Three genera in the Ceratocystidaceae are the respective symbionts of three independent lineages of ambrosia beetles with large, complex mycangia

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\textbf{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 \textit{Ambrosiella xylebori}, \textit{Ambrosiella hartigii}, \textit{Ambrosiella beaveri}, and \textit{Ambrosiella roeperi}, three new species of \textit{Ambrosiella} are described from the ambrosia beetle tribe Xyleborini: \textit{Ambrosiella nakashimae} sp. nov. from \textit{Xylosandrus amputatus}, \textit{Ambrosiella batrae} sp. nov. from \textit{Anisandrus sayi}, and \textit{Ambrosiella grosmaniae} sp. nov. from \textit{Xylosandrus germanus}. The genus Meredithiella gen. nov. is created for symbionts of the tribe Corthylini, based on \textit{Meredithiella norrisii} sp. nov. from \textit{Corthylus punctatissimus}. The genus \textit{Phialophoropsis} is resurrected to accommodate associates of the Xyloterini, including \textit{Phialophoropsis trypodendri} from \textit{Trypodendron scabricollis} and \textit{Phialophoropsis ferruginea} comb. nov. from \textit{Trypodendron lineatum}. Each of the ten named species was distinguished by ITS rDNA barcoding and morphology, and the ITS rDNA sequences of four other putative species were obtained with Ceratocystidaceae-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 TEF1\alpha 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
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 ecologically group of more than 3,400 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 (xylomyctophagy) (Roeper 1995; De Fine Licht & Biedermann 2012). 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 & 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 (Nakashima 1975; Cassier et al. 1996; Marvaldi et al. 2002). In contrast, ambrosia beetles arose at least 10 separate times from bark beetle lineages within the Scolytinae (Farrell et al. 2001; Jordal & 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 (Ophiostomatinae) (Cassar & Blackwell 1996; Harrington & Fraedrich 2010; Harrington et al. 2010, 2014). These small mycangia include oral pouches, pronotal pits, elytral pouches, and coxal enlargements, each of which is simple modifications of the adult beetle’s exoskeleton, with secreting gland cells near the opening of the mycangium (Francke-Grosmann 1967; Beaver 1989). 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 reticulate structure punctuated by gland cells secreting directly into the mycangium (Francke-Grosmann 1956; Finnegan 1963; Schneider & 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 (Francke-Grosmann 1956, 1967; Beaver 1989; Kinuura 1995; Hulcr et al. 2010; Stone et al. 2007; Hulcr & Cognato 2010; Cognato et al. 2011a). In the Cortylinini, *Corthylus* spp. have long, folded tubes that open into the procoxae of adult males (Finnegan 1963; Giese 1967). In the Xyletorini, female *Trypodendron* spp. have large, tubular, pleural-prothoracic mycangia (Francke-Grosmann 1956, 1967; Schneider & Rudinsky 1969b).

*Ambrosiella* initially included ambrosia beetle symbionts with percurrent proliferation of conidigenous cells vs. sympodial proliferation by *Raffaelea* spp. (Brader 1964; von Arx & Hennebert 1965; Batra 1967). Gebhardt et al. (2005) demonstrated that some *Raffaelea* spp. have percurrent and sympodial proliferation, but *Ambrosiella xylebori* Brader ex Arx & Hennebert 1965, *Ambrosiella hartigii* L.R. Batra 1968, and *Ambrosiella furginea* (Math.-Käärik) L.R. Batra 1968 produced conidia from phialides. Harrington et al. (2010) limited *Ambrosiella* spp. to the phialdic ambrosia fungi within the *Ceratocystidaceae* and *Raffaelea* spp. to symbionts within the *Ophiostomatinae*. 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 aleuroconidia with inconspicuous collarettes and/or basipetal chains of cylindrical to barrel-shaped phialoconidia via ring-wall building (Minter et al. 1983; Nag Raj & Kendrick 1993; Riggs & Mims 2000). The presence of deep-seated phialides in the symbiont of *Trypodendron scabri-collis* (tribe *Xyloiterini*), *Ambrosiella 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 synonymy with *Ambrosiella*. However, phylogenetic analyses have generally suggested that the two genera are distinct (Alamouti et al. 2009; Harrington 2009; Six et al. 2009; Harrington et al. 2010; de Beer et al. 2014).

