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

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20 **Abstract:** The genus *Ambrosiella* accommodates species of *Ceratocystidaceae*
21 (*Microascales*) that are obligate, mutualistic symbionts of ambrosia beetles, but the genus
22 appears to be polyphyletic and more diverse than previously recognized. In addition to *A.*
23 *xylebori*, *A. hartigii*, *A. beaveri*, and *A. roeperi*, three new species of *Ambrosiella* are
24 described from the ambrosia beetle tribe *Xyleborini*: *A. nakashimae* sp. nov. from
25 *Xylosandrus amputatus*, *A. batrae* sp. nov. from *Anisandrus sayi*, and *A. grosmanniae* sp.
26 nov. from *X. germanus*. The genus *Meredithiella* gen. nov. is created for symbionts of the
27 tribe *Corthylini*, based on *M. norrisii* sp. nov. from *Corthylus punctatissimus*. The genus
28 *Phialophoropsis* is resurrected to accommodate associates of the *Xyloterini*, including *P.*
29 *trypodendri* from *Trypodendron scabricollis* and *P. ferruginea* comb. nov. from *T.*
30 *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 TEF1 α 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*).

Key words: Ambrosia Fungi, Scolytinae, Symbiosis

1. 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-prothoracic 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* 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* (Batra) T.C. Harr. (2010), was used to erect the monotypic genus *Phialophoropsis* 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, 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.

2. MATERIALS AND METHODS

141 **2.1 Beetle collection and fungal isolation**

142 Most of the adult beetles were caught in flight using Lindgren traps with water or
143 polyethylene glycol in collection cups, while the cups of other traps were dry and had No
144 Pest insecticide strips (vapon, Spectrum Brands, Middleton, Wisconsin). The traps were
145 baited with either ethanol lures or lineatin flexlure (Contech Enterprises, Victoria, British
146 Columbia) in the case of *T. lineatum* and *T. scabricollis*. Some adult beetles were caught
147 in-flight with rotary net traps. Other mature adults were taken directly from fresh
148 galleries by splitting infested wood sections.

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

156 For mycangial examination, adult female *Xyleborini* were dissected in 20% lactic
157 acid on a deep well slide using fine forceps and a scalpel. An incision was made just
158 posterior to the scutellum to expose the interior of the beetle without damaging the
159 mycangium, which sits directly beneath the mesonotum and is attached to the scutellum.
160 Fine forceps were then used to gently tease out the mycangium and scutellum. The
161 mycangium/scutellum was either transferred to a drop of Cotton blue on a slide and
162 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).

2.2 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.

2.3 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 ITS Cer3.1 (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.

2.4 Phylogenetic analysis

Sequences of the small subunit rDNA (SSU, 18S rDNA) and translation elongation factor 1- α (TEF1 α) 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 *Huntia*. 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 TEF1 α) and

206 *Gondwanamyces capensis* (18S rDNA, FJ176834,). Outgroup taxa were *Plectosphaerella*
207 *cucumerina* (18S rDNA, AF176951) and *Neurospora crassa* (18S rDNA, X04971).

208 The SSU sequences were amplified and sequenced using a variety of primers
209 (Vilgalys 2005, White et al. 1990), typically using the overlapping sequences from NS-
210 1/NS-6 and SR-9R/NS-8, but sometimes overlapping sequences were obtained with NS-
211 1/NS-4, NS-3/NS-6, and NS-5/NS-8. These overlapping sequences yielded an aligned
212 sequence of approximately 1700 bp.

213 Amplification of *TEF1 α* used the forward amplification primer EFCF1a (5'
214 AGTGCGGTGGTATCGACAAGCG 3') or EFCF1.5 (5'
215 GCYGAGCTCGGTAAGGGYTC 3') and the reverse primer EFCF6 (5'
216 CATGTCACGGACGGCGAAAC 3') following the protocol of Oliveira et al. (2015).
217 Sequencing was generally performed with the PCR primers as well as the internal
218 primers EFCF2 (5' TGCTCAACGGGTCTGGCCAT 3') and EFCF3 (5'
219 ATGGCCAGACCCGTGAGCA 3'). The aligned sequences were approximately 1200
220 bp.

221 A combined SSU and *TEF1 α* dataset (TreeBase URL:
222 <http://purl.org/phylo/treebase/phyloids/study/TB2:S17680>) of 2781 characters was used
223 for phylogenetic analysis. Model testing using ModelTest 2.1.7 v20141120 (Darriba et al.
224 2012) on both the combined dataset and the separate datasets for each gene showed the
225 GTR+I+G model to be most appropriate. MrBayes 3.2.1 (Ronquist and Huelsenbeck
226 2003) was used for Bayesian analysis with this GTR+I+G model. A single MCMC run
227 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).

2.5 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.)

3. 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.

3.1 Beetle associations

3.1.1 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).

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 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).

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*.)

3.1.2 *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).

3.1.3. *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).

