several of which appeared to be germinating (Figs. 4I, J). The spores exiting from the mycangium, and teased from inside of it (Figs. 4K, L), were composed of a homogenous mix of septate, sometimes branched, thallic-arthric propagules, consistent with mycangium propagules reported previously in *Trypodendron* (Batra 1963; Francke-Grosmann 1956, 1958; Abrahamson et al. 1967).

The main tunnels of *T. retusum* were colored black and were devoid of ambrosia growth. However, the mycelium, conidia and perithecia of Ophiostomatales were sometimes seen. Other Ophiostomatales isolated from *T. retusum* galleries and identified via 28S rDNA sequencing included *Raffaelea tritirachium, Leptographium piriforme* (C3144; **MF399183**), and *Ophiostoma* spp. with sequences similar to *Ophiostoma floccosum* (C3142; **MF399184**) and *Ophiostoma piceae* (C3145; **MF399185**).

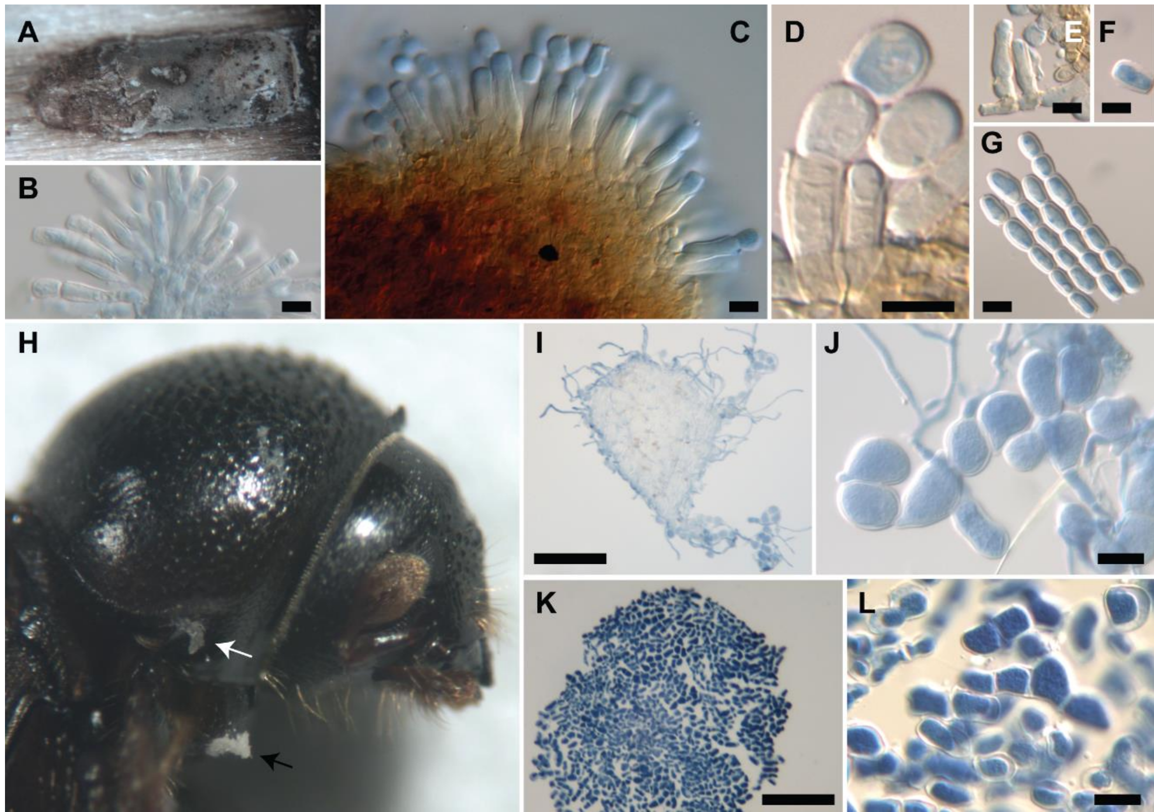

Figure 4. *Phialophoropsis hubbardii* from *Trypodendron retusum.* (A, B, C) Palisades of phialoconidiophores on gallery walls. (D). Phialoconidiophores with phialoconidia produced in shallow phialides. (E). Palisade of phialoconidiophores. (F, G). Detached phialoconidia. (H). Prothorax and head of *T. retusum* with spore mass near mycangium opening (white arrow) and detached spore mass on coxa (black arrow). (I, J). Detached fungal mass attached to coxa showing germinating thick-walled mycangial spores. (K, L). Spore mass teased from mycangium showing thick-walled, septate, arthrospore-like propagules. (A, B, D–G) In gallery of *T. retusum* in *Populus grandidentata* (BPI 910630; holotype). (C). Gallery M597. (H). *T. retusum* female Tre83. All photos except A, H by Nomarski interference microscopy of material stained with cotton blue, Bars = 10 µm, except I, K = 100 µm. A, H by stereo microscope.

Trypodendron betulae

Galleries of *T. betulae* in *Betula papyrifera* were collected in Chippawa Township, Isabella County, Michigan in June 2014. A COI sequence (**MF373740**) obtained from a female found in the gallery was most similar to two sequences in GenBank: from an unknown Curculionidae sp. (**KM850234**) and a sequence attributed to *T. lineatum* (**KU876414**) that was probably misidentified, since the sequence differed greatly from other *T. lineatum* sequences. The only COI sequence in GenBank attributed to *T. betulae* (**KU876412**) matches sequences of *T. lineatum*. However, our studied

beetles morphologically fit the descriptions of *T. betulae*, which is restricted to *Betula* and, rarely, *Alnus* (Wood 1957).

The larval cradles of *T. betulae* had moist carpets of ambrosia growth composed of palisades of phialoconidiophores (Figs. 5A, B), similar to those of other *Trypodendron*. The ITS sequence obtained from the cradles (**MF399193**) was unique within *Phialophoropsis* (Fig. 1A). The cradles yielded two isolates, C3404 (=CBS 141684) and C3405, with characters expected of *Phialophoropsis*; they did not sporulate and tended to sector into a much slower growth form with excessive pigment production. The ITS sequence from both isolates (**MF399191**) was identical to the gallery sequences (Fig. 1A). An 18S rDNA sequence obtained from C3404 differed somewhat (619/630 bp) from the sequence (**U40016**) of a 1996 isolate from *T. betulae* used to represent *P. ferruginea* (Cassar and Blackwell 1996; Blackwell and Jones 1997), but the 20-year-old sequence contained a number of Ns and other single-base differences that may have been due to limitations in sequencing technology.

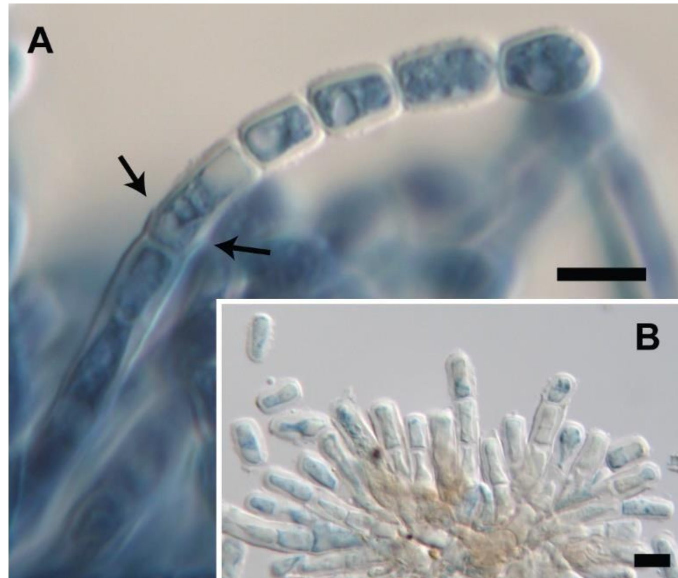

Figure 5. *Phialophoropsis leachii* from *Trypodendron betulae.* (A). Phialoconidiophore bearing chain of phialoconidia, with phialide collar marked with black arrows. (B). Palisade of phialoconidiophores on gallery wall. (A). In gallery of *T. betulae* in *Betula papyrifera* (BPI 910632; holotype). (B).From another *T. betulae* gallery in *B. papyrifera.* Both photos by Nomarski interference micrscopy of material stained with cotton blue, bars = 10 µm.

Ten isolates of a *Raffaelea* sp. were obtained from galleries of *T. betulae.* Four of the isolates (e.g. C3387; **MF399177**) had a 28S sequence identical to that of *Raffaelea* cf. *brunnea* RNB3 isolated from *T. lineatum.*

Indocryphalus pubipennis

Female *I. pubipennis* were collected in Jeju, South Korea from galleries in an unidentified tree in April 2016 and stored in ethanol. The COI sequence from one of the females (**MH042542**) was similar to other *I. pubipennis* sequences on GenBank. Extractions of DNA from dissected mycangia or teased spore masses from five females yielded an ITS sequence (**MH040803**) that was closest to but distinct from *Toshionella* (Fig. 1B)*,* a genus of Asian *Scolytoplatypus* ambrosia symbionts (Chapter 5). Additional female *I. pubipennis* were collected in Senboku, Akita Prefecture, Japan in July 2017 and stored in ethanol. The COI sequence from one of the females (**MH042543**) was also similar to other *I. pubipennis* sequences on GenBank, and it differed from the sequence from South Korea by two base substitutions. The Japanese beetles were caked in a white, waxy material that appeared to be fungal in nature. The ITS sequence obtained from a piece of this material matched *Geosmithia putterillii* (100% identity, match with **HF546347**), a common superficial bark beetle associate (Kolařík et al. 2017). Nevertheless, the mycangia were full of homogenous masses of fungal propagules identical to those in the mycangia of the South Korean *I. pubipennis.* The ITS sequence obtained from a dissected mycangium of *I. pubipennis* in Japan was identical to that from the South Korean *I. pubipennis* (Fig. 1B).

The females from the South Korean and Japanese collections had mycangium openings on the side of the prothorax, positioned just under the ridge separating the

curved dorsal pronotum from the episternal region (Fig. 6A). The openings were horizontal slits that generally tapered on the anterior side and curved slightly dorsally on the posterior side, as expected for *I. pubipennis* (Cognato et al. 2015). The openings were completely covered with a fence of vertical setae that presumably keeps spores inside the cavity (Fig. 6B). Spore masses typically overflowed from the openings in the South Korean beetles (Fig. 6B), and the external masses were often like balls of yarn, composed of long, tangled strings of hyphae (Fig. 6E). Internally, the mycangia were pocket-like, triangular invaginations, shaped like upside-down shark fins with apices that pointed ventrally and anteriorly (Fig. 6C), with walls composed of a sclerotized, reticulated matrix (Fig. 6D). When gentle pressure was applied to the side of the prothorax, homogenous ribbons of fungal propagules (Fig. 6F) protruded from inside the mycangia.