At present, there are six named species of *Ambrosiella*: *A. trypodendri* from *T. scabricollis* (Batra 1967); *A. furginea* from *Trypodendron lineatum* (Batra 1967); *Ambrosiella xylebori* from *Xylosandrus compactus* (Brader 1964); *A. hartigii* from *Anisandrus dispar* (Batra 1967); *Ambrosiella beaveri* *Six*, de Beer & W.D. Stone from *Cnestus mutilatus* (Six et al. 2009); and *Ambrosiella roeperi* T.C. Harr. & McNew from *Xylosandrus crassiusculus* (Harrington et al. 2014). In addition to *X. compactus*, *A. xylebori* has been reported from *Corthylus columbiaen* (Batra 1967; Nord 1972) and *Corthylus punctatissimus* (Roepper 1995). Besides *A. dispar*, *A. hartigii* has been reported from *Anisandrus sayi* and *Anisandrus obesus* (Hazan & Roepper 1980; Roepper &
analyses and observations of cultures from beetles with large phylogenetic analyses may reveal cryptic species and genera (1992) illustrated a fungus from McPherson 1984; Roeper 1996).

Difco yeast extract, 1.5 % agar).

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d autoclaving). Isolations were also attempted directly from am-

The mycangium/scutellum was either transferred to a drop
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scutellum to expose the interior of the beetle without damag-

were grown 4–14 d and extracted using the ProMega Wizard® Genomic DNA Purification Kit (Promega, Madison, WI).

PrepMan® Ultra was used to extract DNA from scraped fun-
gmental jump to ambrosia beetle symbiosis.

Three ambrosial genera in the

Ceratocystidaceae

Xylosandrus germanus (Weber & McPherson 1984; Roeper 1996). A. ferruginea has been reported from several Trypodendron spp., including T. lineatum, Trypo-
dendron domesticum, Trypodendron retusum, Trypodendron ruftar-
sis, and Trypodendron betulæa (Batra 1967; French & Roeper 1972; Roeper and French, 1981; Roeper and French, 1996), and Nakashima et al. (1992) illustrated a fungus from Trypodendron signatum 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 of 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 flexure (Contech Enterprises, Victoria, British Columbia) in the case of Trypodendron lineatum and Trypodendron scab-
ricolis. 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 (Harrington & Frederich 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–Aldrich; 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 for-
ceps and a scalpel. An incision was made just posterior to the scutellum to expose the interior of the beetle without damag-
ing the mycangium, which sits directly beneath the mesono-
tum 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 microsco-
scopic 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 d and DNA was extracted using PrepMan® Ultra, or isolates were grown 4–14 d and extracted using the ProMega Wizard® Genomic DNA Purification Kit (Promega, Madison, WI).

PrepMan® Ultra was used to extract DNA from scraped fun-
gmental 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 & 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’ GCGGAGGGATATTACTTAGG 3’) and ITS Cer 3.7R (5’ GTGAAATGAGCGTCGGACAG 3’) for ITS1 and primer pair ITS Cer 3.1 (5’ CAAACGATCTCTTGGCCTA 3’) and ITS4 (5’ TCCTCCGCTTTAGGATG 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 data-
set. 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 ana-
lyzed by UPGMA in PAUP 4.0b10 (Swofford 2002) using uncor-
rected (“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 (TEFLα) 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 for-
mer members of the Ceratocystis moniliformis complex, now treated as Huntiella. Our Huntiella 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 *Huntiella omanensis* (DQ074739). Other taxa in the Microascales included *Pseudallescheria* spp. (mixed species; *P. trypodendri* sp. CBS 408.68) and *Gondwanamyces* capensis (18S rDNA, AF176951) and *Huntiella omanensis* (18S rDNA, DQ074739). Outgroup taxa were *Flectosphaerella cucumerina* (18S rDNA, AF176951) and *Neurospora crassa* (18S rDNA, FJ176834). Sequencing was generally performed with the PCR primers as well as the internal primer EFCF1a (5'-GCYGAGCTCGGTAAGGGYTC-3') and the reverse primer EFCF6 (5'-GCYGAGCTCGGTAAGGGYTC-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'-GCYGAGCTCGGTAAGGGYTC-3') and EFCF3 (5'-GCYGAGCTCGGTAAGGGYTC-3'). The aligned sequences were approximately 1200 bp.