Trypodendron 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).

3.2 Phylogenetic analysis

A Bayesian consensus tree of the combined SSU and TEF1 α 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 *Huntia* 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 *Huntia* (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.

3.3 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.

417 *Protoperithecia* (Fig 2B, 2C) superficial, spherical, tan to brown, 55-70 µm diam.

418 *Gallery growth* dense, flat, white to grey, becoming brown with age.

419 *Aleurioconidiophores* (Figs 1C) similar to those in culture, 9-70 µm long, ending in a

420 phialide that may have a distinct collarette. *Aleurioconidia* in galleries (Fig 1D) larger

421 than in culture, terminal or in chains, globose to suboblate, thick walled, aseptate,

422 hyaline, may become light brown with age, (8) 9-12.5 (15) × (7.5) 8.5-11.5 (16) µm.

423 *Other cultures examined: USA: Georgia:* Lowndes Co., 30° 49' 06.6" N, 83° 28' 48.3"

424 W, *Xylosandrus amputatus* gallery in *Cinnamomum camphora*, Aug 2014, S. Cameron,

425 CBS 139740 (C3443).

426 *Notes.* The symbiont of *X. amputatus* is similar to *A. beaveri* both in DNA sequences and

427 morphology, especially the aleurioconidiophores, but differs in growth rate (7-32 mm vs.

428 28-36 mm diam after 4 days at 25 C), rarity of phialoconidiophores, and the production

429 of protoperithecia. This is the first report of protoperithecia in ambrosia beetle symbionts,

430 which are thought to be asexual (Harrington et al. 2010). No perithecia or ascospores

431 were seen, but the studied isolates were from galleries of a single infestation and may be

432 of a single mating type. The beetle is native to Asia (Cognato et al. 2011b), and it is

433 possible that only a single mating type of *A. nakashimae* was introduced. It also is

434 possible that *A. nakashimae* is conspecific with *A. beaveri*, but phenotypic differences

435 between the strains warrants distinction at this time.

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

437 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.

458 *Typus: USA: Michigan:* Montcalm County, Alma College Ecological Station, 43° 23'
459 32.31" N, 34° 53' 40.91" W, isolated from *Anisandrus sayi* caught in flight, July 2013, R.
460 *Roeper*, holotype (dried culture, BPI 893131); ex-type living culture C3130 (CBS
461 139735).

462 *Colonies* on malt yeast extract agar 13-51 mm diam. after 4 days at 25° C, odor sweet at
463 4-10 days, surface growth white to buff, fluffy to chalky, growth below surface dense,
464 with irregular colony margins, coloring the media deep rust, darkening with age to
465 chestnut, rust colored liquid drops occasionally seen on surface mycelium. *Conidiophores*
466 scattered on aerial mycelium or concentrated in sporodochia, white to buff, spherical.
467 *Aleurioconidiophores* hyaline to light brown, simple to branched, 25-105 µm tall, either
468 composed of monilioid cells and with a terminal aleurioconidium with subtending
469 collarette (Fig 1L), or rarely composed of non-monilioid cells with chained
470 aleurioconidia from shallow phialides (Fig 1J). *Aleurioconidia* globose to subglobose,
471 thick-walled, aseptate, smooth, hyaline to rarely light brown, 11-16.5 (21) × 11-16 µm,
472 either borne terminally on monilioid aleurioconidiophores, often tearing away along with
473 one, two, or three conidiophore cells attached (Fig 1M, 1N), or borne on non-monilioid
474 aleurioconidiophores in short chains of aleurioconidia that break off either singly (Fig
475 1K) or in short chains.

476 *Other cultures examined: USA: Georgia:* Chattahoochee National Forest, from *An. sayi*
477 caught in flight, 1 July 2014, S. *Fraedrich* (C3415). *Missouri:* Boone County, isolated
478 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 grosmaniae 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).

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

532 *Other specimens examined: USA: Michigan:* Grand Traverse County, Traverse City,
533 Ashton Park, 44° 46' 10.77' N, 85° 38' 59 W, gallery of *X. germanus* in *Acer saccharum*,
534 24 July 2013, *R. Roeper* (BPI 893133).

535 *Other cultures examined: Germany:* Waldeck: near Jena, beech forest, from *X. germanus*
536 caught in flight, 2014, *P. Biedermann* (C3467). **d Netherlands:** Gelderland: near
537 Wageningen, from *X. germanus* caught in flight, 2014, *L. van de Peppel* (C3466). **d**
538 **USA: Georgia:** Clarke Co., Whitehall Forest, from *X. germanus* specimen caught in
539 flight, 21 March 2014, *S. Fraedrich* (C3312). *Michigan:* Grand Traverse Co., Traverse
540 City, Ashton Park, 44° 46' 10.77" N, 85° 38' 59" W, isolated from *X. germanus* taken
541 from gallery (BPI 893133) in *Acer saccharum*, 24 July 2013, *R. Roeper* (CBS 137358,
542 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). **d 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

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

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

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

569

570 Though only one species of *Meredithiella* is described at this time, an ITS sequence from
571 *Corthylus consimilis* and Batra's (1967) illustrations of a similar fungus from *C.*

572 *columbianus* imply that there are other species in *Meredithiella* associated with *Corthylus*
573 spp. The aleurioconidia of *Meredithiella* look similar to those of some *Ambrosiella* spp.,

574 but the aleurioconidiophores of the *C. punctatissimus* (Fig. 2 I, J) and *C. columbianus*
575 symbionts are uniquely branched.