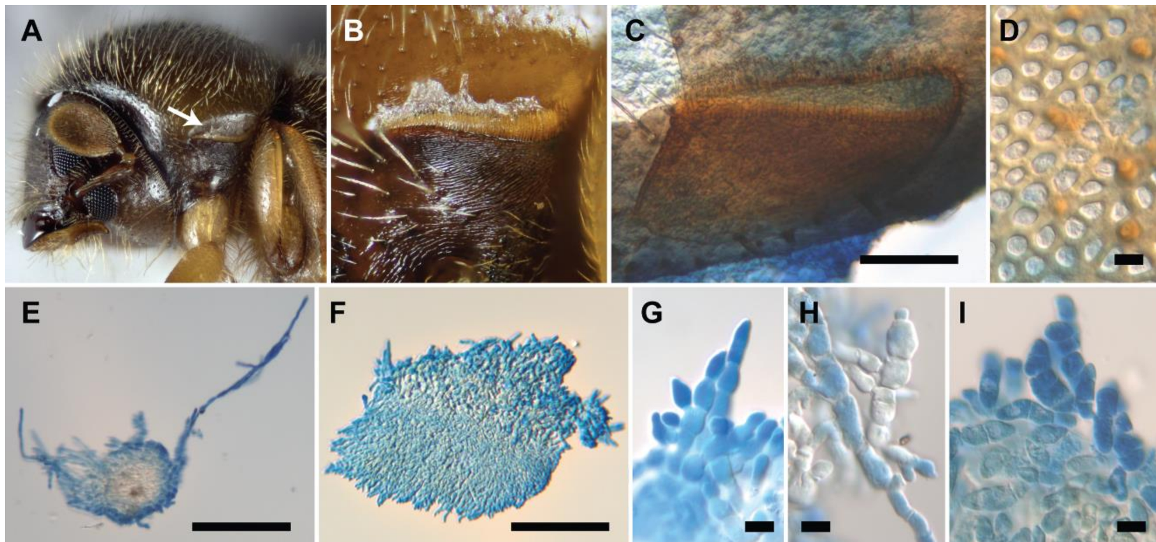

Figure 6. Mycangium of *Indocryphalus pubipennis* and mycangium propagules of its putative *Toshionella* symbiont. (A). Left aspect of female, with mycangium opening indicated by white arrow. (B). Detail of mycangium opening lined with setae, with exiting fungal propagules; beetle anterior is to the left. (C). Dissected left mycangium, viewed from exterior side of exoskeleton; beetle anterior is to the left. (D). Reticulated texture of mycangium wall. (E). Exterior fungal mass, showing long mycelial threads and bundled mass. (F). Spore mass teased from inside mycangium. (G, H). Irregular, chained, branching-arthric propagules in mycangium. (I). Solitary or short-chained propagules in mycangium. All photos except A, B by Nomarski interference micrscopy of material stained with cotton blue, bars = 10 µm, except C, E, F bars = 100 µm. A, B by stereo microscope.

These contents comprised irregularly shaped, thallic-arthric propagules in branching chains (Fig. 6G, H) or the cells were solitary and in short chains (Fig. 6I).

Xyloterinus politus

Galleries of *X. politus* were examined in logs of *Acer rubrum* (August 2013) and *Populus* sp. (May 2014) collected in Montcalm County, Michigan, and in a log of *Betula papyrifera* (June 2014) in Isabella County, Michigan. The COI sequences obtained from two beetles in the 2013 collection (**MF373736** and **MF373735**) matched other sequences of *X. politus* in GenBank. The prothoracic mycangia of female *X. politus* from galleries were shallow basins rimmed with protective setae (Fig. 7O), as previously reported (Abrahamson and Norris 1966, 1969; Abrahamson 1967; Francke-Grosmann 1967). As illustrated by Abrahamson and Norris (1966, 1969) and MacLean and Giese (1968), the prothoracic mycangia contained thick-walled, spherical spores (Figs. 7P, Q). The second type of mycangium, near the mouthparts of both males and females (Abrahamson and Norris 1966, 1969; Abrahamson 1967) was not examined in this study.

Unlike galleries of *Trypodendron* spp., the galleries of *X. politus* were mostly dominated by superficial, darkly pigmented, sparse fungal growth composed of thick, sterile olivaceous hyphae. The mycelium grew along the tunnel walls and intermixed with sawdust and frass to seal or plug the egg niches and larval cradles from the main gallery. Growth in niches and cradles was lighter-colored and thicker than the growth in the main gallery (Fig. 7A) due to the abundance of large, club-shaped conidia (Figs. 7B, C), which appeared similar to the spores in galleries of *X. politus* illustrated by MacLean and Giese (1968). Large, spherical chlamydospores were sometimes observed in the main gallery and cradle plugs (Fig. 7R).

Eight cultures of a very slow-growing, olive-brown fungus were isolated on SMA from *X. politus* galleries and from surface-sterilized female prothoraxes. The cultures initially grew slowly but transitioned to a faster-growing, red-brown phenotype with

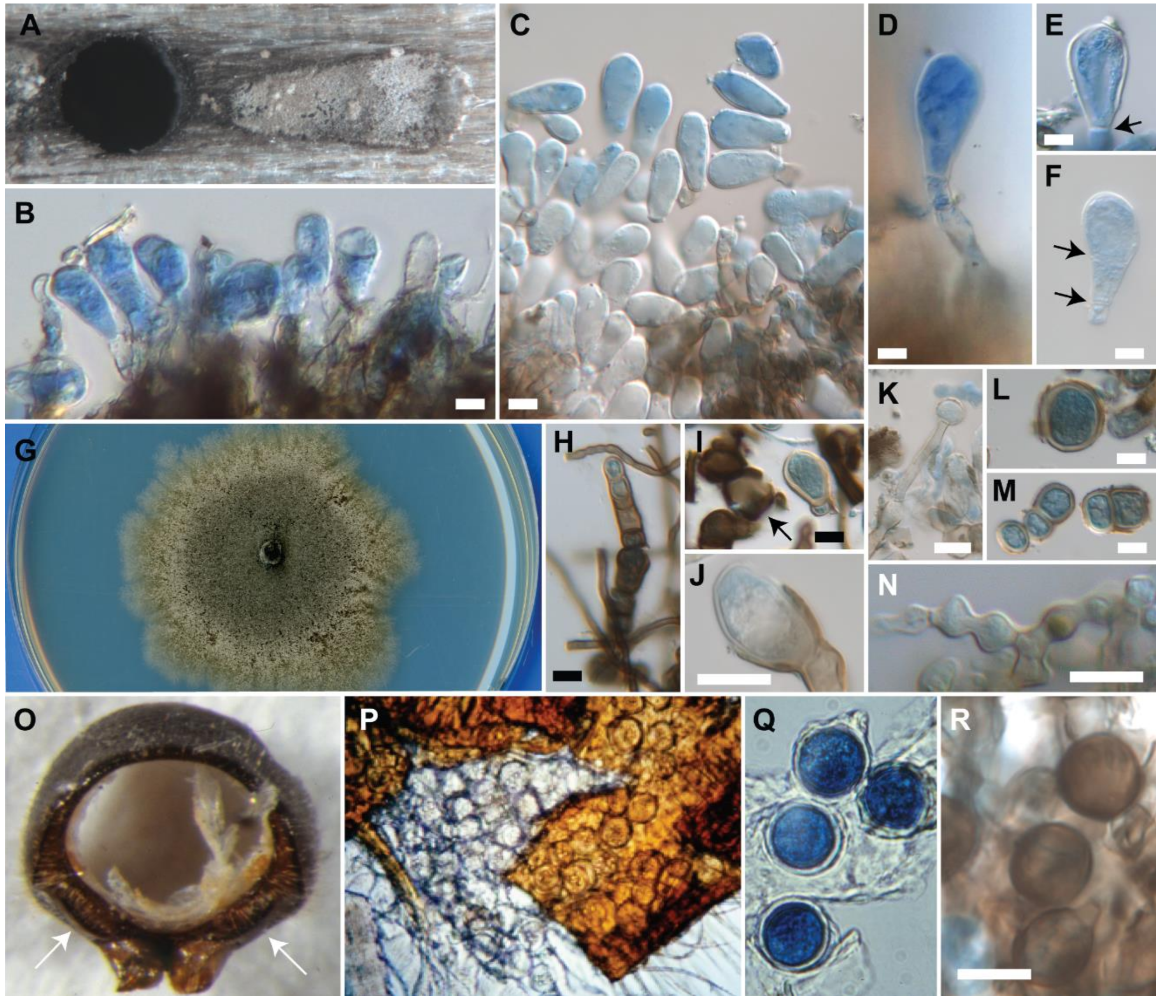

Figure 7. *Kaarikia abrahamsonii* and prothoracic mycangia of *Xyloterinus politus.* (A–F). Conidia and conidiophores in galleries. (A). Larval cradle. (B). Palisade of conidiophores in larval cradle. (C). Detached conidia in egg niche. (D). Simple conidiophore. (E). Terminal conidium with truncate attachment (black arrow). (F). Detached conidium with two septa (black arrows). (G–N). Culture morphology. (G). Growth at 7d on MYEA. (H–M). Chlamydospores. (H). Terminal and intercalary chlamydospores, breaking free. (I). Free chlamydospore on right, with empty pigmented sheath (black arrow) on left. (J). Terminal, with pigmented outer sheath visible. (K). Terminal on simple hypha. (L). Solitary chlamydospore. (M). Chained chlamydospores. (N). Beaded hyphae in culture. (O–Q). Prothoracic mycangium and mycangial propagules. (O). Posterior aspect of *X. politus* prothorax with rest of body removed, showing paired prothoracic basin mycangia (white arrows). (P). Prothoracic basin mycangium cracked under cover slip, with mycangial spores inside. (Q). Spherical mycangial propagules. (R). Spherical chlamydospores at cradle entrance. (A, B, D, F, R) in *Acer rubrum* (BPI 910626). (C) in *Betulae papyrifera* (BPI 910623, holotype). (G–N) isolate A1268 (CBS 142646). (A, O) by stereo microscope. (B–F, H–N, R) by Nomarski Interference Microscopy of material stained with cotton blue, Bar = 10 μm. (O, P) by light microscopy. (P). Stained with Trypan blue. (G). By Epson 10000XL scanner, plate diam. 90mm.

textured aerial mycelium (Fig. 7G) after serial transfer on MYEA. The cultures produced thick, olivaceous hyphae similar to that seen in the galleries, but club-shaped conidia were not observed in culture. Submerged hyphae in older cultures typically produced thick-walled chlamydospores (Figs. 7H–M) that appeared to burst irregularly from within the pigmented hyphae (Fig. 7 H–J), leaving behind pigmented membranes (Fig. 7 I, black arrow). Chlamydospores also were produced terminally on simple hyphae (Fig. 7 K). Knobbed, monilioid hyphal swellings formed in culture (Fig. 7N). Multiple attempts at PCR and direct sequencing with ITS primers were unsuccessful from these isolates, but six isolates yielded an identical 28S sequence (e.g. A1268=CBS 142646; **MF399172**), which had some similarity to the 28S gene of Ophiostomatales but had no close (>91% identity) matches in NCBI BLASTn searches. In both culture morphology and chlamydospore morphology, these isolates resembled UWE-132M, which had been earlier isolated from galleries and prothoracic mycangia of *X. politus* (Abrahamson and Norris 1969).

Seven isolates of a *Raffaelea* sp., '*Raffaelea* cf. *canadensis* RNC5', including C3169 (=CBS 142652), were isolated from galleries, the dissected head of a surface-sterilized female, and two walkabout plates of *X. politus*. The isolates had small, round to pyriform, truncate spores on simple conidiophores, similar to those of *R. canadensis* (Batra 1967). Six of the isolates yielded nearly identical 28S sequences (e.g. C3169, = CBS 142652; **MF399173**) that were similar to that of the ex-type culture of *R. canadensis* (543/555, 98% match, with **EU177458**) but more closely matched those of unnamed *Raffaelea* spp. in the *R. canadensis* complex: *Raffaelea* sp. PL6404 (548/553 bp, 99% match, with **KT803726**) (Bateman et al. 2015), *Raffaelea* sp. C1943 (546/554

bp, 99% match, with **EU177465**) (Harrington et al. 2010), and *Raffaelea* sp. C2711 (544/553 bp, 98% match, with **HQ688665**) (Harrington et al. 2011). Abrahamson and Norris (1969) consistently isolated an unidentified fungus, tentatively designated *Raffaelea* sp. UWE-132L, from heads of male and female *X. politus.* Their illustrations show small, hyaline conidiophores with spherical conidia that were similar to those of *R.* cf. *canadensis* RNC5. However, their representative culture of UWE-132L (CBS 410.68) has a 28S sequence nearly identical to that of *R. tritirachium* (personal communication, Gerard Verkleij, Fungal Collection Curator, CBS), which produces elongated, ellipsoidal spores (Batra 1967).