Amplification of TEF1a used the forward amplification primer EFCF1a (5'-AGTGGCGTGGGTATCAGCAAGGC-3') or EFCF1.5 (5'-GCYGAGCTCGGTAAGGGYTC-3') and the reverse primer EFCF6 (5'-GCYGAGCTCGGTAAGGGYTC-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'-GCYGAGCTCGGTAAGGGYTC-3') and EFCF3 (5'-GCYGAGCTCGGTAAGGGYTC-3'). The aligned sequences were approximately 1200 bp.

A combined SSU and TEF1a dataset (TreeBase URL: http://purl.org/phylo/treebase/phylows/study/TB2:517680) 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 & 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 d. 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 the 14 studied species of ambrosia beetles with

<table>
<thead>
<tr>
<th>Culture or specimen identification numbers</th>
<th>Associated ambrosia beetle</th>
<th>GenBank accession no</th>
</tr>
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<tr>
<td>C3130 (CBS 139735)</td>
<td><em>Anisandrus sayi</em></td>
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<tr>
<td>C3110</td>
<td><em>Phialophoropsis</em></td>
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large, complex mycangia: eight species from tribe Xyleborini (Cnestus mutilatus, Xylosandrus amputatus, Xylosandrus germanus, Xylosandrus crassiusculus, Xylosandrus compactus, Anisandrus dispar, Anisandrus sayi, and Eccoptopterus spinosus), two from tribe Corthylini (Corthylus punctatissimus and Corthylus 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 and 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 (conidiophores 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 *Ambrosiella 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).

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).

*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 homogeneous mass of arthrospore-like cells, similar to those reported by Harrington et al. (2014) from mycangia of *Xylosandrus 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).

*Xylosandrus crassiusculus* adult beetles from Georgia, Missouri, Ohio, and South Carolina yielded isolates with ITS sequences matching that of *Ambrosiella 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 another recovered *A. roeperi* sequence, 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 *Ambrosiella 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 *Ambrosiella hartigi* isolates were obtained from *Anisandrus 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 494.82 = C1573, from Germany). The ITS sequences of all *A. hartigi* 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 aleuroconidiophores and disarticulating aleuropconidia (Fig 4)–N). Additional *A. 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

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The 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.
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 *Anisandrus apicalis* (formerly *Xyleborus 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 2E–F). 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).

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**Fig 4** – Mycangium 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 cover slip. (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.
Male Corthylus 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
Trypodendron 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 Ceratocystidaceae-specific primers, and the sequence was similar to that of the symbionts of other Trypodendron spp. (Fig 3).

Fig 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.
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 Trypodendron spp. (Fig 3).

A culture deposited as Ambrosiella ferruginea (CBS 460.82) was isolated in 1971 from Trypodendron domesticum in Germany. No conidiophores were seen in this culture, but its Trypodendron retusum was isolated in 1971 from Trypodendron domesticum in Germany. No conidiophores were seen in this culture, but its T. scabricollis 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 Trypodendron 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 Ceratocystis adiposa (Butler) Moreau 1952, Ceratocystis fagacearum (Bretz) J. Hunt 1956, Ceratocystis 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 as a monophyletic clade, and all of these species formed aerioconidia on branching conidiophores (Fig 5). The Xyloderma amputatus associate had sequences similar to those of Ambrosiella beaveri, the Anisandrus sayi associate had sequences similar to those of Ambrosiella hartigi, and the Xyloderma germanus associate had sequences most similar to those of Ambrosiella roeperi and Ambrosiella 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 Ceratocystis 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.

**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.


MycoBank MB504757.

Comments – This species was probably introduced to the Southeastern USA with its beetle host, Ceres tus mutilatus, from Asia (Six et al. 2009). The culture from the holotype of Ambrosiella beaveri (CBS 121750) and two isolates (C3180 and C3181) from Ceres tus 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 1C–F).

MycoBank MB812571.