576

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

578 MycoBank MB 812575.

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

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

584 *Colonies* on malt yeast extract agar 20-38 mm diam. after 4 days at 25 C, surface
585 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 monilliod hyphae, bearing a single, terminal aleurioconidium. *Aleurioconidia* terminal, subglobose to globose, thick-walled, aseptate, smooth, hyaline, 9-11 $\times$ 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 $\times$ 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* 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: *P. trypodendri* 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 (Mathiesen-Käärik) T.C. Harr, **comb. nov.**

MycoBank MB 812586.

Basionym: Monilia ferruginea Mathiesen-Käärik, Meddelanden fran Statens

Skogsforskningsinstitut 43: 57 (1953)

649 MycoBank MB 474947

650 *Synonym: Ambrosiella ferruginea* (Mathiesen-Käärik) L.R. Batra, Mycologia 59: 1000
651 (1968)

652 MycoBank MB 326141

653 *Comments* – An ambrosial fungus in the galleries of *T. lineatum* was observed by Hartig
654 (1872) in Germany and later by Leach et al. (1940) in Minnesota. Mathiesen-Käärik
655 (1953) described the fungus as *Monilia ferruginea*, and Batra (1967) moved the species
656 to *Ambrosiella*. Other studies confirmed the relationship between *P. ferruginea* and *T.*
657 *lineatum* (Francke-Grosmann 1967, Funk 1965), but this species was also thought to be
658 the symbiont of *T. domesticum* and *T. retusum* (Batra 1967), *T. betulae* (Roeper and
659 French 1981), and *T. rufitarsis* (French and Roeper 1972). We examined Batra's (1967)
660 Oregon material (BPI 407710) from *T. lineatum* galleries and saw palisades of
661 conidiophores with deep-seated phialides bearing phialoconidia singly or in chains.
662 Isolations were attempted from galleries made by *T. lineatum* in a log of *Picea* sp. from
663 Colorado (BPI 893129, 893130), but the attempts were unsuccessful. Microscopic
664 examination of this gallery growth found phialoconidiophores with deep-seated phialides
665 and chains of conidia (Fig. 2L, 2M) packed densely along the walls of the gallery (Fig.
666 2N). The ITS sequence from *T. lineatum* beetles trapped in Alaska matched the sequence
667 from the *T. lineatum* galleries in Colorado. The ITS, SSU, and TEF1 α sequences from the
668 DNA extracted from *T. lineatum* galleries in Colorado were similar to those of the
669 symbionts from *T. domesticum* and *T. retusum* (Fig. 3, 5), which appear to be different
670 *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 *Phialophoropsis* 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*.

4. 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 be 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 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 *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 *Huntia* (formerly the *C. moniliformis* complex). *Huntia* 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, monilliod cells that may break off with attached, terminal aleurioconidia (Harrington et al. 2014). Distinctive, monilliod 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 monilliod 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 monilliod 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 1989, Nakashima et al. 1987, 1992). *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.

5. ACKNOWLEDGEMENTS

The technical assistance of Caroline Wuest, Rodrigo de Freitas, Yeganeh Gharabigloozare, and Susan Best is greatly appreciated. We thank Doug LeDoux, Robbie Doerhoff, Tom Eager, Thomas Atkinson, Garret Dubois, John Lundquist, Scott Cameron, Mark Bunce, and Lennart van de Peppel for providing beetles or fungal cultures, and Anthony Cognato for identifying beetle specimens. We are grateful to the Forstamt Jena-Holzland (Germany), the Burgergemeinde Bern (Switzerland) and the national park Gesäuse (Austria) for collection permits at their properties. Chase Mayers was supported in part by a fellowship from the Office of Biotechnology, Iowa State University. Peter Biedermann was supported by the Swiss National Science Foundation (P300P3_151134), by a Marie Curie Intra-European Fellowship (IEF) (Project Number 626279) and a field collection grant by the German Entomological Society (DGaaE). Other financial support was provided by the U.S. Forest Service through a cooperative agreement with Iowa State University.

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1236 **Table 1.** Cultures, specimens, and GenBank accessions for representative species.

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

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 μ m.

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 μ m.

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.

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 .

Figure 5. Phylogenetic tree from Bayesian analysis of the combined TEF1 α 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. Diagrammatic 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*.