Six cultures of '*Raffaelea* cf. *lauricola* RNL1' were isolated from *X. politus* galleries and beetles on walkabout plates, but this species was not isolated from surface-sterilized beetles. The six isolates (e.g. C3162) yielded an identical 28S sequence (**MF399178**) that was most similar to that of *R. lauricola* (528/545 bp, 97% match, with **KF515710** from ex-type isolate). Two isolates of a third *Raffaelea* sp. closely-related to or conspecific with *R. tritirachium* were isolated from *X. politus*: C3431 from a larval cradle in *Populus* sp. (28S = **MF399180**, 544/547 bp matching with **EU177464** from ex-type) and C3400 from the exterior of a female in *Betula papyrifera* (28S = **MF399179**, 545/547 bp matching with **EU177464**). A single isolate (C3478; 28S = **MF399186)** with a sequence similar to that of *Ophiostoma karelicum* (467/475 bp, 98% match, with **EU443756**) (Linnakoski et al. 2008) was obtained from the surface of a female.

Phylogenetic analyses

Representatives of the putative *Phialophoropsis* species from *Trypodendron* and the putative *Toshionella* from *I. pubipennis* were included in a six-gene Bayesian tree of the Ceratocystidaceae (Fig. 8). There was strong support for a monophyletic

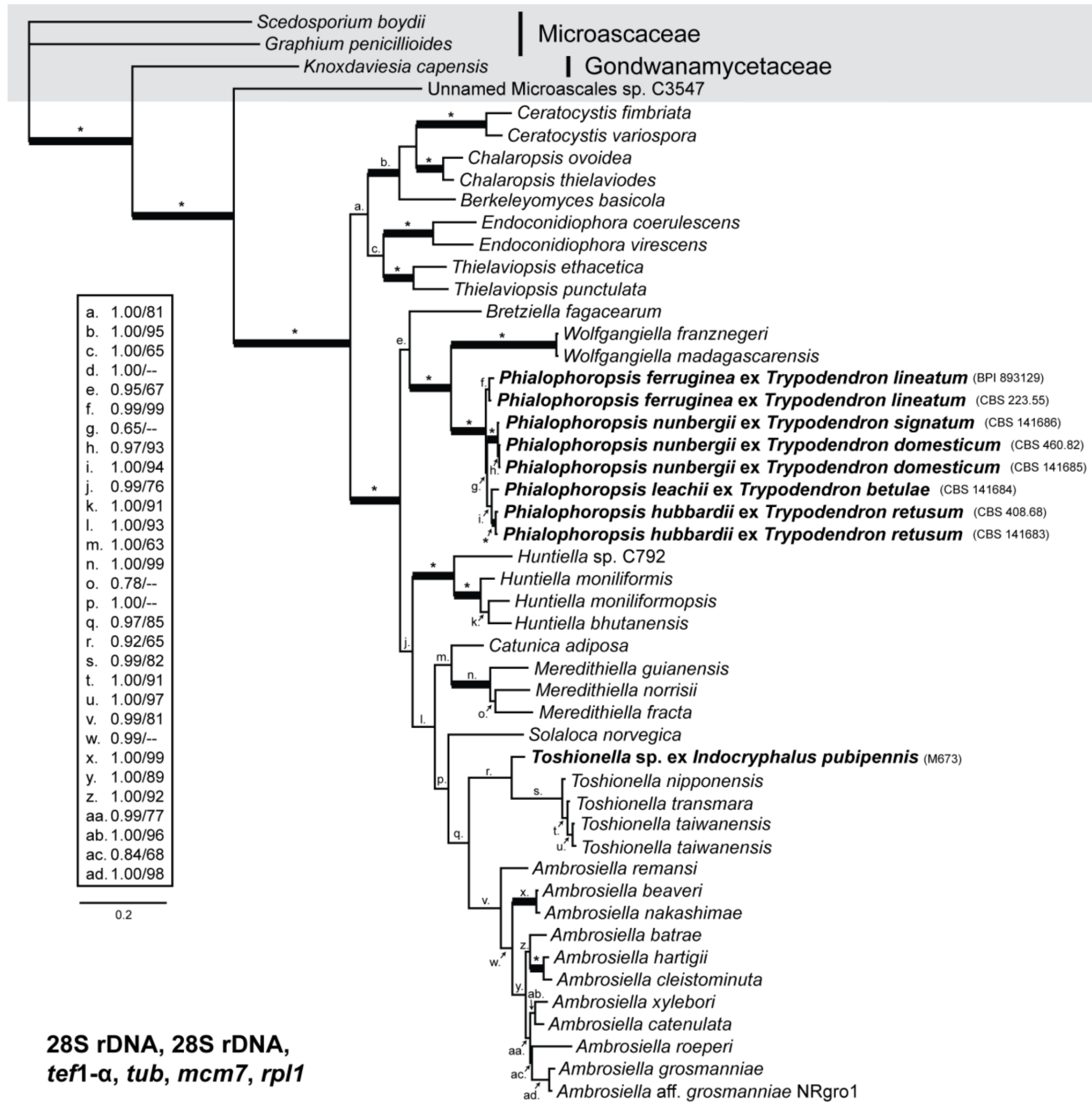

Figure 8. Multi-locus tree from Bayesian analysis of the combined 28S rDNA, 18S rDNA, *tef*1- α*, tub, mcm7,* and *rpl1* dataset of the Ceratocystidaceae and representatives of the Microascales. Mycangial symbionts of Xyloterini ambrosia beetles are in bold. Posterior probabilities from Bayesian analysis and bootstrap support values (>50%) from maximum parsimony analysis are indicated in the key and correspond to lowercase letters on branches. Thickened branches indicate Bayesian posterior probability of 1.0 and maximum parsimony bootstrap support ≥ 95%. Branches with Bayesian posterior probability 1.0, bootstrap support 100% are indicated with asterisks and not included in the key. Microascales representatives outside the Ceratocystidaceae are indicated by the grey-shaded box, with other families indicated with vertical bars. Accession numbers for bolded species in the Iowa State University collection (C) and other collections are given where available; collection information for other species representatives are detailed in Chapter 5. The tree is rooted to *Scedosporium boydii,* which was allowed to collapse into a polytomy with *Graphium penicillioides*. Bar = 0.2 estimated substitutions per site.

Phialophoropsis sister to *Wolfgangiella,* with separation of *P. ferruginea* (from *T. lineatum*) from the symbiont from *T. domesticum* and *T. signatum*, the *T. retusum* symbiont, and the *T. betulae* symbiont. *Phialophoropsis* and *Wolfgangiella* formed a sister group to *Bretziella fagacearum,* as has been reported previously (de Beer et al. 2014; Mayers et al. 2015; Chapter 5). Only the ITS and 28S sequences were obtained from the mycangial symbiont of *I. pubipennis*, and both of these sequences place the fungus in the genus *Toshionella*, which is the mycangial symbiont of Asian *Scolytoplatypus* spp. (Chapter 5).

Representatives of the prothoracic and oral symbionts of *X. politus* were included in a two-gene Bayesian tree of the Diaporthomycetidae, including representatives of the Ophiostomatales (Fig. 9). *Raffaelea* cf. *canadensis* RNC5 was placed clearly within *Raffaelea.* Isolates A1264 (= CBS 144155) and A1268 (= CBS 142646) of the olive-green fungus, which dominated the galleries and was isolated from the prothoracic mycangia of *X. politus,* formed a sister group to the Ophiostomatales on a long branch. There was good posterior probability support for the Ophiostomatales as a monophyletic group excluding the *X. politus* prothoracic symbionts, but there was only moderate support for the branch comprising both the prothoracic symbiont and the Ophiostomatales. The recently-described *Afroraffaelea ambrosiae* (Bateman et al. 2017) was placed in the Ophiostomales clade, but on a very long branch (Fig. 9). When the analysis was repeated without *A. ambrosiae,* the support values for the Ophiostomatales (Fig. 9, branch h.) improved significantly (posterior probability 1.0, MP bootstrap support 91%), whereassupport for the common clade of the prothoracic symbiont and the Ophiostomatales (Fig. 9, branch g.) decreased slightly (posterior probability 0.83,

bootstrap support <50%). It is noteworthy that both *A. ambrosiae* (Bateman et al. 2017) and the prothoracic symbiont of *X. politus* do not tolerate cycloheximide, whereas members of the Ophiostomatales tolerate this antibiotic (Harrington 1981). Thus, the prothoracic symbiont of *X. politus* appears to fall outside of the Ophiostomatales but within the Diaporthomycetidae, with no close relatives known and uncertain placement in order or family.