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, Xyloderma 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 d and 25–50 mm after 6 d 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 d. Sporodochia white to gray on surface, superficial. Aleuriocinidiophores (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 aleuriocinidium or a chain of aleuriocinidia, conidigenous cell with a collarette. Aleuriocinidia (Fig 1F) globose to subglobose, hyaline to light brown with age, thick-walled, asperate, 6–10 (14) × 6–10 μm. Phialociadiophores uncommon, with moderately seated phialides, producing basipetal chains of conidia by ring-wall building. Phialocinidia ellipsoidal to globose, hyaline to light olive brown with age, becoming thick-walled, asperate 6–10 (12) × 5–9 μm. Protoperithecia (Fig 2B, C) superficial, spherical, tan to brown, 55–70 μm diam. Gallery growth dense, flat, white to gray, becoming brown with age. Aleuriociadiophores (Fig 1C) similar to those in culture, 9–70 μm long, ending in a phialide that may have a distinct collarette. Aleuriocinidia in galleries (Fig 1D) larger than in culture, terminal or in chains, globose to subulate, thick walled, asperate, 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, Xyloderma amputatus gallery in C. 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 aleuriociadiophores, but differs in growth rate (7–32 mm vs. 28–36 mm diam after 4 d at 25°C), rarity of phialociadiophores, 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 MB326143.

**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 *A. hartigii*. The fungus has been reported from *Anisandrus dispar* in Michigan, Oregon, and Washington (Batra 1967; Rooper et al. 1980; Roeper & French 1981). We obtained DNA sequences (Fig 3) 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 borne on aleurioconidiophores (Fig 1H), as illustrated by seated phialoconidiophores (Fig 1G) and terminal aleurioconidia in culture: chained phialoconidia from moderately seated heavily on MYEA, and these isolates produced two spore types in culture: chained phialoconidia from moderately seated phialoconidiophores (Fig 1G) and terminal aleurioconidia (Fig 1J) on aleurioconidiophores (Fig 1H), as illustrated by Batra (1967). *A. hartigii* has been reported as the fungal symbiont of *Anisandrus sayi* and *An. obesus* (Hazen & Roeper 1980; Rooper et al. 1980; Roeper & French 1981) and Xylosandrus germanus (Weber & McPherson 1984; Rooper 1996), but the symbionts of *An. sayi* and *X. germanus* are described here as new species of *Ambrosiella*.

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

MycoBank MB812572.

**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, 84° 53′ 40.91″ W, isolated from *An. sayi* caught in flight, July 2013, R. Rooper, holotype (dried culture, BPI 893131); ex-type living culture C3130 (CBS 139735).

Colonies on malt yeast extract agar 13–15 mm diam. after 4 d at 25 °C, odor sweet at 4–10 d, 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 1J, N), 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 & Bright 1992a, 1992b*), *An. sayi*, females of which have mesonotal mycangia (Hazen & Roeper 1980). Cultures of *A. batrcae* 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 aleurioconidia borne in chains (Fig 1J) and terminal aleurioconidia that tear away with conidiophore cells attached (Fig 1M, N).


MycoBank MB326147.

**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 & 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 & Sreedharan 1988) and Japan (Kaneko & 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 d on MYEA) and sporulation. We observed the two types of aleurioconidiophores illustrated by Brader (1964) and von Arx & 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 Corthusius columbianus as *A. xylebori* (Batra 1967) is likely closely related to the new species we describe here from Corthusius punctatissimus, which also was previously reported to be *A. xylebori* (Rooper 1996).


MycoBank MB805798.

**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 3). A culture from *X. crassiusculus* in Taiwan identified as *A. xylebori* by Gebhardt et al. (2005) was likely *A. roeperi*.

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

MycoBank MB812573.

**Etymology**. Named after Helene Francke-Grossmann 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 *X. germanus* caught in flight, 8 May 2013, C. Mayers, holotype (BPI 893132); ex-type C3151 (CBS 137359).

Colonies on malt yeast extract agar 45–60 mm diam. after 4 d 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 d, fading after 8 d. Aleurioconidiophores (Fig 1R) rare, occurring singly or grouped on white to buff sporodochia, hyaline, simple to branched, 20–60 μm long, composed of monilioid 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 aleurioconidiophores (Fig 1O), producing aleurioconidia (Fig 1P, Q) as in culture. Growth in mycangium composed of arthropore-like cells 4.5–8.0 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).


Notes. This new species has been consistently isolated from X. germanus galleries, mycangia, and whole 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 Ambrosiella 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. xylobi, but the olivaceous to brown pigmentation of A. grosmanniae cultures distinguishes it from the white growth of A. xylobi. 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).