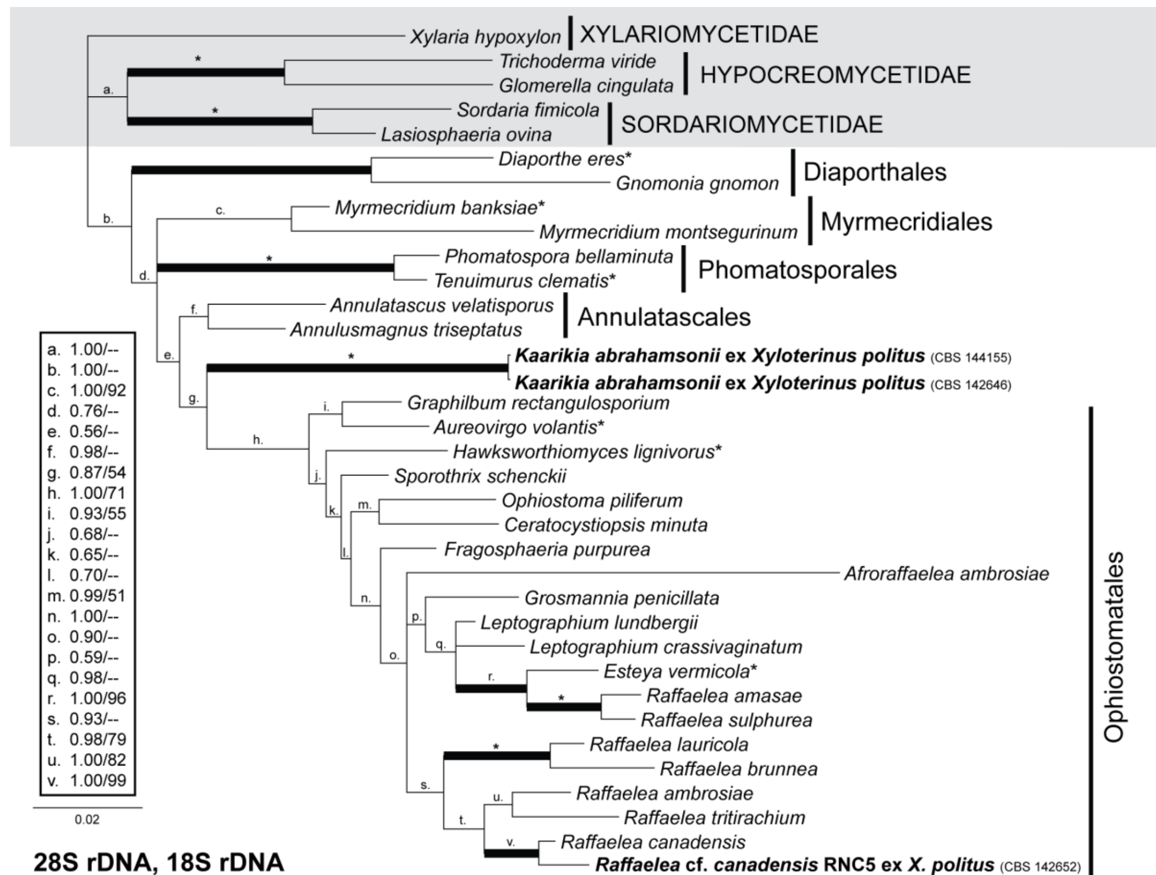

Figure 9. Multi-locus tree from Bayesian analysis of the combined 28S and 18S rDNA dataset of the two mycangial symbionts of *Xyloterinus politus* (*Kaarikia abrahamsonii* and *Raffaelea* cf. *canadensis* RNC5) and other representatives of orders of the Diaporthomycetidae. The two mycangial symbionts are in bold. Posterior probabilities from Bayesian analysis and bootstrap support values (>50%) from maximum parsimony analysis are indicated in the key and correspond to lowercase letters on branches. Thickened branches indicate Bayesian posterior probability of 1.0 and maximum parsimony bootstrap support ≥95%. Branches with Bayesian posterior probability 1.0, bootstrap support 100% are indicated with asterisks and not included in the key. Taxa for which 18S rDNA sequences were not available are marked with asterisks. The tree is rooted to *Xylaria hypoxylon* and includes other Sordariomycetes outside the Diaporthomycetidae (grey box, classes in all caps). Bar = 0.02 estimated substitutions per site.

Taxonomy

Morphological comparisons and phylogenetics support recognition of three new species of *Phialophoropsis* associated with *Trypodendron.* A new genus and species is proposed for the prothoracic symbiont of *X. politus.* The oral symbiont of *X. politus* may be a new *Raffaelea* species, but the species we isolated is closely related to *R. canadensis*, and significant differences in morphology with *R. canadensis* ex-type culture CBS 168.66 were not found.

Disclaimer: The novel taxa proposed in this chapter of the dissertation are not intended to represent validly published names under the International Code of Nomenclature for algae, fungi, and plants.

PHIALOPHOROPSIS L.R. Batra emend. T.C. Harr., Fungal Biology 119: 1086. 2015

TYPE SPECIES – *Phialophoropsis trypodendri* L.R. Batra

Phialophoropsis trypodendri L.R. Batra, Mycologia 59: 1008. 1967 ("1968")

MycoBank MB 336297.

Synonym: Ambrosiella trypodendri (L.R. Batra) T.C. Harr., Mycotaxon 111:355. 2010 MycoBank MB 515299.

Typus. **USA:** *Arkansas:* Newton County, Deer, ambrosia growth in galleries of *T. scabricollis* in *Pinus echinata,* 10 April 1963, *L.R. Batra,* 1952-LRB (holotype, including dried ex-type culture, KANU 394426; isotype BPI 422498; isotype BPI 422499)

Comments –Batra (1967) designated 1952-LRB in KANU (presumably KANU 394426) as the type for *T. trypodendri*, and two isotypes (BPI 422498 or BPI 422499) are also labelled 1952-LRB. We were unsuccessful in finding phialides in the larval cradles or dried cultures contained in KANU 394426. Batra (1967) illustrated phialidic

condiophores and conidia from this material, and Seifert et al. (2011) illustrated the fungus based on Batra's illustration and the BPI material. We could not find conidia or conidiophores in either of the BPI isotypes, but there were three packets labelled LRB-1952 associated with the isotypes that had phialoconidia typical of *Phialophoropsis*. There is no known living culture of *P. trypodendri,* but a dried culture in KANU 394426 was very similar in morphology to other *Phialophoropsis* spp., though no conidia were seen. ITS sequencing was unsuccessful from Batra's holotype material (KANU 394426), but females of *T. scabricollis* caught in Georgia yielded the same ITS sequence as females from eight Missouri locations in an earlier study (Mayers et al. 2015) (Fig. 1).

Phialophoropsis ferruginea (Math.-Käärik) T.C. Harr., Fungal Biology 119: 1087. 2015 (Fig. 2)

MycoBank MB 812586.

Basionym: Monilia ferruginea Math.-Käärik, Meddelanden fran Statens Skogforskningsinstitut 43: 57 (1953)

MycoBank MB 474947.

Synonym: Ambrosiella ferruginea (Math.-Käärik) L.R. Batra, Mycologia 59: 1000 (1967)

MycoBank MB 326141.

Typus. **Sweden:** Regna, from *Trypodendron lineatum* gallery in *Picea abies*, 17 May 1952, *A. Mathiesen-Käärik,* lectotype (dried culture, BPI 910629); ex-type C3549 (living culture CBS 223.55, MUCL 9940).

Comments –Mathiesen-Käärik (1953) did not designate a type specimen when she described *Monilia ferruginea* from *T. lineatum.* Batra (1967) indicated that she deposited

a holotype in the Forest Research Institute, Stockholm, Sweden (now Department of Forest Products, Faculty of Forest Sciences, Swedish University of Agricultural Sciences, Uppsala), but all herbarium specimens from the institute were lost (Geoffrey Daniel, personal communication). Mathiesen-Käärik deposited a culture of *M. ferruginea* from her original description (Mathiesen-Käärik 1953) in CBS (CBS 223.55) (personal communication, Gerard Verkleij, Fungal Collection Curator, CBS), with collection information matching the type: May 17, 1952, gallery of *T. lineatum* in *P. abies,* Regna, Sweden (Batra 1967). This culture had since been lost in CBS, but it was duplicated in MUCL (MUCL 9940) in 1967, likely by G.L. Hennebert (personal communication, Cony Decock, Principal Curator, MUCL). We obtained culture MUCL 9940 (C3549), which had consistent culture morphology to Mathiesen-Käärik's (1953) and Batra's (1967) descriptions. We have deposited a dried specimen of MUCL 9940 in BPI and designate it as the lectotype for *P. ferruginea*, and we deposited MUCL 9940 in CBS to replace the missing CBS 223.55. This culture is similar to our other European and USA collections from *T. lineatum* in morphology and DNA sequences. Ambrosia growth discovered by Hartig (1872a) and illustrated by later authors (Neger 1910, 1911; Hadorn 1933; Funk 1965) in galleries of *T. lineatum* were probably *P. ferruginea.* Isolates CBS 460.82 from *T. domesticum* and CBS 408.68 from *T. retusum,* often cited as *P. ferruginea* in phylogenetic studies (Alamouti et al. 2009; Six et al. 2009; de Beer et al. 2014; Harrington et al. 2014; Mayers et al. 2015), are described below as new species.

Phialophoropsis nunbergii C. Mayers & T.C. Harr., **sp. nov.** (Fig. 3)

MycoBank MB 824943.

Etymology: After Marian Nunberg, who in 1951 first discovered the mycangia of *Trypodendron*.

Typus: **Germany:** Hainich, from female *T. domesticum* caught in flight, April 2015, *P. Biedermann,* holotype (dried culture, BPI 910634); ex-type C3828 (CBS 141685).

Colonies on malt yeast extract agar 13–67 mm diam. after 7 d at 25 C, odor lightly sweet to non-distinctive, surface growth superficial, hyaline, regular, reverse ocherous to umber with chestnut center, reverse becoming chestnut with age, diffusible pigment staining agar bay. *Sporodochium-like* masses sometimes forming in older cultures, white, buff, or greyish-sepia, spherical, scattered or forming a dense carpet, rarely bearing rust-colored liquid drops, without conidiphores. *Phialoconidiophores* rare in culture, borne in small, lone tufts, branching or in palisades, hyaline to red-brown, sometimes flask-shaped and subtended by a stalk of simple hyphae, producing phialoconidia singly or in chains. *Phialoconidia* produced from shallow- to moderately-seated phialides, hyaline to lightly red-brown, thick-walled, smooth, aseptate, cylindrical with rounded apices to globose, 6–13.5 × 7.5–16.5 µm, first conidium larger and somewhat obovoid, subtending conidia shorter and more barrel-shaped, detaching singly or in chains.

Other cultures examined: **Germany:** *Hann-Munden*, from *T. domesticum* gallery in *Sylvatica fagus*, June 1971, *G. Zimmerman*, C2230 (CBS 460.82). *Hainich:* from female *T. domesticum* caught in flight, April 2015, *P. Biedermann,* C3826. *Hainich:* from

female *T. domesticum* caught in flight, April 2015, *P. Biedermann,* C3827. *Hainich:* from female *T. domesticum* caught in flight, April 2015, *P. Biedermann,* C3829. *Hainich:* from female *T. domesticum* caught in flight, April 2015, *P. Biedermann,* C3831. *Hainich:* from female *T. domesticum* caught in flight, June 2015, *P. Biedermann,* C3833. *Hainich:* from female *T. domesticum* caught in flight, June 2015, *P. Biedermann,* C3834. *Hainich:* from female *T. domesticum* caught in flight, July 2015, *P. Biedermann,* C3835. **Netherlands:** *Wageningen:* from female *T. domesticum* caught in flight, June 2015, *L. van de Peppel*, C3832. **Japan:** *Iwate Prefecture:* Morioka, from mycangium of *T. signatum* caught landing on unidentified tree species, 7 April 2015, *H. Masuya*, C3914. *Iwate Prefecture:* Morioka, from mycangium of *T. signatum* caught landing on unidentified tree species, 7 April 2015, *H. Masuya*, C3915. *Iwate prefecture:* Morioka, from mycangium of *T. signatum* caught landing on unidentified tree species, 7 April 2015, *H. Masuya*, C3919. *Iwate Prefecture:* Morioka, from mycangium of *T. signatum* caught landing on unidentified tree species, 7 April 2015, *H. Masuya*, C3920. *Iwate Prefecture:* Morioka, from mycangium of *T. signatum* caught landing on unidentified tree species, 7 April 2015, *H. Masuya*, C4056. *Iwate Prefecture:* Morioka, from mycangium of *T. signatum* caught landing on unidentified tree species, 7 April 2015, *H. Masuya*, C4057.

Notes. This species is the mycangial symbiont of *T. domesticum* and *T. signatum* in Europe and Japan, respectively. The bulging, flask-shaped phialoconidiophores on stalks produced by isolate C3914 from *T. signatum* in Japan are unique within *Phialophoropsis*, but the conidiophores of isolates from *T. domesticum* appeared more typical. The isolates from *T. signatum* also were lighter pigmented. The Japanese *T. signatum* also had distinct ITS sequences (Fig. 1A) and may represent a separate species

in spite of limited DNA sequence differences (Fig. 8). The growth rate of *P. nunbergii* is notably faster than isolates from *T. retusum* and *T. betulae,* and it produces less pigment than isolates from those species and *P. ferruginea*. Previous associations of *Phialophoropsis*-like fungi with *T. domesticum* (Hartig 1872b; Francke-Grosmann 1956, 1958; Batra 1967; Zimmerman 1973) or *T. signatum* (Francke-Grosmann 1952, 1956, 1958; Nakashima et al. 1992) may have been *P. nunbergii*.