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 monilioid 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 Cortlylus consimilus and Batra’s (1967) illustrations of a similar fungus from Cortlylus columbianus imply that there are other species in Meredithiella associated with Cortlylus spp. The aleurioconidia of Meredithiella look similar to those of some Ambrosiella spp., but the aleurioconidiophores of the C. punctatissimus (Fig 21, J) and C. columbianus symbionts are uniquely branched.

Meredithiella norrisii McNew, C. Mayers & T.C. Harr., sp. nov. (Fig 2F–J) MycoBank MB812575.

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

Typus: USA: Iowa: Story Co., McFarland Park, C. punctatissimus gallery in Acer nigrum sapling, 8 August 2013, T. Harrington, holotype (BPI 893135); ex-type C3151 (living culture CBS 139737, dried culture BPI 893136).

Colonies on malt yeast extract agar 20–38 mm diam. after 4 d at 25°C, surface mycelium flat to aerial, white, becoming light gray brown, underside olivaceous, becoming dark brown, odor sweet at 4–10 d 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 21, J) 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 monilioid 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 conidiophore 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 A. saccharum, 8 July 2013, R. Roeper (BPI 893137).


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. columbianus (Batra 1967) were previously identified as A. xylobi, the C. punctatissimus symbiont is morphologically distinct and falls outside of Ambrosiella in phylogenetic analyses (Fig 3). The ITS sequence from C. consimilus is likely that of a distinct species of Meredithiella (Fig 3), and Batra’s (1967) illustrations of A. xylobi from C. columbianus 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. Aleuriocnidia not present. Associated with ambrosia beetles.

Type species: Phialophoropsis trypodendri L.R. Batra.

Batra (1967) originally created Phialophoropsis to accommodate Phialophoropsis trypodendri from Trypodendron scabricollis. Though he placed the Trypodendron lineatum associate in Ambrosiella, both P. trypodendri and Ambrosiella ferruginea form deep-seated phialides, and aleuriocnidia have not been noted in either species. Based on the distinctive phialides, Roeper (1972) suggested that A. ferruginea and A. hartigi 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 aleuriocnidia. 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 MB336297.


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 T. lineatum (BPI 893129, BPI 893130), but the attempts were unsuccessful. Microscopic examination of this gallery growth found phialoconidiophores with deep-seated phialides bearing phialocnidia 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, M) 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 and 5), which appear to be different Phialophoropsis spp. awaiting further study.

**Uncertain or excluded species of Phialophoropsis**


MycoBank MB319858.

Comments — This species was described from a leaf lesion on Embrothium lanceolatum in Wales (Brady & 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 Phialophoropsis cultures, and P. cambrensis was not associated with an ambrosia beetle.


MycoBank MB415852.

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 & 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).
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 independently 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 Xylodorus, Anisandrus, Cenetus, and Eccoptopterus have large mesonotal mycangia (Francke-Grosman 1956, 1967; Happ et al. 1976; Beaver 1989; Hulcr et al. 2007; Hulcr & Cognato 2010) 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) (Francke-Grosman 1967; Beaver 1989) may harbor unrelated species of Raffaelea or Fusarium (Harrington et al. 2010, 2011; Freeman et al. 2013; Kasson et al. 2013; O’Donnell et al. 2015). In Corthylini, male Cythus spp. have long, folded tubes opening into the procoxal cavity (Finnegan 1963; Giese 1967), which apparently harbor Meredithiella spp., while other genera of Cythus (e.g., male Gnatothrixus spp. and female Monarthrum spp.) have simple enlargements of the procoxal cavity (Batra 1963; Farris 1963; Lowe et al. 1967; Schneider & Rudinsky 1969a) and have Raffaelea symbionts (Batra 1967; Funk 1970; Roeger & French 1981; Harrington et al. 2010). Finally, in Xyloterini, Trypodendron spp. carry Phialophoropsis in their complex, tubular, pleural-prothoracic mycangia (Francke-Grosman 1956, 1967; Abrahamson et al. 1967; Batra 1967; Schneider & Rudinsky 1969b; French & Roeger 1972), 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 & 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; Six et al. 2009; Harrington et al. 2010; Harrington 2013; de Beer et al. 2014). Phialophoropsis appears to be closest to Ceratocystis fagaraceum, while our analyses suggest that Ambrosiella and the new genus Meredithiella are more closely related to Ceratocystis adiposa, Ceratocystis norvegica, and the genus Huntiella (formerly the Ceratocystis moniliformis complex). Huntiella spp., C. fagaraceum, and C. adiposa form phialocionidia in deep-seated phialides, but only C. adiposa forms aleuropiconidia (Nag Raj & Kendrick 1975; Harrington 2009). 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). C. fagaraceum, C. adiposa, and C. norvegica are distinct from Ceratocystis sensu stricto (the Ceratocystis 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 arthropore-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 (Norris et al. 1969; Kok 1979; Norris 1979; Six 2012).