Phialophoropsis hubbardii C. Mayers, T.C. Harr., McNew & Roeper, **sp. nov.** (Fig. 4)

MycoBank MB 824944.

Etymology: After Henry Guernsey Hubbard, who in 1897 produced the first comprehensive review on ambrosia beetles and their fungi, which included the first illustration of *Phialophoropsis* from *T. retusum.*

Typus: **USA:** *Michigan:* Montcalm County, Alma College Ecological Tract, ambrosia growth in gallery of *T. retusum* in *Populus grandidentata*, May 2014, *R. Roeper,* holotype M596 (BPI 910630); ex-type C3386 (living culture CBS 141683; dried culture BPI 910631).

Colonies on malt yeast extract agar 11–77 mm diam. after 7 d at 25 C, odor lightly sweet to non-distinctive, strongly sweet to acetone-like when aerial hyphae abundant, surface sepia, becoming umber, rarely with cottony aerial hyphae, white to buff, becoming cinnamon, margin submerged and aerial, hyaline becoming umber, regular, underside umber, becoming chestnut, diffusible pigment staining media umber. *Gallery growth* dense, glistening, umber, in patches in larval cradles, superficial in main tunnel. *Phialoconidiophores* borne in patches in larval cradles but not observed in culture, hyaline to red-brown, bearing terminal phialoconidia singly or in chains.

Phialoconidia produced from shallow- to deeply-seated phialides, hyaline, thick-walled, smooth, aseptate, cylindrical with rounded apices to globose, 5.5–12.5 × (4.5) 7–17 µm, detaching singly or in chains, first conidium somewhat obovoid. *Growth in mycangium* composed of arthrospore-like cells 4.5–18 (20.5) µm in diameter, irregular in shape, thick-walled, single or in septate, rarely branched, chains of two or more cells.

Other cultures examined: **USA:** *Wisconsin:* Madison, from mycangium of *T. retusum,* June 1968, *D. Norris,* C3550 (CBS 408.68). *Michigan:* Montcalm County, Alma College Ecological Tract*,* from egg niche of *T. retusum* in gallery in *Populus grandidentata,* May 2014, *R. Roeper,* C3403.

Specimens examined: **USA:** *Michigan:* Benzie County, gallery of *T. retusum* in *Populus grandidentata,* July 2013, *R. Roeper,* M594, M595. Montcalm County, Alma College Ecological Tract, gallery of *T. retusum* in *Populus* sp., May 2015, *M. Bunce*.

Notes. The mycangial symbiont of *T. retusum* grows more slowly and produces more diffusible pigment than other *Phialophoropsis* spp. with the exception of isolates from *T. betulae*, to which it is closely related (Fig. 8). *P. hubbardii* grows slightly faster than isolates from *T. betulae* and produces more aerial hyphae and less diffusible pigment. Although *T. retusum* and *T. betulae* are sympatric, they have different host preferences; *T. retusum* is restricted to *Populus,* and *T. betulae* to *Betula* and rarely *Alnus* (Wood 1957). Presumably the same is true of their symbionts. Previous associations of *Phialophoropsis*-like fungi with *T. retusum* (Hubbard 1897; Leach et al. 1940; Batra 1967; Roeper et al. 1980; Roeper and French 1981; Cassar 1993) may have been *P. hubbardii*.

Phialophoropsis leachii C. Mayers, T.C. Harr., McNew & Roeper, **sp. nov.** (Fig. 5)

MycoBank MB 824945.

Etymology: After J.G. Leach, who studied fungi associated with many insects, including *T. betulae* and *T. retusum*.

Typus: **USA:** *Michigan:* Isabella County, Chippawa Township, ambrosia growth in larval cradle of *Trypodendron betulae* in *Betula papyrifera*, June 2014, *R. Roeper*, holotype M593/Gal10 (BPI 910632); ex-type C3404 (living culture CBS 141684, dried culture BPI 910633).

Colonies on malt yeast extract agar 8–52 mm diam. after 7 days at 25 C, odor lightly sweet to non-distinctive, surface superficial, umber, dense, rarely ocherous, raised, margin superficial, hyaline, underside ocherous, becoming chestnut to fuscous black, diffusible pigment staining media rust to chestnut. *Gallery growth* dense, glistening, umber carpet forming patches in larval cradles, superficial in main tunnel. *Phialoconidiophores* borne in patches in larval cradles but not observed in culture, hyaline to red-brown, bearing terminal phialoconidia singly or in chains. *Phialoconidia* produced from shallow- to deeply-seated phialides, hyaline, thick-walled, smooth, aseptate, cylindrical with rounded apices to globose, 5–9.5 × 6–17 µm, detaching singly or in chains, first conidium somewhat obovoid.

Other cultures examined: **USA:** *Michigan:* Isabella County, Chippawa Township, larval cradle of *Trypodendron betulae* in *Betula papyrifera*, June 2014, *R. Roeper*, C3405.

Notes. The mycangial symbiont of *T. betulae* is most similar to the symbiont of *T. retusum* (*P. hubbardii*) in morphology and is closely related to it in multi-gene analysis

(Fig. 8), but it has a distinct ITS sequence from other *Phialophoropsis* spp. (Fig. 1A). It grows more slowly, produces almost strictly superficial surface growth, produces more diffuse pigment when compared to *P. hubbardii,* and it tends to produce a dense mycelium in culture that is noticeably harder to pierce with transfer instruments. It has a slower growth rate than the other species in the genus. We observed the culture variation but not the thick, white, highly-sporulating phenotype illustrated by Leach et al. (1940). Previous associations of *Phialophoropsis*-like fungi with *T. betulae* (Leach et al. 1940; Roeper et al. 1980; Roeper and French 1981; Cassar 1993; Kühnholz 2004) may have been *P. leachii.*

KAARIKIA C. Mayers & T.C. Harr., **gen. nov.**

MycoBank MB 824946.

Etymology: After Aino Mathiesen-Käärik, who worked with fungi associated with many forest insects.

Solitary, obovoid, truncate conidia formed terminally on simple conidiophores. Thick-walled chlamydospores, terminal or intercalary, inside pigmented hyphae. Sexual state unknown.

Type species: Kaarikia abrahamsonii C. Mayers & T.C. Harr., sp. nov.

Kaarikia is unique in morphology and DNA sequences, necessitating treatment as a monotypic genus. Phylogenetic analyses place *Kaarikia* within the Diaporthomycetidae, and it may have affinity with the Ophiostomatales and Annulatascales (Fig. 9).

Kaarikia abrahamsonii C. Mayers, T.C. Harr. & Roeper **sp. nov.** (Fig. 7)

MycoBank MB 824947.

Etymology: After Lawrence P. Abrahamson who, along with Dale M. Norris, performed pioneering work on the dual mycangia and ambrosia fungi of *Xyloterinus politus*.

Typus: **USA:** *Michigan:* Isabella County, Ambrosia growth in galleries of *X. politus* in *Betula papyrifera*, June 2014, *R. Roeper,* M599 holotype (BPI 910623); ex-type A1264 (living culture CBS 144155; dried culture BPI 910624).

Colonies on malt yeast extract agar 45–56 mm diam. after 7 days at 25 C, odor earthy, surface superficial, umber with irregular patches of buff to mouse grey aerial hyphae, margin irregular, submerged, dendroid, buff, becoming dense and ochreous, reverse umber to olivaceous black, becoming chestnut, producing non-diffusible red-brown pigment. *Chlamydospores* formed in culture hyaline to red-brown, thick-walled, single celled or rarely septate, globose to irregular, 6.5–22.5 × 8.0–28 µm, borne terminally or intercalary inside red-brown hyphae, separating by tearing of the pigmented hyphal membrane. *Gallery growth* black, superficial in main tunnels; white to green-grey in larval cradles and egg niches, with thick mat composed of palisades of conidiophores. *Conidiophores* simple, unbranched, erect, septate or aseptate, bearing single terminal conidia. *Conidia* obovoid to pyriform, hyaline, thick-walled, 6–18 × 12.5–35.5 µm, truncate, aseptate or rarely one- or two-septate. *Chlamydospores* in gallery as in culture but globose, smaller, 6.0–13.5 um diam. *Mycangium growth* spherical, thick-walled, 10–17.5 µm diam.

Other cultures examined: **USA:** *Michigan,* Montcalm County, Alma College Ecological Tract, from prothorax of female *X. politus* in *Populus* sp., 21 May 2014, *R. Roeper,* A1268 (CBS 142646; dried culture BPI 910625). Isabella County, from gallery of *X. politus* in *Betula papyrifera*, June 2014, *R. Roeper,* A1262. From gallery of *X. politus* in *B. papyrifera*, June 2014, *R. Roeper,* A1263. From prothorax of female *X. politus* in *B. papyrifera*, June 2014, *R. Roeper,* A1265. From prothorax of female *X. politus* in *B. papyrifera*, June 2014, *R. Roeper,* A1266. From prothorax of female *X. politus* in *B. papyrifera*, June 2014, *R. Roeper,* A1267. From prothorax of female *X. politus* in *B. papyrifera*, June 2014, *R. Roeper,* A1269.

Other specimens examined: **USA:** *Michigan:* Montcalm County, Alma College Ecological Tract, galleries of *X. politus* in *Acer rubrum,* 20 August 2013, *R. Roeper,* M598 (BPI 910626).

Notes. The prothoracic symbiont of *X. politus* and dominant ambrosia form is a unique fungus that appears to be unrelated to other known symbionts of ambrosia beetles. The unknown fungus from *X. politus* illustrated by MacLean and Giese (1968) and the *X. politus* prothoracic symbiont UWE-132M studied by Abrahamson and Norris (1969) are undoubtedly *K. abrahamsonii*.

Discussion

A surprising diversity of ambrosia fungi were identified from *Trypodendron* spp., *Indocryphalus pubipennis*, and *Xyloterinus politus*. The well-developed prothoracic mycangium of *Trypdoendron* was closely tied to *Phialophoropsis* spp. We confirmed the association of *P. ferruginea* with European and American specimens of *T. lineatum*, but other *Trypodendron* spp. were found to harbor novel *Phialophoropsis* symbionts. The prothoracic mycangium of *I. pubipennis* was illustrated for the first time, and an unnamed

species of *Toshnionella* was associated with its mycangium. We confirmed previous findings that *X. politus* carries different symbionts (*Raffaelea* cf. *canadensis* RNC 5 and *Kaarikia ambrahamsonii*) in its two types of mycangia, oral and prothoracic. Thus, four genera of mycangial symbionts were identified for the tribe, and numerous non-mycangial species in the Ophiostomatales were also found associated with these beetles.

The Xyloterini are among the earliest ambrosia beetle tribes in the Scolytinae (Gohli et al. 2017; Pistone et al. 2017). The crown age of tribe Xyloterini is estimated at 41 Ma (Gohli et al. 2017) to 50 Ma (Pistone et al. 2017), similar to the estimated origin of the Scolytoplatypodini (estimated by the same authors at 34 and 52 Ma, respectively), which may have been the first tribe to domesticate Ceratocystidaceae as mycangial symbionts (Chapter 5). In contrast to the Scolytoplatypodini, the range of mycangium types and wide range of mycangial symbionts suggest the symbiosis was not well developed or specialized in the early evolutionary history of the Xyloterini. Given its age, it is also surprising that there are so few genera and species in the Xyletorini. The circumboreal *Trypodendron* has 14 species (Robideau et al. 2016), the Asian *Indocryphalus* has eight (Cognato et al. 2015), and the North American *Xyloterinus* is monotypic (MacLean and Giese 1967). This stands in stark contrast to the much younger and more speciose tribe Xyleborini, for instance (Gohli et al. 2017; Pistone et al. 2017).

Indocryphalus

Indocryphalus has not been well studied, but it may not be monophyletic (Cognato et al. 2015), and some species may have split from the other Xyloterini early in the history of the tribe (Gohli et al. 2017; Pistone et al. 2017). The prothoracic mycangium openings in *Indocryphalus* vary and are either vertically- or horizontally-oriented (Wood 1957; Beaver 2000; Cognato et al. 2015). The single species included in

this study, *I. pubipennis,* had a horizontal mycangium opening (Cognato et al. 2015), similar to but wider than those of *Trypodendron*. The *I. pubipennis* mycangia are significantly smaller in volume than the mycangia of *Trypodendron* but have similarly reticulated walls. The smaller mycangium of *I. pubipennis* carries a *Toshionella* symbiont, which are otherwise associated with *Scolytoplatypus* ambrosia beetles in Asia (Chapter 5). *Indocryphalus pubipennis* is sympatric with several *Scolytoplatypus* species in Asia (Beaver and Gebhardt 2006; Cognato et al. 2015), and it is possible that *Toshionella* is a recent acquisition from *Scolytoplatypus* or vice versa. Obviously, the ambrosia fungi of more *Indocryphalus* species need to be studied, especially those with different mycangium openings and from different locations to determine if *Toshionella* symbionts are generally the primary symbionts for the genus. The only other *Indocryphalus* species included so far in phylogenetic analyses, *I. aceris,* is more divergent from *I. pubipennis* than *Trypodendron* is from *X. politus,* and the estimated crown age of *Indocryphalus* is older than the estimate for the divergence of the other two genera (Gohli et al. 2017).

Xyloterinus

The monotypic genus *Xyletorinus* is unique, and its phylogenetic relationship to *Indocryphalus* and *Trypodendron* is not clear (Cognato et al. 2015). *Xyloterinus politus* is the only known Scolytid ambrosia beetle to have two sets of mycangia: a pair of small oral mycangia in both sexes and prothoracic mycangia in females (Abrahamson and Norris 1966; Abrahamson 1967; MacLean and Giese 1968), and each mycangium type is specific for their respective fungi. The prothoracic mycangia of *X. politus* are simple, shallow excavations in the base of the prothorax that are trimmed with setae and do not lead to large internal cavities. The vertical mycangium openings of some species of

Indocryphalus (Beaver 2000; Cognato et al. 2015) may be homologous to the vertical openings of *X. politus* (Wood 1957; Cognato et al. 2015). However, *X. politus* appears to be sister to *Trypodendron* (Gohli et al. 2017; Pistone et al. 2017), and its prothoracic basin mycangium may be a reduced organ derived from an ancester with mycangia more similar to those of *Trypodendron* or *Indocryphalus*. In contrast, Wood (1957) considered *X. politus* to have the most primitive characters among the Xyloterini, with *Trypodendron* having the most specialized characters, and *Indocryphalus* with intermediate characters. Perhaps the common ancestor of the Xyloterini had a non-selective, prothoracic mycangium (or no mycangium), and the three genera represent three different, separate adaptations to fungus-farming in the tribe, with separate mycangium developments and symbiont captures.

Abrahamson and Norris (1969) carefully dissected *X. politus* males and females, and they isolated UWE-132M from 0/62 oral mycangia and 62/62 prothoracic mycangia of 32 females, but they were unable to isolate UWE-132M from seven males. The morphology of UWE-132M is consistent with the morphology of *K. abrahamsonii* recovered in this study, including the large, darkly pigmented hyphae, club-shaped aleurioconidia in galleries, and large, spherical, thick-walled chlamydospores. A fungus isolated from *X. politus* by MacLean and Giese (1968) also appears to be *K. abrahamsonii,* though the other fungi they isolated are common contaminating molds and not important associates (Abrahamson and Norris 1969). It is unclear if the spherical chlamydospores of *K. abrahamsonii* actually grow in the prothoracic mycangia of *X. politus* or are just scooped up from the gallery walls and stored.

We isolated three different *Raffaelea* species from *X. politus: Raffaelea* cf. *canadensis* RNC5*, Raffaelea* cf. *lauricola* RNL1, and *Raffaelea tritirachium.* Batra (1967) implicated an unnamed *Raffaelea* sp., '*Raffaelea* taxonomic sp.-1', as the primary symbiont of *X. politus* and considered it separate from *R. tritirachium*. Abrahamson and Norris (1969) isolated a *Raffaelea* sp., UWE-132L, from 62/62 oral mycangia of females and 7/7 oral mycangia of males, but it was only isolated from one of the 62 sampled prothoracic mycangia of 32 females. The illustrations of globose conidia of UWE-132L provided by Abrahamson and Norris (1969) would be consistent with the conidia of *R.* cf. *lauricola* RNL1 or *R.* cf. *canadensis* RNC5, which also produces truncate conidia. However, the culture deposited by Abrahamson and Norris in CBS proved to be near or conspecific with *R. tritirachium,* which produces distinctive elongated conidia (Batra 1967). Batra (1967) considered *R. tritirachium* an auxiliary rather than a primary ambrosia fungus in association with *Monarthrum fasciatum,* and we also isolated *R. tritirachium* from *X. politus* as a superficial contaminant. Alternatively, *X. politus* may harbor several *Raffaelea* spp. in its oral mycangia, as has been found with *Xyleborus glabratus* (Harrington and Fraedrich 2010). Paired preoral mycangia are not known in other genera of the Xyloterini, but they have evolved multiple times in the Scolytinae (Hulcr and Stelinski 2017), and they are often associated with *Raffaelea* spp. that are non-specific in their beetle associations (Harrington et al. 2010, 2011; Vanderpool et al. 2017). Regardless, *Raffaelea* cf. *canadensis* RNC5 was the most commonly isolated *Raffaelea* in this study, and it was the only symbiont recovered from surface-sterilized beetle heads. If the ancestor of the Xyletorini had a prothoracic mycangium and *Indocryphalus* diverged early from *Trypodendron* and *Xyletorinus* (Gohli et al. 2017;

Pistone et al. 2017), then the oral mycangia in *X. politus* arose later. *Kaarikia abrahamsonii* could be a parasitic or ineffective symbiont of a reduced mycangium, and *R. canadensis* may serve as the primary symbiont of *X. politus*, though both fungi were consistently found as ambrosia in galleries of *X. politus* and *K. abrahamsonii* was more conspicuous.

Trypodendron

The most developed Xyletorini mycangium is found in *Trypodendron*, whose mycangium openings are horizontal slits on the side of the prothorax that lead to a pair of large, folded, prothoracic pleural mycangia with sclerotized, reticulated walls (Nunberg 1951; Francke-Grosmann 1956, 1958, 1967; Abrahamson et al. 1969; Schneider and Rudinsky 1969). Although a fossil specimen initially labeled as *Trypodendron* was given a date of 35 Ma (Hulcr et al. 2015), the specimen was unlikely a Scolytid (Hopkins 1900; Wood and Bright 1992). Extant *Trypodendron* species have an estimated crown age of only 9.0 Ma (Pistone et al. 2017) to 11.2 Ma (Gohli et al. (2017), which corresponds to the relatively young crown age of their *Phialophoropsis* symbionts (Chapter 5). Thus, *Trypodendron* may represent a young lineage with an advanced mycangium and a specialized, co-adapted fungal symbiont.

Trypodendron mycangia differ slightly in cavity diameter, shape, and in the degree to which the terminus curls (Francke-Grosmann 1956), and these subtle differences may or may not correlate with beetle phylogeny and fungal associates. The mycangia of *T. domesticum* and *T. signatum* both have greatly-enlarged ascending cavities when compared to the mycangia of *T. lineatum* (Francke-Grosmann 1956), which may explain why *T. domesticum* and *T. signatum* appear to carry the same or closely related *Phialophoropsis* sp. The mycangial propagules might be somehow

adapted to the particular shape and size of the mycangium, which may confer some degree of specificity.

Early studies on the ambrosia fungi of *Trypodendron* (Hubbard 1897; Neger 1910, 1911; Hadorn 1933; Leach 1940; Funk 1965) recognized that they produced beaded, monilioid chains of cells, now recognized as the chained phialoconidia of *Phialophoropsis* (Mayers et al. 2015). Each species of *Trypodendron* may carry a particular *Phialophoropsis* symbiont in its prothoracic mycangium. As presented here, *P. nunbergii* is associated with both *T. domesticum* and *T. signatum*, but the symbionts from these two beetle species differ slightly and may represent cryptic species. Different *Phialophoropsis* symbionts of sympatric *Trypodendron* species may be explained by differences in host tree preferences. For example, the geographic ranges of *T. retusum* and *T. betulae* overlap in the USA, but the former is limited to *Populus* and the latter is limited to *Betula* and *Alnus incana* (Wood 1957). Populations of *T. lineatum* or *T. signatum* consistently yielded their specific fungal symbiont, whether the sampled insects were European, Asian, or North American.

Superficial fungi

A wide variety of fungi have been associated loosely with *Trypodendron* spp. (Kühnholz 2004). Besides ubiquitous contaminating molds such as *Trichoderma* and *Aspergillus,* several genera of common insect-associated fungi have been isolated from *Trypodendron* galleries (Bakshi 1950, 1952; Mathiesen-Käärik 1953; Funk 1965; Hinds and Davidson 1972; Zimmerman 1973; Babuder and Pohleven 1993; Carlier et al. 2006; Jankowiak et al. 2017). We isolated many superficial fungi from *Trypodendron* galleries and beetle surfaces, including Ophiostomatales in *Raffaelea*, *Leptographium*, and *Ophiostoma*. We isolated at least one *Raffaelea* from most of the sampled Xyloterine

galleries, including *Raffaelea tritirachium* from *T. retusum* and *X. politus* galleries. These auxiliary species may or may not be effectively transmitted by *Trypodendron* beetles, but if carried by the beetles they would most likely be superficial passengers similar to fungi carried by bark beetles (Harrington 2005) or as interlopers in the mycangium (Bateman et al. 2016). Haanstad and Norris (1985) and Suh & Zhou (2010) isolated several yeasts from *X. politus* adults and galleries, and they suggested they may play important roles in the beetles' ecology, but such yeasts are not likely a part of the ambrosia symbiosis as they would not form a thick layer of sporulation for grazing (Mayers et al. 2018).

The ambrosia growth of *Phialophoropsis* in *Trypodendron* larval cradles is generally thin, pigmented, and consists of a single palisade layer bearing chained phialoconidia, unlike the thick, luxurious, white ambrosia growth of *Ambrosiella* and *Meredithiella* (Mayers et al. 2015, 2017, 2018). *Phialophoropsis* typically does not produce ambrosia growth in the main tunnels, which are usually sparse and blackened. Healthy ambrosia growth in *Trypodendron* galleries consistently show *Phialophoropsis* as the dominant fungus, especially in cradles (Leach 1940; Mathiesen-Käärik 1953; Batra 1967). However, *Phialophoropsis* spp. are slow growing and difficult to isolate (Mathiesen-Käärik 1953; Batra 1963, 1967), and it is not surprising that some studies failed to detect *Phialophoropsis* or considered other fungi to be the primary symbionts (e.g. Bakshi 1950, 1952; Zimmerman 1973; Linnakoski et al. 2010; Oranen 2013). Kühnholz (2004) reported difficulties isolating *Phialophoropsis* from *Trypodendron* but had greater success suspending mycangial contents in water before spreading. We found that *Phialophoropsis* propagules from females and galleries are very susceptible to desiccation, and isolations were unsuccessful if the material dried in storage or shipping.