Production of phialoconidia from deep-seated phialides by ring-wall building is found throughout the Ceratocystidaceae (Paulin-Mahady et al. 2002; Harrington 2009), 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 phialoconidophores of Phialophoropsis 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 phialocionidia are produced in dense columns. The chlamydospore-like hyphal swellings of P. ferruginea described by Mathiesen-Käärick (1953) were not observed in our cultures of Phialophoropsis, nor were aleuropiconidia seen in galleries or cultures of Phialophoropsis spp.

Gebhardt et al. (2005) reported phialidic conidiogenous cells in P. ferruginea, Ambrosiella hartigii, and Ambrosiella xylebori, but we have found only aleuropiconidophores with inconspicuous collarettes in A. xylebori. Moderately-seated phialides were observed in Ambrosiella beaveri, A. hartigii, and perhaps Ambrosiella nakashimeae, 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 aleuropiconidia 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 Ambrosiella 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, monilioid cells that may break off with attached, terminal aleuropiconidia (Harrington et al. 2014). Distinctive, monilioid conidiophores have been illustrated in A. xylebori (Batra 1967; Brader 1964; von Arx & Hennebert 1965; Kaneko 1967), Ambrosiella roperi (Kaneko 1967; Harrington et al. 2014), and Ambrosiella grosmanniae (Nakashimeae et al. 1992), and we observed them in A. batrae. In addition to disarticulating aleuropiconidophores, A. xylebori produces single aleuropiconidia from simple, hyphae-like
Aleuroconidophores (Batra 1967; von Arx & Hennebert 1965), which likely do not disarticulate (Harrington et al. 2014).

**Meredithiella norrisii** produces terminal aleuroconidia on monilioid hyphae that tear away with one or more conidiophore cells attached, similar to the third type of Ambrosiella conidiophore. However, the **M. norrisii** aleuroconidia are borne on short side branches that arise from a central monilioid hypha, as opposed to the branched aleuroconidophores 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 aleuroconidia (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 (Kessler 1974; Harrington & Fraedrich 2010; Harrington et al. 2011; Mendel et al. 2012; Ploetz et al. 2013; Kusumoto et al. 2015).

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 **Corthys** spp. and **Trypodendron** spp. found similarly tight associations with Meredithiella and Phialophoropsis spp., respectively.

Ambrosiella spp. appear to be associated with only the genus 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 ferruginus and Xyleborus affinis using PCR. The amplified ITS sequence from X. ferruginus matched most closely to the ITS sequence of A. grossmanniae from Xylodendrocarpus germanus (HQ538467), referred to as “Ceratocystis sp., CapXger3” (Kostovcik et al. 2014). It is possible that contaminating DNA of an Ambrosiella sp., such as A. roperi from Xylodendrocarpus crassiusculus, was amplified by Kostovcik et al. (2014). They failed to detect a Raffaelea sp. in X. ferruginus 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 X. germanus, X. crassiusculus, and Xylodendrocarpus compactus. Also, related species within beetle genera carry related fungal symbionts, even if the beetle species are native to different continents. The American Anisandrus sayi harbors A. batrae, while the closely related A. hartigii is associated with the European Anisandrus 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. 2011a) 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 Corthys 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 Scoytoplatypus spp., which have large mycangial pockets in the pronotum (Schedl 1962) and have been associated with fungi with monilioid chains of spores (Nakashima et al. 1987, 1992; Nakashima 1989; Kinuura 1995). Microcorthys spp. apparently have mycangia similar to Corthys spp. (Schedl 1962) and may harbor species similar to M. norrisii. 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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