Nonetheless, we have consistently detected *Phialophoropsis* with sequencing of DNA extracted from adult female prothoraxes with Ceratocystidaceae-specific primers, even when isolation was not successful.

Compared to the galleries of other genera with Ceratocystidaceae symbionts, galleries of *Trypodendron* are frequently inhabited by auxiliary fungi. *Trypodendron* spp. overwinter in leaf litter rather than in their natal galleries in wood (Hadorn 1933; Border 1988), so the autumn microflora may not be well tended and the adult beetles may acquire various fungi during the winter and spring (Schneider and Rudinsky 1969). Zimmerman (1973) mentions that *Phialophoropsis* was only isolated from *T. domesticum* galleries in the spring. The mycangia of *Trypodendron* are full of debris during winter, which might include non-ambrosial fungi (Schneider and Rudinsky 1969), and different fungi are isolated from *Trypodendron* depending on if they are attacking, dispersing, or overwintering (Oranen 2013). During winter months, propagules of *Phialophoropsis* may be stored deep in the crook of the mycangium, growing in spring when the nutrient-producing gland cells are activated (Schneider 1975) and pushing other debris out of the mycangium. The mycangia are apparently full of homogenous fungal propagules during the first flight and gallery initiation (Schneider and Rudinsky 1969), so the secretions from the gland cells around the mycangium must be favoring the growth of *Phialophoropsis*.

Future directions

New *Phialophoropsis* spp. may be associated with unstudied *Trypodendron* beetles. Targets include *T. laeve,* a central European species with habits similar to *T. domesticum* (Lukášová and Holuša 2014), and the North American *T. rufitarsis,* which has similar mycangia to other *Trypodendron* (French and Roeper 1972) and may harbor a

Phialophoropsis symbiont (French and Roeper 1972; Roeper et al. 1980; Roeper and French 1981; Cassar 1993; Kühnholz 2004). However, the seven unstudied species of *Indocryphalus* may prove to be the most interesting for their variation in mycangium openings (Wood 1957; Beaver 2000; Cognato et al. 2015) and symbionts. Further study might determine if *Toshionella* was a recent acquisition from Asian *Scolytoplatypus* or if *Toshionella* co-adapted with *Indocryphalus* and was later acquired by Asian *Scolytoplatypus*.

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Tables

Table 1. Selected representative isolates of mycangial symbionts of the Xyloterini, with GenBank accession numbers.

	Associated ambrosia beetle	Locality	Culture or specimen number(s)[1]	GenBank Accession No.						
				ITS rDNA	28S rDNA	18S rDNA	*tef-1a*	*tub*	*mcm7*	*rpl1*
Phialophoropsis L.R. Batra emend. T.C. Harr.										
P. ferruginea (Math.-Käärik) T.C. Harr.	*Trypodendron lineatum*	Regna, Sweden	C3549 (CBS 223.55)	MF399187	MF399166	MF398168	MF375458	MG269947	MG270166	MG272458
	T. lineatum	Colorado, USA	M243 (BPI 893129)	—	KY744224	KR673889	KT318387	—	—	—
	T. lineatum	Colorado, USA	M240 (BPI 893130)	KR611328	—	—	—	—	—	—
	T. lineatum	Colorado, USA	M241	MF399197	—	—	—	—	—	—
	T. proximum	Korea	C4275 (CBS 144148)	MF399198	—	—	—	—	—	—
P. nunbergii C. Mayers & T.C. Harr.	*T. domesticum*	Hann-Münden, Germany	C2230 (CBS 460.82)	KC305146	KF646766	KR673890	KT318388	—	KM495406	KM495493
	T. domesticum	Hainich, Germany	C3828 (CBS 141685)	MF399188	MF399167	MF398169	MF375459	MG269948	MG270164	MG272460
	T. signatum	Morioka, Iwate, Japan	C3914 (CBS 141686)	MF399189	MF399168	MF398170	MF375460	MH042544	—	—
	T. signatum	The Netherlands	M560	MF399196	—	—	—	—	—	—
P. hubbardii C. Mayers, T.C. Harr., McNew & Roeper	*T. retusum*	Wisconsin, USA	C3550 (CBS 408.68)	KC305145	AF275505	EU984254	HM569641	MH042545	—	—
	T. retusum	Michigan, USA	C3386 (CBS 141683)	MF399190	MF399169	MF398171	MF375461	MG269949	MG270165	MG272459
	T. retusum	Michigan, USA	M594	MF399194	—	—	—	—	—	—
	T. retusum	Michigan, USA	M596 (BPI 910630)	=MF399194	—	—	—	—	—	—
P. leachii C. Mayers, T.C. Harr., McNew & Roeper	*T. betulae*	Michigan, USA	C3404 (CBS 141684)	MF399191	MF399170	MF398172	MF375462	MH042546	MH042549	MH042548
	T. betulae	Michigan, USA	M593 (BPI 910632)	MF399193	—	—	—	—	—	—
P. trypodendri L.R. Batra	*T. scabricollis*	Georgia, USA	M577	MF399195	—	—	—	—	—	—

Table 1 continued

Toshionella C. Mayers & T.C. Harr.										
Toshionella sp.	*Indocryphalus pubipennis*	Jeju, South Korea	M673	MH040803	MH040911	—	—	—	—	—
Raffaelea Arx & Hennebert										
R. cf. *canadensis* RNC5	*Xyloterinus politus*	Michigan, USA	C3169 (CBS 142652)	—	MF399173	MF398175	—	—	—	—
Kaarikia C. Mayers & T.C. Harr.										
K. abrahamsonii C. Mayers, T.C. Harr., & Roeper	*X. politus*	Michigan, USA	A1264 (CBS 144155)	—	=MF399172	=MF398174	—	—	—	—
	X. politus	Michigan, USA	A1268 (CBS 142646)	—	MF399172	MF398174	MF375464	MH042547	—	—

[1] Collection abbreviations include: Iowa State University collection (C, M); Westerdijk Fungal Biodiversity Institute (CBS); U.S. National Fungus Collections (BPI). [2]Accesion numbers preceded by ''=' represent an identical (100% identity) match with that accession.

Supplemental Tables

Table S1. GenBank accessions for other Microascales fungi included in phylogenetic analyses.

Species	Culture collection [1]	GenBank Accession No. [2]					
		28S	18S	*tef-1a*	*tub*	*mcm7*	*rpl1*
Ceratocystidaceae representatives and outgroups							
Ambrosiella batrae C. Mayers, McNew & T.C. Harr.	C3130 (CBS 139735)	KY744584	KR673881	KT290320	MG269932	MG270152	MG272445
A. beaveri Six, de Beer & W.D. Stone	C2749 (CBS 121750)	KF646765	KR673882	KT318380	MG269938	MG270156	MG272448
A. catenulata Y.T. Lin & H.H. Shih	C3913 (CBS 142649)	MG269982	MG950189	MG944394	MG269937	MG270154	MG272446
A. cleistominuta C. Mayers & T.C. Harr.	C3843 (CBS 141682)	KY744585	KX925304	KX925309	MG269936	MG270153	MG272443
A. grosmanniae C. Mayers, McNew & T.C. Harr.	C3151 (CBS 137359)	KY744587	KR673884	KT318382	MG269933	MG270150	MG272444
Ambrosiella aff. *grosmanniae* NRgro1	C3899 (CBS 142648)	MG269983	MG950190	MG944395	MG269934	MG270155	MG272447
A. hartigii L.R. Batra	C1573 (CBS 404.82)	KY744588	KR673885	KT318383	MG269931	MG270157	MG272442
A. nakashimae McNew, C. Mayers & T.C. Harr.	C3445 (CBS 139739)	KY744586	KR673883	KT318381	MG269939	MG270158	MG272450
A. remansi C. Mayers & T.C. Harr.	M290 (BPI 910622)	—	KX342072	KX354426	—	—	—
A. roeperi T.C. Harr. & McNew	C2448 (CBS 135864)	KF646767	KR673886	KT318384	MG269935	MG270151	MG272449
A. xylebori Brader ex Arx & Hennebert	C3051 (CBS 110.61)	KM495318	KR673887	KT318385	MG269930	KM495407	KM495495
Berkeleyomyces basicola (Berk. & Broome) W.J. Nel, Z.W. de Beer, T.A. Duong, & M.J. Wingf.	C1372 (CBS 414.52, MUCL 8363)	AF222458	KX925307	HM569628	MG269963	—	—
	CMW7068 (CBS 413.52)	—	—	—	—	KM495484	KM495574
Bretziella. fagacearum (Bretz) Z.W.deBeer, Marinc., T.A.Duong & M.J.Wingf.	C927 (CBS 129242)	=AF222483	KR673892	KT318389	MG269953	MG270170	MG953416
Catunica adiposa (Butler) C. Mayers & T.C. Harr.	C999 (CBS 183.86)	=KM495320	KR673891	HM569644	MG269952	MG270169	MG953415
Ceratocystis fimbriata Ellis & Halst	C1099 (ICMP 8579)	=KR347445	KR673893	HM569615	MG269954	—	—
	CMW 15049 (CBS 141.37)	—	—	—	—	KM495432	KM495520
C. variospora (R.W. Davidson) C. Moreau emend. J.A. Johnson & T.C. Harr.	C1963 (CBS 135862)	KF646773	KX925305	KR347450	MG269956	—	—
	CMW 20935, C1843 (CBS 114715)	—	—	—	—	KM495471	KM495561
Chalaropsis ovoidea (Nag Raj & W.B. Kendr.) A.E. Paulin, T.C. Harr. & McNew	C1375, CMW 22733 (CBS 354.76)	AF275502	KY744595	HM569625	MG269957	KM495487	KM495577

Table S1 continued

Ch. thielavioides (Peyronel) A.E. Paulin, T.C. Harr. & McNew	C1378 (CBS 130.39)	AF222480	MF398184	HM569627	MG269958	—	—
	CMW 22736 (CBS 148.37, MUCL 6235)	—	—	—	—	KM495489	KM495579
Endoconidiophora coerulescens Münch.	C301 (CBS 100198)	AF275510	KR673895	HM569653	MG269960	—	—
	CMW26365, C313, C695 (CBS 140.37, MUCL 9511)	—	—	—	—	KM495418	KM495506
E. virescens R.W. Davidson	C252 (CBS 128998)	=KM495385	KX925306	HM569645	MG269959	—	—
	CMW17339, C261 (CBS 130772)	—	—	—	—	KM495472	KM495562
Huntiella bhutanensis (M. van Wyk, M.J. Wingf. & Kirisits) Z.W. de Beer, T.A. Duong & M.J. Wingf.	CMW8217 (CBS 114289)	All six genes were extracted from genome assembly MJMS00000000					
H. moniliformis (Hedgc.) Z.W. de Beer, T.A. Duong & M.J. Wingf	CMW10134 (CBS 118127)	All six genes were extracted from genome assembly MJMS00000000					
H. moniliformopsis (Yuan & Mohammed) Z.W. de Beer, T.A. Duong & M.J. Wingf.	C1934 (DAR 74609)	KF646769	KR673898	HM569638	MG269962	—	—
Huntiella sp. C792	C792	KY744592	KR673897	KT318392	MG269961	MG270172	MG272465
Knoxdaviesia capensis M.J. Wingf. & P.S. van Wyk	C1960, CMW997	MG269985	FJ176834	HM569657	MG269967	KM495478	KM495568
Meredithiella norrisii McNew, C. Mayers & T.C. Harr.	C3152 (CBS 139737)	KY744589	KR673888	KT318386	MG269944	MG270162	MG272456
M. fracta C. Mayers, C. Bateman & T.C. Harr.	C4171 (CBS 142645)	KY744590	KY744594	KY773179	MG269945	MG270163	MG272457
M. guianensis C. Mayers, C. Bateman & T.C. Harr.	M552 (BPI 910532)	KY744223	KY744227	KY773180	MG269946	—	—
Solaloca norvegica (J. Reid & Hausner) T.C. Harr.	C3124 (UAMH 9778)	KY744591	KR673894	KT318390	MG269955	MG270171	MG272455
Thielaviopsis ethacetica Went	C1107	KY744593	KR673899	HM569632	MG269964	—	—
	CMW 37775 (IMI 50560, MUCL 2170)	—	—	—	—	KM495426	KM495514
T. punctulata (Hennebert) A.E. Paulin, T.C. Harr. & McNew	C869, CMW 1032 (CBS 114.47, 146. MUCL 9526)	AF275513	KX925308	KX925310	=MG269965	KM495459	KM495548
Unnamed Microascales sp. C3547	C3547 (CBS 142647)	MF399171	MF398173	MF375463	MG269966	MG270148	MG272463

[1]Includes isolate or specimen numbers from the culture collection of T.C. Harrington, Iowa State University (C or M), Westerdijk Fungal Biodiversity Institute (CBS), U.S. National Fungus Collections (BPI), New South Wales Plant Pathology Herbarium (DAR), UAMH Centre for Global Microfungal Diversity (UAMH), International Collection of Microorganisms from Plants (ICMP), Royal Botanic Gardens Kew HerbIMI (IMI), and the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria (CMW). [2]Accession numbers preceded by ''=' represent an identical (100% identity) match with that accession.

Table S2. GenBank accessions for other Diaporthomycetidae fungi included in phylogenetic analysis.

	GenBank Accession No.	
	28S rDNA	18S rDNA
Annulatascus velatisporus	KX977086	KX977089
Annulusmagnus triseptatus	GQ996540	JQ429242
Aureovirgo volantis	KR051131	—
Ceratocystiopsis minuta	EU913656	MF398176
Diaporthe eres	AF362565	—
Esteya vermicola	EU627684	—
Fragosphaeria purpurea	AF096191	AF096176
Glomerella cingulata	DQ286199	M55640
Gnomonia gnomon	AF408361	DQ471019
Graphilbum rectangulosporium	AB235158	AB235159
Grosmannia penicillata	DQ294385	HQ634822
Hawksworthiomyces lignivorus	EF139119	—
Lasiosphaeria ovina	AF064643	AY083799
Leptographium crassivaginatum	MF399175	MF398183
Le. lundbergii	DQ294388	AH008988
Myrmecridium banksiae	JX069855	—
Myrmecridium montsegurinum	KT991664	KT991645
Ophiostoma piliferum	AY281094	AJ243295
Phomatospora bellaminuta	FJ176857	FJ176803
Raffaelea amasae	MF399174	MF398177
R. ambrosiae	EU177453	EU170278
R. brunnea	EU177457	MF398180
R. canadensis	EU177458	MF398181
R. lauricola	KF515710	MF398179
R. sulphurea	EU177463	MF398178
R. tritirachium	EU177464	MF398182
Sordaria fimicola	AY780079	X69851
Sporothrix schenckii	KX590890	M85053
Tenuimurus clematis	MFLUCC14	—
Trichoderma viride	AY489726	AF525230
Xylaria hypoxylon	AY544648	AY544692

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CHAPTER 7. GENERAL CONCLUSIONS

The results of this dissertation make it clear that ambrosia fungi in the family Ceratocystidaceae are tightly linked with ambrosia beetle hosts that have uniquely large and complex mycangia. There appears to be a mycangium hierarchy, in which mycangia with Ceratocystidaceae symbionts are set apart from the smaller and simpler mycangia that typically carry *Raffaelea* symbionts. Some ambrosia beetle lineages may have started with *Raffaelea* cultivars and later developed more sophisticated mycangia and switched to Ceratocystidaceae symbionts. The surprisingly consistent associations of genera of Ceratocystidaceae with these mycangium types implies a cultivar hierarchy as well, and these fungi must provide some advantage over other available fungal cultivars such as *Raffaelea*. This work sets a basic foundation for further ambrosia research, for which many questions remain unanswered.

Despite a wide variety of ubiquitous saprophytes and superficial passengers available for domestication, all of the large and complex mycangia are associated with species in five genera of a single family, Ceratocystidaceae: *Ambrosiella, Meredithiella, Phialophoropsis, Toshionella,* and *Wolfgangiella*. Despite frequent host switching in *Raffaelea* between beetles of completely different species, genera, tribes, and subfamilies, and despite the frequent presence of *Raffaelea* in galleries of *Trypodendron,* the mycangia that carry Ceratocystidaceae fungi were never observed to have taken up *Raffaelea* symbionts. The Ceratocystidaceae cultivars must provide some superior benefit to their farmers, such as luxuriant ambrosia growth and grazing efficiency, an abundance of specific nutrients, or more effective exclusion of weed fungi. It is noteworthy that the conidiophores and conidia

of many of the ambrosia symbionts of the Ceracystidaceae contain an abundance of lipid bodies, and fats have been implied as an important nutrient source for ambrosia beetles.

The mycangia that carry Ceratocystidaceae fungi are relatively large in relation to the beetles' size, and these mycangia are complex structures in the form of winding tubes or folded pouches rather than simple invaginations. However, these aspects alone cannot explain the success of these mycangia at selectively maintaining symbioses with Ceratocystidaceae. An abundance of gland cells often line the walls of these mycangia, and are usually held in reticulated scaffolds. Specificity is unlikely to be through the action of antifungal or antibacterial compounds produced by these glands, because the compounds would have to be exceedingly broad in their targets and potent in their effect yet leave the mycangial symbiont unharmed. More likely is that some specific nutrient produced by these mycangia is uniquely metabolized by Ceratocystidaceae, perhaps an unusual amino acid. The mycangial secretions must at least provide a competitive edge for the growth of Ceratocystidaceae fungi, which could then push other fungi out of the mycangium.

The multiple origins of fungus farming in the Scolytinae may have been enabled by horizontal transfer of *Raffaelea* cultivars that were domesticated by the Platypodinae (Vanderpool et al. 2017), followed by additional transfers between Scolytinae tribes. These *Raffaelea* species remain the dominant symbionts of ambrosia beetles, but there is little evidence for their specificity among beetle tribes or co-evolution with their beetle hosts. The first Ceratocystidaceae symbiont was likely domesticated by a Scolytoplatypodini ancestor, arguably the first tribe of ambrosia beetles in the Scolytinae. A tight co-adaptation between a nutritionally superior symbiont and its host insect may have led to a highly derived and carefully tuned mycangium, which is found in all three lineages of the Scolytoplatypodini.

Fungal lineages derived from a *Toshnionella*/*Ambrosiella*-like ancestor survive as symbionts of Asian *Scolytoplatypus* and *Remansus.* Later, the *Xylosandrus* complex in the Xyleborini may have developed its highly specialized mesonotal mycangium to accommodate the unique nutritional requirements of an *Ambrosiella* from the Scolytoplatopodini, and a similar transfer may have occurred in *Indocryphalus* (Xyloterini)*,* which was associated with a *Toshionella*-like symbiont.

The American *Corthylus* likely evolved and developed its specialized mycangia in the absence of Scolytoplatypodini symbionts, because the latter tribe is strictly Old World. The coiled prothoracic mycangium in males of *Corthylus* may be derived from the simpler mycangium of a *Monarthrum*-like ancestor that had *Raffaelea* symbionts. The elaboration of the *Corthylus* mycangium was likely a co-adaption with a saprophytic or plant parasitic Ceratocystidaceae, such as close relative *Catunica adiposa*. *Catunica adiposa* forms aleurioconidia similar to those of *Toshionella* and *Ambrosiella*, and such conidia may have been produced by the common ancestor of *Meredihtiella, Toshionella,* and *Ambrosiella*, which could explain the morphological similarity of these separately-domesticated genera.

What, then, caused the other ambrosia beetle groups (African *Scolytoplatypus* and *Trypodendron*) to independently domesticate other unrelated Ceratocystidaceae lineages? African *Scolytoplatypus* may have originally had *Toshnionella*/*Ambrosiella*-like symbionts but later switched to a new lineage in the Ceratocystidaceae with a similar physiology. *Trypodendron*, too, may have had a *Toshionella*-like symbiont (as in *Indocryphalus*) but later independently co-adapted with a new Ceratocystidicaceae, giving rise to *Phialophoropsis*. However, the relative sparseness of the ambrosia growth of *Wolfgangiella* and *Phialophoropsis* and their lack of aleurioconidia with large lipid bodies does not suggest that

these genera are superior food sources for the ambrosia beetles. A much broader taxonomic and geographic sampling of ambrosia beetles would be necessary test these hypotheses. It is noteworthy that Central Asian species of *Scolytoplatypus* and Xyloterini remain unstudied, yet this region may have played a critical role in the origins of the fungal symbionts and horizontal exchange of symbionts among the tribes.

A hierarchy of ambrosia symbionts, and historical replacement of fungal cultivars between insect sublineages lineages, would mirror similar patterns in fungus-growing ants (Schultz and Brady 2008) and fungus-growing termites (Aanen et al. 2002), though in each of these systems the origin of fungus farming occurred only once in the insects.

There is still much that remains unknown about ambrosia fungi, ambrosia beetles, and the symbiosis as a whole. The ambrosia fungi of multiple ambrosia beetle lineages remain completely unknown (Hulcr and Stelinski 2017). Work also needs to be done on the molecular and chemical basis of the symbiosis. Which nutrients are secreted by mycangia to support fungal growth, and which nutrients are provided by ambrosia fungi that make them a superior food source? What triggers an ambrosia fungus to switch from a yeast-like phase in the mycangium to a filamentous form in wood or a sporulating form in galleries? What is the biological role of a sexual state in obligate mycangial symbionts, and is it widespread among such fungi or do the sexual species discovered in this dissertation represent exceptions? How did ambrosia fungi co-evolve or co-adapt with their beetle hosts? Deeper studies of evolution require genome sequencing, and the first ambrosia fungus genomes are now becoming available, such as the genomes of several *Raffaelea* spp. and *Ambrosiella xylebori* (Vanderpool et al. 2017).

The ambrosia symbiosis must be appreciated as three equally-important points of a triangle, each of which must co-adapt in response to the other two: the beetle's biology, the fungus, and the mycangium. Of the three, mycangia are greatly underappreciated. I strongly urge future authors describing species of ambrosia beetles, especially in lineages where mycangia are not well known, to examine and describe their mycangia. They are essential components in the beetles' lives and their successful reproduction, and must be assumed to have taxonomic and biological significance. Ambrosia fungi should also be examined, where possible, in studies of ambrosia beetles. Other components of the symbiosis, such as host tree, locality, and other microbes in the galleries, appear to be relatively minor players in evolutionary history. The ambrosial triangle forms the foundation of the most diverse system of insect agriculture on the planet, which serves as a promising model system for the study of mutualism. This dissertation significantly added to our understanding of Ceratocystidaceae ambrosia fungi, but other fungal lineages associated with unstudied beetles and mycangia wait to be discovered. Vast and unexplored aspects of this symbiosis remain unknown and were previously hindered by incomplete understanding of the beetles and especially of their fungi. Perhaps the last pillars of basic research will soon be completed, and deeper questions about the ecology and evolution of this fascinating symbiosis can finally be addressed.

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